SCIENTIFIC OPINION

Scientific Opinion on application (EFSA-GMO-DE-2010-82) for the placing on the market of insect-resistant genetically modified maize MIR162 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta[1]

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

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ABSTRACT

This scientific opinion reports an evaluation of a risk assessment for placing on the market the genetically modified (GM) insect-resistant maize MIR162 for food and feed uses, import and processing. Maize MIR162 contains a single insert consisting of the *vip3Aa20* and *pmi* expression cassettes. The Vip3Aa20 protein confers resistance against certain lepidopteran pests and phosphomannose isomerase (PMI) serves as a selection marker. Bioinformatic analyses of the inserted DNA and flanking regions do not raise safety concerns. The levels of Vip3Aa20 and PMI proteins in maize MIR162 have been sufficiently analysed. The stability of the genetic modification has been demonstrated. Comparative analyses established that there are no biologically relevant differences in the compositional, agronomic or phenotypic characteristics of maize MIR162 compared with its conventional counterpart, and its composition falls within the range of non-GM commercial varieties, except for the expression of the Vip3Aa20 and PMI proteins. The safety assessment identified no concerns regarding potential toxicity and allergenicity of Vip3Aa20 and PMI proteins or of maize MIR162. A feeding study on broiler chickens confirmed that grain produced by maize MIR162 is as nutritious as that produced by its conventional counterpart and a non-GM commercial variety. There are no indications of an increased likelihood of establishment and spread of feral maize plants. Considering its intended use as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with an unlikely but theoretically possible horizontal gene transfer from maize MIR162 to bacteria have not been identified. The monitoring plan and reporting intervals are in line with the intended uses of maize MIR162. In conclusion, the EFSA GMO Panel considers that information available for maize MIR162 addresses scientific comments raised by Member States and that maize MIR162, as described in this application, is as safe as its conventional counterpart and non-GM commercial varieties with respect to potential effects on human and animal health and the environment in the context of its intended uses.

[1] On request from the Competent Authority of Germany on an application (EFSA-GMO-DE-2010-82) submitted by Syngenta, Question No EFSA-Q-2010-00972, adopted on 31 May 2012.

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KEY WORDS

GMO, maize (*Zea mays*), MIR162, insect resistance, Vip3Aa20, PMI, human and animal health, import and processing, Regulation (EC) No 1829/2003.

SUMMARY

Following the submission of an application (EFSA-GMO-DE-2010-82) under Regulation (EC) No 1829/2003[4] from Syngenta, the Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA GMO Panel) was asked to deliver a scientific opinion on the safety of insect-resistant genetically modified (GM) maize MIR162 (unique identifier SYN-IR162-4) for food and feed uses, import and processing.

In delivering its scientific opinion, the EFSA GMO Panel considered the application EFSA-GMO-DE-2010-82, additional information supplied by the applicant, scientific comments submitted by European Union (EU) Member States and relevant scientific publications. The scope of the application EFSA-GMO-DE-2010-82 is for food and feed uses, and import and processing of maize MIR162 within the EU in the same way as any non-GM maize, but excludes cultivation in the EU. The EFSA GMO Panel evaluated maize MIR162 with reference to the intended uses and appropriate principles described in the guidance documents of the EFSA GMO Panel for the risk assessment of GM plants and derived food and feed (EFSA, 2006, 2011a). The scientific evaluation of the risk assessment included molecular characterisation of the inserted DNA and expression of the corresponding proteins. An evaluation of the comparative analyses of composition, agronomic and phenotypic traits was undertaken, and the safety of the new proteins and of the whole food/feed was evaluated with respect to potential toxicity, allergenicity and nutritional wholesomeness. An evaluation of environmental impacts and the post-market environmental monitoring plan was also undertaken.

The molecular characterisation data establish that the genetically modified maize MIR162 contains one copy of an intact *vip3Aa20* expression cassette and a *pmi* cassette in a single locus. No other parts of the plasmid used for transformation are present in maize MIR162. The results of the bioinformatic analyses of the inserted DNA and the flanking regions do not raise safety concerns. The levels of Vip3Aa20 and PMI proteins in maize MIR162 have been sufficiently analysed and the stability of the genetic modification has been demonstrated.

The EFSA GMO Panel compared the composition and phenotypic and agronomic characteristics of maize MIR162 with those of its conventional counterpart and assessed all statistically significant differences identified. The Panel came to the conclusion that there are no biologically relevant differences in the composition or agronomic or phenotypic characteristics of maize MIR162 compared with its conventional counterpart, and that the composition falls within the range of non-GM commercial varieties, except for the expression of the Vip3Aa20 and PMI proteins. The safety assessment of the newly expressed proteins and the whole crop included an analysis of data from analytical and bioinformatics studies, as well as *in vitro* and *in vivo* studies. The Panel concluded that maize MIR162 is as safe as its conventional counterpart and that the overall allergenicity of the whole plant is not changed. A feeding study with broiler chickens confirmed that grain produced by maize MIR162 is as nutritious as that produced by its conventional counterpart and a non-GM commercial variety. In conclusion, the EFSA GMO Panel is of the opinion that maize MIR162 is as safe and as nutritious as its conventional counterpart and non-GM commercial varieties, and concludes that this maize and derived products are unlikely to have adverse effects on human and animal health, in the context of its intended uses.

The application EFSA-GMO-DE-2010-82 concerns food and feed uses, import and processing. Therefore, there is no requirement for scientific information on possible environmental effects associated with the cultivation of maize MIR162. There are no indications of an increased likelihood of establishment and spread of feral maize plants in case of accidental release into the environment of viable maize MIR162 grains during transport and processing for food and feed uses. Considering its intended use as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with an unlikely but theoretically possible horizontal gene transfer from maize MIR162 to bacteria have not been identified. The scope of the post-market environmental monitoring plan provided by the applicant is in line with the intended uses of maize MIR162. Furthermore, the EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the general surveillance plan.

In conclusion, the EFSA GMO Panel considers that the information available for maize MIR162 addresses scientific issues indicated by the guidance documents of the EFSA GMO Panel and the scientific comments raised by the Member States, and that the maize MIR162, as described in this application, is as safe as its

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

conventional counterpart and non-GM commercial varieties with respect to potential effects on human and animal health and the environment in the context of its intended uses.

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BACKGROUND

On 23 July 2010, the European Food Safety Authority received from the Competent Authority of Germany an application (Reference EFSA-GMO-DE-2010-82) for authorisation of genetically modified (GM) maize MIR162 (Unique Identifier SYN-IR162-4), submitted by Syngenta within the framework of Regulation (EC) No 1829/2003 on GM food and feed. After receiving the application EFSA-GMO-DE-2010-82 and in accordance with Articles 5(2)(b) and 17(2)(b) of the Regulation (EC) No 1829/2003, EFSA informed the Member States and the European Commission, and made the summary of the application publicly available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of the Regulation. On 13 August 2010, EFSA received additional information requested under completeness check (requested on 23 July 2010). On 24 August 2010, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to the Member States and the European Commission, and consulted nominated risk assessment bodies of the Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[5] following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Member States had three months after the date of receipt of the valid application (until 24 November 2010) within which to make their opinion known.

The Scientific Panel on Genetically Modified Organisms of EFSA (EFSA GMO Panel) carried out an evaluation of the scientific risk assessment of the GM maize MIR162 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. The EFSA GMO Panel carried out the safety evaluation in accordance with the appropriate principles described in the EFSA GMO Panel Guidance Documents for the risk assessment of GM plants and derived food and feed (EFSA, 2006, 2011a). In addition, the scientific comments of the Member States, the additional information provided by the applicant, and relevant scientific publications were taken into consideration.

On 24 August 2010 and 21 January 2011 the EFSA GMO Panel requested additional information from the applicant. The applicant provided the requested information on 6 October 2010 and 1 February 2012, respectively.

In giving its scientific opinion on maize MIR162 to the European Commission, the Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of six months from the acknowledgement of the valid application. As additional information was requested by the EFSA GMO Panel, the time limit of six months was extended accordingly, in line with Articles 6(1), 6(2), 18(1), and 18(2) of Regulation (EC) No 1829/2003.

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).

TERMS OF REFERENCE

The EFSA GMO Panel was requested to carry out a scientific assessment of maize MIR162 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environment and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The EFSA GMO Panel was not requested to give a scientific opinion on information required under Annex II to the Cartagena Protocol. Furthermore, the EFSA GMO Panel did also not consider proposals for labelling and

[5] 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. Official Journal of the European Communities, L106, 1–38.

methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to risk management.

ASSESSMENT

1. INTRODUCTION

The genetically modified (GM) maize MIR162 (unique identifier SYN-IR162-4) was evaluated with reference to its intended uses, taking account of the appropriate principles described in the EFSA GMO Panel Guidance Documents for the risk assessment of GM plants and derived food and feed (EFSA, 2006). The evaluation of the risk assessment presented here is based on the information provided in the application relating to maize MIR162 submitted in the EU, as well as scientific comments submitted by the Member States and relevant scientific publications.

