Bioveiligheidsraad
Conseil de Biosécurité

23-02-2016

Secretariaat
Secrétariat

O./ref.: WIV-ISP/41/BAC/2016_0122

Title: Advice of the Belgian Biosafety Advisory Council on application EFSA/GMO/DE/2009/66 from Syngenta under Regulation (EC) No. 1829/2003

Context

Application EFSA/GMO/DE/2009/66 was submitted by Syngenta on 4 March 2009 for the marketing of genetically modified (GM) maize Bt11 × MIR162 × MIR604 × GA21 for food and feed uses, import and processing, excluding cultivation within the European Union (EU), within the framework of Regulation (EC) No. 1829/2003[1].

The four-event stack maize Bt11 × MIR162 × MIR604 × GA21 was obtained by conventional crossing (no new genetic modification involved) of the corresponding single events:
- Bt11 genetically modified with the *cry1Ab* and *PAT* genes;
- MIR162 genetically modified with the *vip3Aa20* and *pmi* genes;
- MIR604 genetically modified with the *mcry3A* and *pmi* genes;
- GA21 genetically modified with the *mepsps* gene.

It was therefore developed to achieve insect resistance (conferring protection against specific lepidopteran pests and coleopteran pests through expression of the cry1Ab, vip3Aa20 and mcry3A proteins) and herbicide tolerance to glyphosate-based (mEPSPS protein) and glufosinate ammonium-based (PAT protein) herbicides. It also expresses the PMI protein that was used as a selectable marker in maize MIR162 and MIR604.

The application was officially acknowledged by EFSA on 13 July 2009. On 21 July 2009 EFSA started the formal three-month consultation period of the Member States, in accordance with Articles 6.4 and 18.4 of Regulation (EC) No. 1829/2003 (consultation of national Competent Authorities within the meaning of Directive 2001/18/EC designated by each Member State in the case of genetically modified organisms being part of the products).

Within the framework of this consultation, the Belgian Biosafety Advisory Council (BAC), under the supervision of a coordinator and with the assistance of its Secretariat, contacted experts to evaluate the dossier, chosen from the common list of experts drawn up by the BAC and the Biosafety and Biotechnology Unit (SBB). Six experts answered positively to this request, and formulated a number of comments to the dossier, which were edited by the coordinator. See Annex I for an overview of all the comments and for the list of comments actually submitted to EFSA on 21 October 2009.
Since the dossier submitted by the applicant consisted of the complete data package for maize Bt11 x MIR162 x MIR604 x GA21 (application EFSA/GMO/DE/2009/66) and the complete data package for the triple-event stack maize Bt11 x MIR162 x GA21 (registered by EFSA as a separate application EFSA/GMO/DE/2009/67) the comments provided by the experts covered both applications.

[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).

The opinion of the EFSA Scientific Panel on GMOs was adopted on 29 October 2015 (EFSA Journal 2015;13(12):4297[2]), and published on 7 December 2015 together with the responses from the EFSA GMO Panel to comments submitted by the Member States during the three-month consultation period.
On 14 December 2015 the opinion of EFSA was forwarded to the Belgian experts. They were invited to give comments and to react if needed to the answers given by the EFSA GMO Panel, in particular in case comments formulated in their initial assessment of the dossier were not taken into account in the opinion of EFSA.

It is important to note that the EFSA opinion on application EFSA/GMO/DE/2009/66 covers the four-event stack maize Bt11 × MIR162 × MIR604 × GA21 but also the ten subcombinations independently of their origin resulting from the combination of any of the single events Bt11, MIR162, MIR604 and GA21. Subcombinations occur as segregating progeny in the harvested grains of Bt11 × MIR162 × MIR604 × GA21 (embryo and albumen), and refer also to any combination of up to three of the events Bt11, MIR162, MIR604 or GA21 that has either been or could be produced by conventional crossing, through targeted breeding approaches. These are maize stacks that can be bred, produced and marketed independently of the four-event stack Bt11 × MIR162 × MIR604 × GA21.
Concerning these 10 subcombinations, the EFSA GMO Panel previously assessed four of them and did not identify safety concerns. For the remaining six subcombinations, with the exception of Bt11 × MIR162 × GA21, the applicant provided no experimental data. The EFSA GMO Panel used a weight-of-evidence approach to conclude on the safety of these six subcombinations, considering information from: (i) the previous assessments of the four single maize events, (ii) the assessment of the four-event stack maize, and (iii) the four subcombinations previous assessed and the newly available data.