2. ISSUES RAISED BY THE MEMBER STATES

The comments raised by the Member States are addressed in Annex G of the EFSA overall opinion[6] and have been considered in this scientific opinion.

3. MOLECULAR CHARACTERISATION

MIR162 is a genetically modified maize which confers protection against a number of significant lepidopteran insect pests feeding as larvae on maize. Protection is provided through the expression of a vegetative insecticidal protein (Vip) derived from the native Vip3Aa1 protein found in *Bacillus thuringiensis* (*Bt*) strain AB88. Vips are insecticidal proteins produced by *Bt* (Estruch et al., 1996).

3.1. Evaluation of relevant scientific data

3.1.1. Transformation process and vector constructs[7]

Maize MIR162 was developed through *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation of immature maize embryos derived from a proprietary maize line using the pNOV1300 plasmid and *A. tumefaciens* strain LBA4404. The region intended for insertion contains two expression cassettes:

i) *vip3Aa19* gene cassette

The *vip3Aa19* gene is a synthetic, maize-optimised, *vip3Aa1* gene derived from *B. thuringiensis* strain AB88. The vip3Aa19 protein differs from the Vip3Aa1 protein by a single amino acid at position 284 (L284Q). The *vip3Aa19* gene is under the control of the maize polyubiquitin promoter and the intron #9 from the maize phosphoenolpyruvate carboxylase gene. The transcription is terminated by the 35S terminator from the *Cauliflower mosaic virus* (CaMV).

ii) *pmi* gene cassette (used as selectable marker)

The *pmi* gene is derived from *Escherichia coli* and encodes phosphomannose isomerase (PMI) enzyme. The gene is under the control of the maize polyubiquitin promoter and the transcription is terminated by the nopaline synthase (NOS) terminator from *A. tumefaciens.* Expression of PMI enables transformed maize cells to utilise mannose and therefore to survive on media in which mannose is the sole source of carbon.

3.1.2. Transgene constructs in the genetically modified plant[8]

Data from Southern analyses demonstrated that maize MIR162 contains a single insert, single copies of the *vip3Aa19* and *pmi* genes, two copies of the maize polyubiquitin promoter (in addition to the endogenous polyubiquitin promoters) corresponding to the two copies of the promoter present in plasmid pNOV1300 used for transformation, one copy of the NOS terminator and none of the backbone sequences from pNOV1300.[9]

[6] http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2010-00972
[7] Technical dossier/sections C and D1.
[8] Technical dossier/section D2.
[9] Additional information, February 2012.

Sequence analysis confirmed that the sequences of functional elements in pNOV1300 are essentially retained within the insert in MIR162. However, compared with the sequence of vip3Aa19 present in the transformation plasmid, there were two single nucleotide changes in the coding sequence of the maize MIR162 insert. The new gene incorporated into maize MIR162 genome was therefore designated vip3Aa20. One of these transformation-induced nucleotide changes resulted in an amino acid change in the encoded protein: methionine at position 129 has been replaced by isoleucine. The other nucleotide change resulted in an altered codon but did not result in an amino acid substitution. The Vip3Aa20 protein thus differs from the Vip3Aa1 protein from *B. thuringiensis* strain AB88 by two amino acids, at positions 129 and 284.

Some truncation occurred at the right and left borders of the T-DNA during the transformation process that resulted in maize MIR162. The entire right border, along with two base pairs of non-coding sequence, was truncated, and the entire left border along with 32 base pairs of non-coding sequence was truncated. These deletions did not affect the functionality of the T-DNA insert.

Updated bioinformatic analysis was performed.[10] Putative *in silico* translation products of all reading frames of the MIR162 T-DNA were evaluated for similarities with toxins and allergens using BLASTX and either FARRP or NCBI Entrez® Protein databases. There were no hits with known toxins. The search for putative allergens showed no alignments with the FARRP allergen database that exceeded the minimum 35 % shared identity over a minimum of 80 amino acids for the BLASTX alignment. There was a match of eight identical amino acids between the sequence encoding PMI and α-parvalbumin from *Rana* species CH2001, which is described in the assessment of the sequence similarity of PMI to known and putative allergens.[11] Another single match of eight identical amino acids between an alternate (putative) reading frame of the MIR162 insert and three putative allergens (Asp f9/16) from *Aspergillus fumigatus* was detected. However, the sequence match does not fall within a known immunologically relevant epitope of the putative allergens. The open reading frame (ORF) sequence is located in an alternative frame compared with the *vip3Aa20* gene product, between two stop codons, and is not downstream of a potential translation start sequence. Expression of this putative peptide in maize MIR162 is extremely unlikely. Western blot analysis of proteins extracted from kernels of MIR162 using polyclonal antibodies raised against a synthetic peptide derived from the sequence of the putative translation product of this ORF did not detect any expression in kernels.

Bioinformatic analysis of the sequences spanning the junctions between the maize genomic sequence and the MIR162 insert identified 12 nucleotide sequences that are contained between stop codons. BLASTP analysis revealed no similarities to known toxins or allergens.

BLASTX analysis of maize genomic sequences flanking the insert resulted in two alignments for the 5′ region. The first was to a hypothetical protein sequence from *Chryseobacterium gleum* and the second to a hypothetical protein sequence from *Harpegnathos*. These sequences have no known function. The region of the 5′ flanking sequence that aligned with these sequences was not immediately adjacent to the maize MIR162 genome to insert junction. The genomic sequence flanking the 3′ region of the maize MIR162 insert did not align to these same sequences. BLASTN analysis of the maize genomic sequence flanking the 5′ region of the MIR162 insert revealed similarity with Dissociation1 (Ds1)-related transposable elements. The region of similarity between the 5′ flanking sequence and the Ds1-related elements is located over 500 bp upstream of the genome to insert junction; thus, the Ds1 element is not disrupted. BLASTN analysis of the maize genomic sequence flanking the 3′ region of the insert revealed similarity to several plant sequences, but these were either un-annotated plant genomic sequences or outside annotated gene coding sequences. Alignments between these sequences and the 3′ flanking sequence started at least 300 bp downstream of the genome–insert junction, and no similarity was observed with the 5′ flanking sequence. Thus, there was no evidence that endogenous maize genes were interrupted by the MIR162 insert.

3.1.3. Information on expression of the insert[12]

The levels of Vip3Aa20 and PMI were determined by enzyme-linked immunosorbent assay (ELISA) in several plant tissues and whole plants at various growth stages in US field trials in 2005 and 2006 and in Brazilian field trials in 2007. Two MIR162 hybrids, designated MIR162-A and MIR162-B, were analysed in 2005. The hybrid

[10] Additional information, February 2012.
[11] Additional information/Appendix 12, February 2012.
[12] Technical dossier/section D3.

MIR162-A was grown in Bloomington, Illinois, and MIR162-B in York, Nebraska. The corresponding near-isogenic, non-transgenic hybrids were used as controls. Given that the scope of the application is for import and for food and feed uses, the EFSA GMO Panel considers protein expression data for grain as the most relevant data, and they are summarised in Table 1.

Table 1. Summary of protein levels in grain produced by maize MIR162 (µg/g dry weight)

Location	Vip3Aa20 mean (range)	PMI mean (range)
2005 Bloomington, Illinois		
Hybrid A	45.72 (41.10–50.53)	2.23 (2.08–2.54)
2005 York, Nebraska		
Hybrid B	41.40 (40.47 – 42.32)	1.62 (1.33–2.38)
2006 Bloomington, Illinois		
Hybrid A	123.8 (54.25 – 165.94)	2.48 (1.08–3.16)
Hybrid B	83.8 (56.41 – 108.27)	1.84 (1.11–2.58)
2007 Brazil		
Ituiutaba location	61.07 (49.5–71.98)	1.18 (1.11–2.58)
Uberlandia location	58.99 (45.86–64.53)	1.43 (1.18–1.70)

Although year-to-year and site-to-site variation is evident, this does not raise a safety issue per se.

3.1.4. Inheritance and stability of inserted DNA[13]

The inheritance pattern of the maize MIR162 event was investigated using individual plants from three backcrosses. Southern analyses confirmed the genetic stability of the insert over several generations. Plants were also assayed for the presence of the *vip3Aa20* and *pmi* genes by real-time polymerase chain reaction (PCR) analysis. Statistical analysis of segregation patterns over three generations of maize MIR162 confirmed the expected Mendelian inheritance ratio for both the *vip3Aa20* and *pmi* genes. The results showed that insertion had taken place in the nuclear genome.

Stability of Vip3Aa20 and PMI protein expression was evaluated over several generations of maize MIR162-derived plants. Plants derived from three backcross generations were grown under greenhouse conditions, and leaves hemizygous for the transgenes were assayed by ELISA. Overall, levels were similar across the three generations analysed and there was no evidence of any significant trend either up or down, indicating the stable expression of the Vip3Aa20 and PMI proteins.

3.2. Conclusion

Appropriate analysis of the insert and integration site, including flanking sequences and bioinformatic analysis, has been performed to characterise the transformation event MIR162, which do not raise safety concerns. The levels of the Vip3Aa20 and PMI proteins have been analysed sufficiently and the stability of the genetic modification has been demonstrated over several generations.

4. COMPARATIVE ANALYSIS

4.1. Evaluation of relevant scientific data

4.1.1. Choice of comparator and production of material for the compositional assessment[14]

For the comparative analysis of the compositional characteristics of forage and grain of maize MIR162, field studies were undertaken in the USA in 2005. Maize MIR162 was grown alongside its conventional counterpart. The field study was carried out at six locations spread over five US states representing the major maize-growing regions there. The field study was performed based on a replicated block design. Both maize MIR162 and its conventional counterpart underwent the same agronomic treatment (e.g. pesticides were applied when needed). Samples of forage and grain were taken at developmental stages that are typical for harvesting these tissues for

[13] Technical dossier/section D5.
[14] Technical dossier/section D7.2 and additional information, October 2010, and additional information, February 2012.

food and/or feed purposes. In the field trial in Brazil (2007), maize MIR162 was grown alongside its conventional counterpart; this trial also included two non-GM reference varieties. Samples of forage and grain were obtained from field trials at two locations in Brazil during one season (2007).

At the EFSA GMO Panel's request, the applicant provided additional data on the composition of maize obtained from field trials carried out during additional growing season. These trials provided data on the composition of forage and grains of maize MIR162, its conventional counterpart and non-GM reference varieties. In the field trial in the USA (2009), maize MIR162 was grown alongside its conventional counterpart; this trial also included, in total, eight non-GM reference varieties. Samples of forage and grain were obtained from field trials at six locations in the USA in 2009[15].

For the analysis of agronomic and phenotypic characteristics, maize MIR162 and its conventional counterpart were grown at various locations in the USA during two seasons (six locations in 2005 and 10 locations in 2006). At these locations, the agronomic treatments consisted of normal practices for weed, pest and disease management.

Data from these locations, taken together, in the EFSA GMO Panel's opinion, are considered acceptable for the comparative analyses of maize MIR162 and its conventional counterpart. The GMO Panel considered the information on the pedigrees of maize MIR162 for the various field trials and found the selection of the corresponding conventional counterpart and non-GM reference varieties appropriate. The Panel was also satisfied with the number of growing seasons and the selection of locations included in the experimental design of the comparative assessment.

4.1.2. Compositional analysis[16]

The compositional parameters that were measured for the comparative analysis of forage and grain of maize MIR162 were in agreement with those recommended by the Consensus Document on key compositional parameters of maize published by the OECD Task Force on the Safety of Novel Food and Feed (OECD, 2002).

In the samples of forage and grain obtained from the field trials in the USA in 2005 and 2009, the parameters that were analysed included proximates (protein, fat, ash, moisture, and carbohydrates by calculation), fibre (acid-detergent fibre, ADF; neutral-detergent fibre, NDF; total dietary fibre, TDF), and minerals (calcium, phosphorus). Grain was additionally analysed for fatty acids, amino acids, additional minerals, (pro-)vitamins (β-carotene, cryptoxanthin, B1, B2, niacin, pyridoxine, folic acid, C, α-, β-, γ-, and δ-tocopherol) and secondary metabolites including antinutrients (ferulic acid, *p*-coumaric acid, furfural, inositol, raffinose, phytic acid, trypsin inhibitor). In 2005 field trials, grain was further analysed for phytosterols (cholesterol, campesterol, stigmasterol, β-sitosterol).

All materials from the Brazilian field trials were analysed for proximates (fat, protein, ash, moisture, carbohydrates by calculation), fibre (ADF, NDF), energy (by calculation), calcium and phosphorus. Forage was additionally analysed for minerals, while grain was also analysed for starch, fatty acids and amino acids.

The outcome of the compositional analysis on forage and grain samples of maize MIR162 and its conventional counterpart that had been grown in the USA in 2005 revealed statistically significant differences across locations for some constituents. Parameters showing differences included NDF in forage, ash, calcium, β-carotene, ferulic acid, iron, linoleic acid, linolenic acid, NDF, *p*-coumaric acid, phosphorus, pyridoxine, β-sitosterol, starch and vitamin E in grain. Differences were generally small and not consistent across locations. Levels observed for MIR162 maize fell within the background range of values obtained from the literature (OECD, 2002), including the ILSI Crop Composition Database.

In the compositional analysis of samples obtained from the Brazilian field trial (2007), maize MIR162 showed statistically significant differences from its conventional counterpart in some compositional parameters, including ADF and phosphorus in forage, and cystine and glycine in kernels. The values of these parameters differed only slightly between MIR162 and its conventional counterpart, and also from the values of the non-GM reference varieties and, therefore, are not considered biologically relevant. Moreover, they fell within the

[15] Additional information, October 2010.
[16] Technical dossier/section D7.1 and additional information, October 2010, and additional information, February 2012.

background ranges of values for the same parameters obtained from the literature (OECD, 2002), including the ILSI Crop Composition Database.

In the compositional analysis of forage obtained from US field trials in 2009, no statistically significant differences were observed in the comparison across locations between maize MIR162 and its conventional counterpart. Comparative analysis of grain revealed one statistically significant difference: between the proportion of linoleic acid in maize MIR162 and its conventional counterpart. As the difference was marginal (57.6 % in MIR162 vs. 57.3 % in its conventional counterpart), it was not considered biologically relevant. For all components, all across-location mean levels were within the ranges determined for eight non-GM reference varieties grown simultaneously at the same locations and were within the ranges reported in the ILSI Crop Composition Database.

Given that PMI is an enzyme involved in carbohydrate metabolism, information on the levels of specific compounds linked to the mode of action of PMI in maize MIR162 and in its conventional counterpart was requested by the EFSA GMO Panel, in order to assess the likelihood of the occurrence of unintended effects. In the additional field trial in 2009, an analysis of monosaccharides and disaccharides, sugar alcohols, and their phosphorylated forms, was carried out on grain derived from maize MIR162 and its conventional counterpart. No statistically significant differences were observed in the levels of fructose, glucose, mannitol, mannose, sucrose, maltose, trehalose, myo-inositol, fructose-6-phosphate, glucose-6-phosphate, mannose-6-phosphate, mannose-1-phosphate, fructose-1,6-diphosphate, sucrose-6-phosphate, trehalose-6-phosphate and various unidentified saccharides.

The EFSA GMO Panel considered the observed compositional differences between forage and grain produced by maize MIR162 and its conventional counterpart in the light of the field trial design, measured biological variation and the level of the studied compounds in non-GM commercial varieties, and concluded that no biological relevant differences were identified in the compositional characteristics of forage and grain produced by maize MIR162 compared with its conventional counterpart, and that its composition falls within the range of non-GM commercial varieties, except for the expression of the VIP3Aa20 and PMI proteins.

4.1.3. Agronomic traits and GM phenotype[17]

The analysis of agronomic and phenotypic characteristics of maize MIR162 compared with its conventional counterpart included a range of parameters related to crop physiology, morphology, development, yield and biotic stress.

The outcomes of these observations revealed some statistically significant differences between maize MIR162 and its conventional counterpart (emergence, test weight and grain moisture) when analysed across all locations. These differences were inconsistent because they did not occur at each location.

As the magnitudes of the differences in agronomic parameters were small, and they fell within the ranges observed for commercial varieties, the GMO Panel found these differences to be of no biological relevance.

4.2. Conclusion

Based on the results of the comparative analysis, the EFSA GMO Panel concludes that there are no biologically relevant differences in the compositional, agronomic or phenotypic characteristics of maize MIR162 compared with its conventional counterpart and that the composition of maize MIR162 falls within the range of commercial varieties, except for the expression of the Vip3Aa20 and PMI proteins.

5. FOOD/FEED SAFETY ASSESSMENT

5.1. Evaluation of relevant scientific data

5.1.1. Product description and intended use[18]

The scope of application EFSA-GMO-DE-2010-82 is for food and feed uses, import and processing of maize MIR162 and all derived products in the EU.

[17] Technical dossier/section D4 and additional information, October 2010.
[18] Technical dossier/section D7.7.

The genetic modification results in the expression of the Vip3Aa20 protein in maize MIR162, which confers on the plants resistance to some lepidopteran species. The PMI protein was used as a selectable marker allowing maize cells to use mannose as a sole carbon source. Thus, the genetic modification in maize MIR162 is intended to improve agronomic performance only and is not intended to alter the nutritional properties, the processing characteristics or overall use of maize as a crop. Maize MIR162 is intended to be processed like any conventional maize. The primary use of maize is for animal feed, but it is also processed into valuable food products such as starch, syrups and oils, etc.