In delivering the present advice the Biosafety Advisory Council considered in particular the information below:
- The comments formulated by the experts on applications EFSA/GMO/DE/2009/66 and EFSA/GMO/DE/2009/67;
- The opinion of EFSA including the answers of the EFSA GMO Panel to these comments;
- The advices already adopted by the BAC on the four single events and four of the possible subcombinations. The conclusions of the BAC were as follows:

Event	Application number	BAC advice	Conclusions
GA21	EFSA/GMO/UK/2005/19 EFSA/GMO/RX-GA21	BAC/2007/SC614 (07/12/2007)	On the basis of the compositional analysis, the BAC agreed with the overall conclusion of the GMO panel of EFSA that: "it is unlikely that maize GA21 will have any adverse effects on human and animal health or on the environment in the context of its intended uses". The BAC was also of the view that EFSA should systematically request from the applicants the evaluation of the potential allergenicity of the whole GM plant or kernels, and that the power of the statistical analysis and/or the sensitivity of the tests performed on animals for toxicological and nutritional assessment need to comply with standards of good statistics in order to allow scientifically sound conclusions. Because of these remarks, some members of the BAC were not convinced that the health safety of this GM maize has been proven.

[2] See http://www.efsa.europa.eu/en/efsajournal/pub/4297

Bt11	EFSA/GMO/RX-Bt11	BAC/2009/0904 (17/03/2009)	No major risks for human and animal health or concerning the environment were identified.
MIR604	EFSA/GMO/UK/2005/11	BAC/2009/01365 (02/10/2009)	No major risks for animal health or concerning the environment were identified. The BAC disagreed with the GMO panel of EFSA that no risks for human health were identified, since identified potential allergenicity of the transgene protein (PMI) had not been tested *in vivo*. The BAC therefore gave a negative advice for the placing on the market of GM maize MIR604.
MIR162	EFSA/GMO/DE/2010/82	BAC/2012/0785 (29/08/2012)	No major risks for animal health or concerning the environment were identified. A minority of the members of the BAC agreed with the GMO panel of EFSA that maize MIR162 was unlikely to have an adverse effect on human health in the context of its intended uses. A majority disagreed, since identified potential allergenicity of the transgene PMI protein had not been appropriately tested with *in vitro* and/or *in vivo* tests. Therefore the BAC advised a conditional approval provided a tough monitoring on human health is conducted.
Bt11 × GA21	EFSA/GMO/UK/2007/49	BAC/2009/01493 (06/11/2009)	No major risks for human and animal health or concerning the environment were identified.
MIR604 × GA21	EFSA/GMO/UK/2007/48	BAC/2010/0952 (05/10/2010)	No major risks for animal health or concerning the environment were identified. A minority of the members of the BAC agreed with the GMO panel of EFSA that the maize MIR604 x GA21 was unlikely to have an adverse effect on human health in the context of its intended uses. A majority disagreed, since identified potential allergenicity of the transgene PMI protein had not been tested *in vitro* on serum of patients allergic to latex nor by appropriate *in vivo* tests. The BAC therefore could not give a univocal conclusive advice for the placing on the market of GM maize MIR604 x GA21.
Bt11 × MIR604	EFSA/GMO/UK/2007/50	BAC/2010/0956 (05/10/2010)	Same conclusion as for GM maize MIR604 x GA21.
Bt11 x GA21 × MIR604	EFSA/GMO/UK/2008/56	BAC/2010/0958 (05/10/2010)	Same conclusion as for GM maize MIR604 x GA21.

The eight GM maizes mentioned in the table above are all authorised in the EU for food and feed uses[3].

[3] See EU register of GM food and feed: http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

Scientific evaluation

1. Environmental risk assessment

According to the Biosafety Advisory Council no major risks were identified concerning the European environment[4].

2. Molecular characterisation

With regard to the molecular characterisation, the Biosafety Advisory Council is of the opinion that the information provided is sufficient and does not raise safety concerns.

3. Assessment of food/feed safety and nutritional value

3.1. Assessment of compositional analysis

The Biosafety Advisory Council agrees with the GMO panel of EFSA that the compositional data of GM maize Bt11 × MIR162 × MIR604 × GA21, in comparison with its conventional counterpart, do not raise safety concerns.