5.1.2. Effect of processing[19]

Maize MIR162 will be used for production and manufacturing of food and feed products in the same way as any other commercial maize variety. As no biological relevant differences were identified in the compositional characteristics of forage and grains produced by maize MIR162 compared with its conventional counterpart, and its composition falls within the range of non-GM commercial varieties, except for the expression of the VIP3Aa20 and PMI proteins (see section 4.2), the effect of processing on maize MIR162 is not expected to be different from the effect on conventional maize.

The influence of temperature on the Vip3Aa20 protein (batch MIR162VIP3A-0106; see section 5.1.3.1) was studied in a bioassay by determining its insecticidal activity on insect larvae after incubation of the enzyme at 4, 25, 37, 65 and 95 °C for 30 minutes. After incubation at 65 ºC for 30 minutes at pH 10.5, no activity was detected.

The incubation of the microbially derived PMI protein (PMI-0105; see section 5.1.3.1) at 25 °C and 37 °C resulted in no loss of enzymatic activity. Incubation at 65 °C resulted in loss of enzymatic activity below the enzymatic assay limit of quantitation, and incubation at 95 °C resulted in no detectable enzymatic activity. The enzymatic activity of the microbially derived PMI protein (PMI-0198; see section 5.1.3.1) was almost completely lost (98 % reduction) after incubation at pH 7.0 and 65 °C for 30 minutes.

5.1.3. Toxicology[20]

5.1.3.1. Protein used for safety assessment

Because of the low levels of newly expressed proteins Vip3Aa20 and PMI in tissues of maize MIR162, analogues of these proteins have been produced in and purified from recombinant *Escherichia coli* bacteria. These microbially produced analogues of Vip3Aa20 and PMI have been used in safety testing of the newly expressed proteins. The two batches of the *Escherichia coli*-produced Vip3Aa20 analogue used in safety testing were designated batch MIR162VIP3A-0106 and batch MIR162VIP3A-0108, while the two PMI analogues were designated PMI-0198 and PMI-0105.

The Vip3Aa20 protein produced by maize MIR162 and its microbially produced analogue present in batch MIR162VIP3A-0106 were compared with each other using various analytical methods, including Western blotting; mass spectrometry of peptides derived from these proteins through degradation by trypsin; and insecticidal bioactivity assay using larvae of *Spodoptera frugiperda*. In addition, Vip3Aa20 from both sources was analysed for glycosylation through glycan staining after gel electrophoresis. The microbially produced protein was also analysed for its N-terminal sequence. The results showed that, in Western blots, the position of the main band, corresponding to the size of intact Vip3Aa20, was identical in the plant-expressed and microbially produced Vip3Aa proteins. Most of the additional bands seen in both plant and microbial preparations probably corresponded to immunoreactive degradation products of Vip3Aa20. The insecticidal bioassays showed that the activities (LC_{50} values) of both preparations were very similar and not statistically significantly different. Peptide mapping through mass spectrometry of the proteins after degradation into peptides by trypsin resulted in the identification of peptides corresponding to the Vip3Aa20 proteins tested, further confirming the identity of these proteins. The N-terminal sequence of the microbially produced analogue of Vip3Aa20 corresponded to the amino acid sequence of the plant-produced protein.

[19] Technical dossier/section D7.6.
[20] Technical dossier/section D7.8 and additional information, February 2012.

The microbially produced analogue present in batch MIR162VIP3A-0108 was characterised with respect to solubility, purity and insecticidal activity. Additionally, the identity was confirmed by examining the apparent molecular weight, immunoreactivity, N-terminal amino acid sequence and total mass of the protein.

The sequence encoding the microbially produced PMI-0198 contained an extension comprising 16 additional amino acid residues at the N-terminus of the PMI protein as a consequence of DNA sequence fusion used for production of the DNA encoding the recombinant protein. These 16 amino acids were encoded by additional DNA sequences derived from a T7 tag sequence (13 residues) and a polylinker sequence (three residues). The amino acid sequence encoding the microbially produced PMI-0105 is the same as that of the native PMI protein encoded by the *E. coli manA* gene (also known as the *pmi* gene), and is the same as that of the PMI protein expressed in maize MIR162.

PMI produced in maize MIR162 was isolated from leaves and compared with the microbially produced PMI-0198 through Western blotting (molecular weight determination and immunoreactivity) and enzymatic activity assay. Both the plant-expressed and microbially produced PMI-0198 showed an immunoreactive band in Western blots corresponding to the expected molecular weight. In addition, the microbially produced PMI-0198 showed a second, faint, band, probably corresponding to a dimer of PMI. The specific enzymatic activities of the plant-expressed and microbially produced PMI-0198 were very similar.

PMI from maize MIR162 leaf material and microbially produced PMI-0105 showed the same mobility in the Western blot analysis, and confirmed an apparent molecular weight consistent with the predicted molecular weight of approximately 42.8 kDa. PMI proteins from both sources immunologically cross-reacted with anti-PMI antibodies, as shown by the Western blot analysis, confirming the identity and integrity of the PMI proteins. In an enzymatic activity assay, both the plant-produced PMI and the microbially produced test substance PMI-0105 were active.

The EFSA GMO Panel accepts the use of the microbially produced Vip3Aa20 and PMI proteins for safety testing.

5.1.3.2. Toxicological assessment of expressed novel proteins in maize MIR162

On request of the EFSA GMO Panel the applicant provided some additional information[21] on the background of the class of proteins, i.e. the vegetative insecticidal protein (Vip), to which the Vip3Aa20 protein belongs. Vips occur widely in isolates of *B. thuringiensis*. They are known to act in target insects via receptors that are different from those to which Cry proteins bind, and to cause pore formation in insect intestinal cells. Vips contain a carbohydrate-binding domain and an active C-terminal, trypsin-resistant, part of molecular weight 62 kDa.

Also provided by the applicant, at the EFSA GMO Panel's request, were data on the occurrence of Vips in commercial preparations of *B. thuringiensis*-based biopesticides, as well as data on the volumes of such biopesticides being used in agriculture and horticulture. The data showed that the tested biopesticides indeed contained Vip3A proteins and that substantial amounts of these biopesticides are sold and used in European agriculture. One biopesticide contained an immunoreactive protein with a molecular weight equal to that of Vip3Aa20 as present in maize MIR162. This leads the EFSA GMO Panel to conclude that the data indicate that humans and animals, including consumers and agricultural workers, may already have been exposed to low levels of Vips.

PMI enzymes have been purified from many organisms, including bacteria, yeast, rats, pigs and humans (Proudfoot et al., 1994), and have been demonstrated to be essential for many organisms, including *E. coli* (Markovitz et al., 1967) and fungi (Proudfoot et al., 1994). PMI activity is present in many mammalian tissues, including skeletal muscle, brain, heart, liver, spleen, lung and placenta (Freeze, 2002).

PMI catalyses the conversion of mannose-6-phosphate to fructose-6-phosphate and vice versa, and these two compounds are the only known substrates of PMI enzymes (Freeze, 2002). PMI as contained in test substance PMI-0105 shows a broad pH dependence curve within the pH range tested, with the optimum activity observed at pH 7.5. Substrate specificity of PMI has been further confirmed by a study performed by the applicant, in which various structurally similar saccharides were incubated with the microbially produced PMI-0105.

[21] Additional information, February 2012

Although PMI catalysed the interconversion between fructose-6-phosphate and mannose-6-phosphate at pH 7.5, no reaction occurred when other sugars or sugar phosphates were added as substrates.

A) Acute toxicity testing[22]

The microbially produced proteins Vip3Aa20 (MIR162VIP3A-0106) and PMI (PMI-0198) did not induce adverse effects in acute oral toxicity studies using mice after administration of a single dose of 1 250 mg Vip3Aa20/kg body weight (bw) and 3030 mg PMI/kg bw, respectively.

The EFSA GMO Panel considers that acute toxicity testing of the newly expressed proteins is of little additional value for the risk assessment of the repeated human and animal consumption of food and feed derived from GM plants.

B) Repeated dose oral toxicity study[23]

Considering the available information on occurrence and the additional studies as described in this section, the EFSA GMO Panel did not require a 28-day oral toxicity study with the newly expressed PMI protein.

The applicant provided a study published in literature which describes a 28-day oral toxicity study in rats with a bacterial preparation containing a Vip that shows 99.7 % homology to the Vip3Aa20 protein present in MIR162 maize (Peng et al., 2007). In this 28-day repeat dose oral toxicity study, using five male and five female animals per group, doses of 0, 200, 1 000 and 5 000 mg/kg bw were administered. Administration of the highest dose of 5 000 mg/kg bw did not result in any deaths or any statistically significant effects on body weight, food or water consumption, haematological or serum chemistry parameters, organ weights or histopathological findings that could be attributed to treatment with the test material. Peng et al. (2007) concluded, based on their study, that there are no toxic effects resulting from repeated dose exposure.

With respect to this study performed by Peng et al. (2007), the EFSA GMO Panel noted that (1) the study was performed with a bacterial preparation (BMB696B) containing a Vip3Aa protein that was not identical to the one expressed in maize MlR162; (2) the concentration of the Vip3Aa protein in the bacterial preparation used for the 28-day toxicity study was unknown; and (3) no comprehensive histopathological examination as recommended in OECD Guideline 407 including tissues of the intestinal tracts of the tested animals was performed. Therefore, the EFSA GMO Panel requested a 28-day repeated dose toxicity study in rodents using purified Vip3Aa20 protein that was identical to the one expressed in maize MIR162.

The applicant provided a repeated-dose oral toxicity study with the Vip3Aa20 protein (MIR162VIP3A-0108 batch) conducted in accordance with the OECD guideline 407. Groups of five male and five female Han Wistar Crl: WI(Han) rats received the test material by oral gavage at dose levels equating to 5, 50 or 500 mg/kg bw/day Vip3Aa20 protein daily over a period of 28 consecutive days. Two control groups were used: one received the vehicle alone (water for injection), while the other received bovine serum albumin (BSA) at the highest Vip3Aa20 protein dose, i.e. 500 mg/kg bw/day repeatedly for 28 days. The animals were observed regularly for clinical signs, and body weight and food consumption were recorded. At the end of the treatment period, haematology and clinical chemistry analyses were performed. All animals were sacrificed after completion of 28 days of treatment and underwent a detailed necropsy examination with selected organs weighed. Tissues from all animals in the high-dose group and from both control groups were subjected to a comprehensive histological examination. Data for the test groups were compared with those of the control groups and, where relevant, to historical control data.

The daily oral gavage administration of Vip3Aa20 to rats for 28 consecutive days was well tolerated. No statistically significant treatment-related differences in animals' food and water consumption were observed among the various test groups. No differences were observed in mean body weights or body weight gain, except during the day 4–7 period in females receiving 50 or 500 mg Vip3Aa20/kg bw/day, in which mean body weight gain was statistically significantly lower than in the vehicle-treated control group. The difference in the mid-dose group, which also showed a lower total body weight gain at the end of the treatment period (not significant), was accompanied by a somewhat lower food consumption during days 4–7 and at later time

[22] Technical dossier/section 7.8/Appendices 24 and 25
[23] Technical dossier/section 7.8/Appendix 36 and additional information, February 2012.

intervals. In the high-dose group, total body weight gain did not differ significantly from that of the other groups; therefore, the difference observed in females during days 4–7 was considered incidental and not of biological relevance.

Absolute thymus weights were statistically significantly lower in the female group administered 50 mg Vip3Aa20/kg bw/day than in the vehicle-treated control group, and relative thymus weights were also lower (not statistically analysed). However, thymus weights were not different from those of the control group given BSA protein. Furthermore, no dose–response relationship was observed, individual animal data for thymus weights were within historical control range, and there were no histopathological findings in this organ. Thus, the observed differences were not considered biologically relevant.

Absolute liver weights were statistically significantly higher in the female group that received 500 mg Vip3Aa20/kg bw/day and also in the BSA control group (relative weights were also slightly higher in these groups). However, the observed values fell within the range of historical control data. In addition, there were no changes in parameters indicative of liver toxicity (e.g. alkaline aminotransferase or aspartate aminotransferase activity) and no histopathological changes in this organ. Therefore, the higher liver weights in female animals were not considered toxicologically relevant.

No effects were observed on the clinical condition of the test animals (including neurotoxicity parameters), on ophthalmoscopic, haematological or blood chemistry parameters or on macroscopic and microscopic pathology parameters, including the gastrointestinal tract at dose levels up to and including 500 mg Vip3Aa20/kg bw/day. The EFSA GMO Panel concludes that there were no indications of adverse effects up to the highest dose tested.

The Panel accepts the results of the study provided by the applicant since (i) no adverse effects were observed at the highest dose tested, (ii) the Vip3Aa20 protein is rapidly degraded under *in vitro* conditions, (iii) no sequence homology has been observed between Vip3Aa20 protein and known toxins and (iv) the highest dose tested is much higher than the calculated daily consumption by humans of the Vip3Aa20 protein present in maize MIR162.

C) Degradation in simulated digestive fluids[24]

The resistance to degradation of the newly expressed Vip3Aa20 and PMI proteins in maize MIR162 and of the microbially produced enzymes was tested *in vitro* by exposing the proteins to protein-degrading enzymes. During incubations of the plant-expressed Vip3Aa20 protein and its microbially produced analogue (MIR162VIP3A-0106) with pepsin at pH 1.2, the protein was rapidly degraded (within 1 minute), while a minor band at 8 kDa was still visible following Western blotting after 60 minutes of incubation (last sampling time). An additional study was performed at pH 1.2, 2.0, 2.5 and 3.5 to determine the effect of pH on the digestibility of Vip3Aa20, and particularly on the 8-kDa fragment. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were used in this essay to evaluate the *in vitro* digestibility of Vip3Aa20 over a 60-minute time course at 37 °C. The study confirmed the results of the previous *in vitro* digestibility study of Vip3Aa20 in simulated gastric fluid (SGF) at pH 1.2. The data for the various pH values suggest that the 8-kDa Vip3Aa20-derived fragment is more readily degraded by pepsin at pH 2.5 than at pH 1.2 or 2.0. No degradation of Vip3Aa20 in SGF was observed at pH 3.5, which may be due to decreased activity of pepsin at this pH.

On request of the Panel, the applicant performed quantification and identification of the 8-kDa band from the *in vitro* digestion of Vip3Aa20 protein under simulated mammalian gastric conditions. The 8-kDa band remaining after digestion of Vip3Aa20 was between 0.63 % and 0.31 % of the initial amount of Vip3Aa20 in the SGF digestibility assay, with the highest proportion observed at pH 1.2. The N-terminal sequencing analysis confirmed that the 8-kDa band is a degradation fragment of Vip3Aa20.

When the microbially produced analogue (MIR162VIP3A-0106) was incubated in solutions containing pancreatin, at pH 7.5, the main band corresponding to the intact protein (approximately 89 kDa) rapidly disappeared (within 5 minutes), whereas minor, indistinct bands, corresponding to degradation products with sizes of approximately 55 and 62 kDa, remained visible in Western blots in samples that had been taken until 48 hours after the start of incubation (i.e. at the last sampling time).

[24] Technical dossier/section 7.9, and additional information, October 2010.

The PMI-0198 protein was rapidly degraded in SGF. The PMI protein was also shown to be rapidly digested in simulated mammalian intestinal fluid.

D) Potential binding of Vip3Aa20 to mammalian intestinal epithelial cells[25]

Considering the mode of action of the Vips in insects, the EFSA GMO Panel requested the applicant to deliver any available information on the potential binding of Vip3A proteins to, and/or effects of Vip3A proteins on, mammalian intestinal epithelial cells. The potential effect on cell membrane disruption was assessed using an *in vitro* culture of the human epithelial colorectal adenocarcinoma cell line Caco-2. After exposure to Vip3Aa20, two cytotoxicity/cell viability assays (lactate dehydrogenase (LDH) release and Neutral Red (NR) uptake) were used to determine if the Vip3Aa20 protein had an effect on the cell membrane of the Caco-2 cells compared with a negative control (cell culture medium only). The LDH and NR assay results demonstrate that Vip3Aa20 is not cytotoxic to Caco-2 cells at the concentrations, up to 50 µg/mL, tested in this study. The concentrations tested during the *in vitro* cytotoxicity studies were within the range of the maximum exposure for humans consuming maize MIR162 directly. The EFSA GMO Panel did not find this *in vitro* study of great relevance with regard to potential binding of Vip3A proteins to mammalian intestinal cells.

E) Bioinformatic studies[26]

With regard to the safety of the newly expressed proteins Vip3Aa20 and PMI in maize MIR162, bioinformatics-supported comparisons of the sequences of these proteins with the sequences of known toxic proteins in a general protein database (NCBI Entrez Protein Database) were performed. During the course of the evaluation, the applicant provided updated bioinformatic studies for these newly expressed proteins present in maize MIR162. No relevant similarities between PMI or Vip3Aa20 and known toxins were identified apart from the similarity of Vip3Aa20 to other vegetative insecticidal proteins (Vip) from *Bacillus thuringiensis.*

5.1.3.3. Toxicological assessment of new constituents other than proteins

No new constituents other than Vip3Aa20 and PMI are expressed in maize MIR162. No biologically relevant changes in the composition of maize MIR162 were detected (see section 4.1.3). Therefore, a toxicological assessment of new constituents is not applicable.

5.1.3.4. Toxicological assessment of the whole GM food/feed[27]

A 90-day rat feeding study of grain from maize MIR162 was carried out in rats of a Wistar-derived strain (Alpk:APfSD). There were four groups of rats, each consisting of 12 animals of either gender, two of which received diets containing grain from maize MIR162 at 10 % or 41.5 % (w/w) inclusion rates, while the other two groups received diets containing grain from the conventional counterpart at the same inclusion levels. During the experimental period, animals were checked daily for clinical signs. Food consumption and body weight were recorded weekly, and functional capability and motor activity tests were carried out at the end of the treatment period. Clinical pathology measurements at study termination included haematology, serum chemistry, organ weight determinations and macroscopic and microscopic examinations.

All animals survived the treatment period. There were no relevant differences between the test and control groups with regard to clinical signs, ophthalmoscopic findings, parameters of a functional observation battery (FOB) and motor activity. Body weights, total body weight gain, food consumption and food utilisation were similar in all groups. Regarding haematology and clinical chemistry examinations as well as organ weight determinations, there were a number of statistically significant differences compared with the controls, some of which occurred in the group fed diets a diet containing 10 % maize MIR162 (compared with the 10 % control group) and not in the groups fed a diet containing 41.5 % maize MIR162, i.e. a higher red blood cell count higher plasma alkaline phosphatase activity and lower relative kidney weights in males and a lower plasma glucose level in females. The EFSA GMO Panel considers that these differences, which occurred only between the 10 % groups, were not dose related, and therefore not related to the administration of maize MIR162. Differences that occurred in the groups fed 41.5 % GM maize compared with the controls included lower basophil counts in the serum of females (also observed in the 10 % group; however, some control animals

[25] Additional information, October 2010.
[26] Additional information October 2010.
[27] Technical dossier/section 7.8 and additional information, February 2012.

showed very high counts; most of the ranges of control and test groups overlapped); and increased activated partial thromboplastin time in males, which were still within the historical ranges. Each of these findings was limited to one sex. Furthermore, the differences were small and not accompanied by changes in related haematology and coagulation parameters. Therefore, the EFSA GMO Panel does not consider the observed statistically significant differences as toxicologically relevant. Macroscopic and microscopic examinations of selected organs and tissues did not reveal relevant differences in findings between the test and control groups.

In conclusion, there were no indications of adverse effects after administration of grain from maize MIR162 to rats for 90 days.

5.1.4. Allergenicity[28]

The strategies used when assessing potential allergenic risk focus on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons and whether the transformation may have altered the allergenic properties of the modified food. A weight-of-evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence of allergenicity (Codex Alimentarius, 2009; EFSA, 2006, 2010).

5.1.4.1. Assessment of allergenicity of the newly expressed proteins

The Vip3Aa20 and PMI proteins originate from sources that have no documented history of allergenicity. In addition, these proteins are rapidly degraded in simulated gastric fluid (see section 5.1.3.2C).

When the criterion of an identical contiguous stretch of eight amino acids was applied in bioinformatic-supported studies using databases of known allergens, the Vip3Aa20 sequence yielded no positive outcomes, whereas one identical stretch of eight amino acids was observed to occur in both PMI and a frog leg allergen. However, immunoblotting demonstrated no cross-reaction between the PMI protein and IgE serum from a human subject known to be allergic to frog legs. This serum was taken from the same subject reported in the scientific literature to react to the frog allergen in question (Hilger et al., 2002). A positive control with the frog leg allergen reacted positively.

Another bioinformatic analysis was carried out on possible similarities of the sequences of the plant-expressed Vip3Aa20 and PMI proteins to known allergens. Codex Alimentarius (CAC, 2003), as referenced by the EFSA Guidance Document, recommends considering potential IgE cross-reactivity if there is more than 35 % identity in a segment of 80 or more amino acids (EFSA, 2006). No peptides having 35 % identity in an 80-amino-acid window to an allergen sequence of similar size were identified for Vip3Aa20 and PMI as expressed in maize MIR162.

The EFSA GMO Panel also noted that PMI derived from the *manA* gene in *E. coli* is a member of the superfamily of "cupins", which are proteins with a specific three-dimensional structure. Some members of this superfamily are known allergens (Dunwell et al., 2001; Breiteneder and Radauer, 2004; Mills et al., 2004). The EFSA GMO Panel noted that bioinformatic analysis did not reveal any relevant sequence homology between the PMI expressed in maize MIR162 and known allergens of the cupin superfamily (see above). At the EFSA GMO Panel's request, the applicant provided a risk assessment of the potential allergenicity, including the capacity for sensitisation, of the newly expressed PMI protein, being a member of the cupin superfamily. This included the construction of a three-dimensional, spatial structure of the newly expressed PMI protein using a computer algorithm, and a comparison of this spatial structure with that of the cupin allergen Ara h 1, which naturally occurs in peanut. A comparison of these spatial structures showed that, whilst the proteins share a core with the typical barrel structure inherent to cupins, the remainder of the structures was different. For example, various parts of the Ara h 1 protein that are known to act as IgE-binding epitopes do not have corresponding counterparts in the spatial structure of the newly expressed PMI.

The EFSA GMO Panel concludes that it is unlikely that these newly expressed proteins are allergenic.

[28] Technical dossier/section D7.9.

5.1.4.2. Assessment of allergenicity of the whole GM plant

The issue of a potentially increased allergenicity of maize MIR162 compared with conventional maize varieties does not appear relevant to the EFSA GMO Panel, since maize is not considered a common allergenic food. However, rare cases of occupational allergy to maize dust have been reported in the scientific literature (Jeebhay and Quirce, 2007; Bardana, 2008). The EFSA GMO Panel is also aware that a few cases of food allergy to maize have been specifically observed in some geographically restricted areas where maize is a common food and that, in the few cases reported, the major maize allergens have been identified. In the context of the present application, the EFSA GMO Panel considers it unlikely that the newly expressed proteins would alter the pattern of expression of endogenous proteins/potential allergens and thereby significantly change the overall allergenicity of the whole plant. In addition, given all the available information, the EFSA GMO Panel sees no reason to expect that the use of maize MIR162 would significantly increase the intake and exposure to maize.

5.1.5. Nutritional assessment of GM food/feed[29]

The applicant provided a 44-day broiler chicken feeding study to test the nutritional properties of maize MIR162. A total of 540 chicks (Ross 344 males crossed with feather-sexable Ross 508 females) were allocated to three groups of 180, 90 animals of each gender, subdivided into six pens per sex with 15 chicks per pen (initial body weight 44 g/animal). Three lots of maize grain were used to prepare broiler diets: (1) GM maize MIR162; (2) the conventional counterpart; and (3) non-GM commercial variety NC 2006. Starter (up to day 17; 50.1–51.6 % maize), grower (up to day 32; 56.6–58.3 % maize) and finisher diets (62.2–64.1 % maize; up to slaughtering) were formulated and adjusted for metabolisable energy and amino acids. Maize and broiler diets were analysed for nutrients and mycotoxins. During the experimental period, the animals were checked for survival, feed intake, body weight and feed conversion rate (FCR). At 45 days, a random sample of two birds from each pen (altogether 72 broilers) was slaughtered in order to determine carcass yield. There were no statistically significant differences ($P > 0.05$) between treatments in terms of mortality of broilers. The cumulative mortality of the faster-growing males amounted to 3.7 % and of the females to 1.5 %. The average final weight of the males was 3 048 g, and of the females 2 477 g. Maize sources did not significantly influence weight gain, FCR (cumulative: 1.63 kg/kg for males and 1.73 kg/kg for females) or slaughtering results. No other statistically significant changes were noted in the comparison of broilers fed adjusted diets containing maize MIR162, its conventional counterpart or the non-GM commercial variety NC 2006.

In conclusion, the broiler feeding study identified no relevant difference in broiler performance, carcass yield or meat composition between chickens fed diets containing grains produced from maize MIR162 or the conventional counterpart or non-GM commercial varieties. The data confirm the results of the comparative compositional analysis, which identified no biologically relevant differences in the compositional, agronomic or phenotypic characteristics of maize MIR162 compared with its conventional counterpart, and that showed its composition falls within the range of non-GM commercial varieties and, therefore, implicitly it is as nutritious as the conventional counterpart and a non-GM commercial variety.

5.1.6. Post-market monitoring of GM food/feed

No biologically relevant compositional, agronomic and phenotypic changes were identified in maize MIR162 when compared with its conventional counterpart, and its composition fell within the range of non-GM commercial varieties, except for the expression of the VIP3Aa20 and PMI proteins. Furthermore, the overall intake or exposure is not expected to change because of the introduction of maize MIR162 into the market. The EFSA GMO Panel therefore considers maize MIR162 to be as safe as its conventional counterpart and non-GM commercial varieties and that post-market monitoring (EFSA, 2006, 2011a) of the food/feed derived from maize MIR162 is not necessary.

5.2. Conclusion

The proteins Vip3Aa20 and PMI expressed in maize MIR162 have been assessed in this application and no safety concerns were identified for human and animals. The Vip3Aa20 and PMI proteins are degraded in simulated digestive and intestinal fluids, and bioinformatics-supported studies demonstrated that these proteins show no homology to known toxic and allergenic proteins. No toxicity of the Vip3Aa20 protein was observed in a repeated dose 28-day toxicity study in rats in which the protein was administered orally at a high dose. A subchronic (90-day) feeding study found no indications of adverse effects in rats fed diets containing grains

[29] Technical dossier/section D7.10.

from maize MIR162. The EFSA GMO Panel considers it unlikely that the overall allergenicity of maize MIR162 has been altered. Grain produced by maize MIR162 was tested in a broiler chicken feeding study, which shows that this maize is as nutritious as its conventional counterpart and one non-GM commercial variety. In conclusion, the EFSA GMO Panel is of the opinion that maize MIR162 is as safe and as nutritious as its conventional counterpart and a non-GM commercial variety, and concludes that this maize and derived products are unlikely to have adverse effects on human and animal health, in the context of its intended uses.

6. ENVIRONMENTAL RISK ASSESSMENT AND MONITORING PLAN

6.1. Evaluation of relevant scientific data

The scope of this application, EFSA-GMO-DE-2010-82, is for food and feed uses, import and processing and does not include cultivation. Considering the intended uses of maize MIR162, the environmental risk assessment is concerned with the exposure through manure and faeces from animals fed grains (F_2 generation) from maize MIR162 and with the accidental release into the environment of viable grains of maize MIR162 during transportation and processing.

Maize MIR162 has been developed for protection against specific lepidopteran pests (e.g. *Heliothis zea* (corn earworm), *Spodoptera frugiperda* (fall armyworm), *Agrotis ipsilon* (black cutworm), *Striacosta albicosta* (western bean cutworm) and other pests of the family Noctuidae). The insect resistance is achieved by expression of the modified insecticidal VIP3Aa20 protein from *Bacillus thuringiensis*. In addition, maize MIR162 was engineered with the *pmi* (*manA*) gene from *E. coli*, which encodes the enzyme PMI (phosphomannose isomerase) as a selectable marker. Expression of PMI enables transformed maize cells to utilise mannose and therefore to survive on media in which mannose is the sole source of carbon (see section 3).

6.1.1. Environmental risk assessment

6.1.1.1. Potential unintended effects on plant fitness due to the genetic modification

Maize is highly domesticated and generally unable to survive in the environment without management intervention. Maize plants are not winter hardy in many regions of Europe; furthermore, they have lost their ability to release seeds from the cob and they do not occur outside cultivated land or disturbed habitats in agricultural landscapes of Europe, despite cultivation for many years. In cultivation, maize volunteers may arise under some environmental conditions (mild winters). Observations made on cobs, cob fragments or isolated grains shed in the field during harvesting indicate that grains may survive and overwinter in some regions, resulting in volunteers in subsequent crops. The occurrence of maize volunteers has been reported in Spain and other European regions (e.g. Gruber et al., 2008). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009).

Field trials carried out by the applicant found no indications of an altered fitness of maize MIR162 relative to its conventional counterpart. A series of field trials with maize MIR162 were carried out across 16 field site locations in the USA over two consecutive years: six locations in 2005 and 10 locations in 2006. Information on phenotypic (e.g. crop physiology, morphology, development) and agronomic (e.g. grain yield) characteristics was provided to assess the agronomic performance of maize MIR162 in comparison with its conventional counterpart (for further details, see section 4.1.2). Although these field trial data show some statistically significant differences in some parameters (i.e. number of emerged plants recorded 14 days after planting, grain test weight and percentage grain moisture), the EFSA GMO Panel recognises that these differences, which are small in magnitude, are not always present, and thus the Panel is of the opinion that they do not raise any environmental safety concern.

The data presented in the application do not indicate an increased weed potential of maize MIR162 compared with conventional maize, and it can be considered that the survival, multiplication and dissemination characteristics of maize MIR162 are not altered compared with its conventional counterpart.

In addition to the data presented by the applicant, the EFSA GMO Panel is not aware of any scientific report of increased spread and establishment of maize MIR162 or maize with comparable properties or of any change in survival capacity, including overwintering. In addition, the ability to utilise mannose can be regarded as selective advantage only where and when mannose is available as a carbon source, which is not the case in soils.

Survival of maize plants outside cultivation or other areas is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions. Since these general characteristics are unchanged in maize MIR162, the EFSA GMO Panel is of the opinion that the likelihood of unintended environmental effects due to the accidental release into the environment of viable grains from maize MIR162 will not differ from that of conventional maize varieties.

6.1.1.2. Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either horizontal gene transfer of DNA or vertical gene transfer via seed spillage followed by cross-pollination.

(A) Plant-to-bacteria gene transfer

Genomic plant DNA is a component of several food and feed products derived from maize. It is well documented that DNA present in food and feed becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, a low level of exposure of fragments of ingested DNA, including the recombinant fraction of such DNA, to bacteria in the digestive tract of humans, domesticated animals and other environments exposed to the GM plant or plant material is expected.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally located DNA fragments between unrelated organisms (such as plants to bacteria) is not likely occur at detectable frequencies under natural conditions (see EFSA, 2009, for further details).

A successful horizontal transfer would require stable insertion of the transgene sequences into a bacterial genome and a selective advantage to be conferred on the transformed host. The only mechanism known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes is homologous recombination. This requires the presence of stretches of DNA sequences that are similar in the recombining DNA molecules and, in addition to substitutive gene replacement, facilitates the insertion of non-homologous DNA sequences if their flanking regions share sequence similarity with bacterial sequences in the recipient.

Maize MIR162 was developed through *Agrobacterium tumefaciens*-mediated transformation and contains genetic elements with identity or high similarity to those of bacteria. These are the modified *vip3Aa20* gene, a synthetic gene which is highly similar to corresponding gene *vip3Aa1* from *Bacillus thuringiensis*, and the *pmi* gene derived from *E. coli*. The flanking regions of the recombinant gene insert do not contain sequences of the right or left border of the Ti-plasmid of *Agrobacterium tumefaciens*.

Whereas natural strains of *E. coli* are typical inhabitants of the gastrointestinal tract of humans and animals, *B. thuringiensis* is not considered to be prevalent in these main receiving environments considering the intended use of maize MIR162 as food and feed. Natural strains of *B. thuringiensis*, among them those producing vegetative insecticidal proteins (VIPs), e.g. VIP3A, may occur in soil, and can frequently be isolated from the guts of insects (Jensen et al., 2003). It is to be expected that natural strains of *E. coli* or other gut bacteria with homologous genes encoding for PMI will come into contact with recombinant genes of maize MIR162 when used as food and feed. Furthermore, it cannot be ruled out that, outside this main route of exposure, recombinant DNA of maize MIR162 may also come in contact with *B. thuringiensis* harbouring natural genes encoding for insecticidal VIPs, e.g. as a result of insects in soil coming into contact with faecal material. Therefore, various routes of exposure have been considered here.

On a theoretical basis (i.e. in the absence of experimental evidence of horizontal gene transfer in GM food and feed derived from maize MIR162 or any other GM plant), it can be assumed that, as an extremely rare event, homologous recombination may occur in the environment between the recombinant *vip3Aa20* and *pmi* genes and their natural variants as they may occur in *B. thuringiensis* and *E. coli*, respectively. Such recombination events would only replace natural variants (substitutive recombination) and are therefore unlikely to provide any new property connected to a selective advantage for the recipient organisms (EFSA, 2009).

In addition to homology-based recombination processes, illegitimate recombination that does not require similarity between the recombining DNA molecules is theoretically possible. However, transformation rates for illegitimate recombination are considered to be 10^{10}-fold lower than for homologous recombination (Hülter and Wackernagel, 2008; EFSA, 2009). Illegitimate recombination events have not been detected in studies that have exposed bacteria to high concentrations of GM-plant DNA (EFSA, 2009). Thus, this process, compared with

homologous recombination, is considered not to contribute significantly to horizontal gene transfer events. In comparison with the above-described homology-facilitated recombination processes, the contribution of illegitimate recombination is extremely low.

Both protein-encoding genes from bacteria are regulated in maize MIR162 by the eukaryotic promoter of the maize polyubiquitin (see section 3). The expression of eukaryotic promoters in bacteria is generally inefficient (Warren et al., 2008).

Owing to the natural occurrence of *vip3A* and *pmi* genes in the environment, a low-level gene transfer to *B. thuringiensis* (for *vip3Aa20*) and to *E. coli* (for *pmi*) is thought not to confer a new trait and selective advantage. Considering its intended use as food and feed and the above assessment, the EFSA GMO Panel has therefore not identified a concern associated with a horizontal gene transfer from maize MIR162 to bacteria.

(B) Plant-to-plant gene transfer

Considering the intended uses of maize MIR162 and the physical characteristics of maize seeds, possible pathways of gene dispersal are grain spillage and the dispersal of pollen from occasional feral GM maize plants originating from accidental grain spillage during transportation and/or processing.

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

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

Although GM maize plants outside cropped area have been reported in Korea, as a result of grain spillage during import, transportation, storage, handling and processing (Kim et al., 2006; Lee et al., 2009; Park et al., 2010), survival of maize plants outside cultivation in Europe is mainly limited by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and frost. As for any other maize varieties, GM maize plants would only survive in subsequent seasons in warmer regions of Europe and are not likely to establish feral populations under European environmental conditions.

The EFSA GMO Panel takes into account that this application does not include cultivation of maize MIR162 within the EU so that the likelihood of cross-pollination between cultivated maize and the occasional feral maize plants resulting from grain spillage is considered extremely low. In conclusion, as maize MIR162 has no altered survival, multiplication or dissemination characteristics, the EFSA GMO Panel is of the opinion that the likelihood of unintended environmental effects as a consequence of spread of genes from this GM maize in Europe will not differ from that of conventional maize varieties.

6.1.1.3. Potential interactions of the GM plant with target organisms

Owing to the intended uses of maize MIR162, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with target organisms were not considered an issue by the EFSA GMO Panel.

6.1.1.4. Potential interactions of the GM plant with non-target organisms

Owing to the intended uses of maize MIR162, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with non-target organisms were not considered an issue by the EFSA GMO Panel.

6.1.1.5. Potential interactions with the abiotic environment and biochemical cycles

Owing to the intended uses of maize MIR162, which exclude cultivation, and the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles were not considered an issue by the EFSA GMO Panel.

6.1.2. Post-market environmental monitoring

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

Monitoring is related to risk management, and thus a final adoption of the monitoring plan falls outside the mandate of EFSA. However, the EFSA GMO Panel gives its opinion on the scientific content of the monitoring plan provided by the applicant (EFSA, 2011b). The potential exposure to the environment of maize MIR162 would be through manure and faeces from animals fed maize MIR162 grains and/or through accidental release into the environment of GM maize grains (e.g. during transportation and processing). The scope of the monitoring plan provided by the applicant is in line with the intended uses. As the environmental risk assessment did not identify potential adverse environmental effects due to maize MIR162, no case-specific monitoring is required.

The general surveillance plan proposed by the applicant 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 established by EuropaBio for the collection of the information recorded by the various operators; and (3) the use of networks of existing surveillance systems (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a general surveillance report on an annual basis.

The EFSA GMO Panel is of the opinion that the scope of the monitoring plan proposed by the applicant is in line with the intended uses of maize MIR162 as the environmental risk assessment did not cover cultivation and identified no potential adverse environmental effects. The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the general surveillance plan.

6.2. Conclusion

The scope of the application includes food and feed uses, import and processing of maize MIR162 and excludes cultivation. Considering the intended uses of maize MIR162, the environmental risk assessment is concerned with indirect exposure, mainly through manure and faeces from animals fed grains from maize MIR162, and with the accidental release into the environment of viable maize MIR162 grains (e.g. during transportation and processing).

In the case of accidental release into the environment of viable seeds of maize MIR162, there are no indications of an increased likelihood of spread and establishment of feral maize MIR162 plants. Considering its intended use as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with an unlikely but theoretically possible horizontal gene transfer from maize MIR162 to bacteria have not been identified. The scope of the post-market environmental monitoring plan provided by the applicant and the reporting intervals are in line with the intended uses of maize MIR162 and the guidance document of the EFSA GMO Panel on post-market environmental monitoring of GM plants (EFSA, 2011). The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the general surveillance plan.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The EFSA GMO Panel was requested to carry out an evaluation of a scientific risk assessment for food and feed uses, import and processing in accordance with Regulation (EC) No 1829/2003 of maize MIR162. The EFSA GMO Panel evaluated the risk assessment of maize MIR162 performed by the applicant. The evaluation of the risk assessment presented here is based on the information provided in the application relating to maize MIR162 submitted in the EU, including additional information provided by the applicant, scientific comments raised by the Member States and relevant scientific publications.

The EFSA GMO Panel is of the opinion that the molecular characterisation data provided for maize MIR162 are sufficient. The results of the bioinformatic analyses of the inserted DNA and the flanking regions do not raise safety concern. The levels of Vip3Aa20 and PMI proteins in maize MIR162 have been sufficiently analysed and the stability of the genetic modification has been demonstrated.

The EFSA GMO Panel compared the composition and phenotypic and agronomic characteristics of maize MIR162 with those of its conventional counterpart and assessed all statistically significant differences identified. The Panel came to the conclusion that there are no biologically relevant differences in the composition or agronomic or phenotypic characteristics of maize MIR162 compared with its conventional counterpart, and its composition falls within the range of non-GM commercial varieties, except for the expression of the Vip3Aa20 and PMI proteins. The safety assessment of the newly expressed proteins and the whole crop included an analysis of data from analytical and bioinformatics studies, as well as *in vitro* and *in vivo* studies. The Panel concluded that maize MIR162 is as safe as its conventional counterpart and that the overall allergenicity of the whole plant is not changed. A feeding study with broiler chickens confirmed that grain produced by maize MIR162 is as nutritious as that produced by its conventional counterpart and a non-GM commercial variety. In conclusion, the EFSA GMO Panel is of the opinion that maize MIR162 is as safe and as nutritious as its conventional counterpart and a non-GM commercial variety, and concludes that this maize and derived products are unlikely to have adverse effects on human and animal health, in the context of its intended uses.

Considering the intended uses of maize MIR162, which exclude cultivation, there is no requirement for a scientific assessment of possible environmental effects associated with the cultivation of this GM maize. In the case of accidental release into the environment of viable maize MIR162 grains during transportation and processing, there are no indications of an increased likelihood of spread and establishment of feral maize plants. Considering its intended use as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with an unlikely but theoretically possible horizontal gene transfer from maize MIR162 to bacteria have not been identified. The scope of the post-market environmental monitoring plan provided by the applicant is in line with the intended uses of maize MIR162 and the guidance document of the EFSA GMO Panel on post-market environmental monitoring of GM plants (EFSA, 2011). In addition, the EFSA GMO Panel acknowledges the approach proposed by the applicant to put in place appropriate management systems to restrict environmental exposure in cases of accidental release of viable seeds of maize MIR162. The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the general surveillance plan.

In conclusion, the EFSA GMO Panel considers that the information available for maize MIR162 addresses the scientific comments raised by the Member States and that maize MIR162, as described in this application, is as safe as its conventional counterpart and non-GM commercial varieties with respect to potential effects on human and animal health and the environment in the context of its intended uses.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Competent Authority of Germany, received on 12 July 2010, concerning a request for placing on the market of maize MIR162 in accordance with Regulation (EC) No 1829/2003.
2. Acknowledgement letter, dated 23 July 2010, from EFSA to the Competent Authority of Germany (Ref. CGL/RM/PB/KL/mt (2010) 5013037).
3. Letter from EFSA to the applicant, dated 23 July 2010, requesting additional information under completeness check (Ref. PB/KL/CE/lg (2010) 5017321).
4. Letter from the applicant, received on 13 August 2010, providing additional information under completeness check.
5. Letter from EFSA to the applicant, dated 24 August 2010, delivering the "Statement of Validity" for application EFSA-GMO-DE-2010-82, maize MIR162 submitted by Syngenta under Regulation (EC) No 1829/2003 (Ref. PB/KL/CE/lg (2010) 5071810).
6. Letter from the applicant, received on 8 September 2010, providing EFSA with an updated version of the application EFSA-GMO-DE-2010-82 submitted by Syngenta under Regulation (EC) No 1829/2003.
7. Letter from EFSA to the applicant, dated 24 August 2010, requesting additional information and stopping the clock (ref. PB/KL/YL/lg (2010) 5071902).
8. Letter from the applicant to EFSA, received on 6 October 2010, providing additional information.
9. Letter from EFSA to the applicant, dated 21 January 2011, with request for additional information (ref. PB/KL/YL/lg (2011) 5493042).
10. Letter from the applicant to EFSA, received on 1 February 2012, providing additional information.
11. Letter from EFSA to the applicant, dated 17 April 2012, restarting the clock (Ref. EW/ZD/YL/lg (2012) 6493900).

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