The Biosafety Advisory Council also considers that, although not required by the OECD document on compositional considerations for new varieties of maize (OECD, 2002[5]), it lacks the analysis on dietary fibre. The Biosafety Advisory Council recommends the analysis on dietary fibre since this concept is widely accepted in human food studies.

3.2. Assessment of toxicity

The Biosafety Advisory Council has evaluated the safety of the newly expressed Cry1Ab, mCry3A, Vip3Aa20, mEPSPS, PAT and PMI proteins in the context of previous applications, and no safety concerns were identified. Taking into account the updated information considered in the current application, the Council is of the opinion that its previous conclusions remain valid.
The Biosafety Advisory Council is also of the opinion that the combined expression of the newly expressed proteins in the stacked event should not raise toxicological concerns.

3.3. Assessment of allergenicity

The Biosafety Advisory Council has evaluated the safety of the newly expressed Cry1Ab, mCry3A, Vip3Aa20, mEPSPS and PAT proteins in the context of previous applications, and no concerns were identified. Since no new information on allergenicity of these proteins has become available, the Council is of the opinion that its previous conclusions remain valid.

Previous advices of the Biosafety Council on GM maizes expressing the PMI protein (see applications EFSA/GMO/UK/2005/11, EFSA/GMO/UK/2007/48, EFSA/GMO/UK/2007/50, EFSA/GMO/UK/2008/56 and EFSA/GMO/DE/2010/82) reflected the concerns expressed by some of the members about the potential allergenicity of the PMI protein due to a possible cross-reactivity with a moderately important latex allergen, Hev b13.

[4] As the application doesn't imply a cultivation of the GM crop in the EU, a full environmental assessment is not required according to EFSA procedure and was therefore not achieved.
[5] OECD, 2002. Consensus Document on Compositional Considerations for New Varieties of Maize (Zea Mays): Key Food and Feed Nutrients, Anti-Nutrients and Secondary Plant Metabolites.

To elaborate further on this issue the Biosafety Advisory Council collected, in the frame of the evaluation of this application, the scientific opinions of Prof. Bart Lambrecht (UZ Gent and UGent) and Prof. Johan Grooten (UGent), specialists in allergology.
On the basis of these opinions, the Council came to a common agreement that further testing of the potential allergenicity of the PMI protein in humans is not needed from the safety viewpoint.
The Biosafety Advisory Council is also of the opinion that the combined expression of the newly expressed proteins in the stacked event does not raise concerns regarding the allergenicity.

With regard to the allergenicity of the whole GM plant, maize is not considered to be a common allergenic food. Based on the available information, the Biosafety Advisory Council considers that there is no evidence that the overall allergenicity of maize Bt11 × MIR162 × MIR604 × GA21 is changed as a result of the genetic modifications.

3.4. Nutritional value

The Biosafety Advisory Council is of the opinion that the information provided is sufficient to conclude that the nutritional characteristics of maize Bt11 × MIR162 × MIR604 × GA21-derived food and feed are not expected to differ from those of conventional maize varieties.

4. Monitoring

Since the allergenicity of the whole GM maize has not been assessed, it is recommended to take up monitoring of allergenicity as part of the general surveillance.

Conclusion

Based on the scientific assessment of the dossier done by the Belgian experts, taking into account the opinion of EFSA, the advices already adopted by the BAC on the four single events and four of the possible subcombinations, and considering the data presently available, the Biosafety Advisory Council:

1) Agrees with the GMO panel of EFSA that no major risks concerning the environment were identified;
2) Agrees with the GMO panel of EFSA that there is no reason to expect interactions between the newly expressed proteins that could impact on the food or feed safety;
3) Agrees with the GMO panel of EFSA that no major risks for animal and human health were identified;
4) Considers that the conclusions of the Biosafety Advisory Council on the four stacks that have been assessed previously (maizes Bt11 × MIR604 × GA21, Bt11 × GA21, MIR604 × GA21 and Bt11 × MIR604 – see table on pages 2-3 for further information) remain unchanged.

Prof. Maurice De Proft
President of the Belgian Biosafety Advisory Council

Annex I: Compilation of comments of experts in charge of evaluating the applications EFSA/GMO/DE/2009/66 & EFSA/GMO/DE/2009/67 and comments submitted on the EFSAnet on mandate of the Biosafety Council (ref. BAC_2009_01427)

Bioveiligheidsraad
Conseil de Biosécurité

21-10-2009

Secretariaat
Secrétariat

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Compilation of comments of experts in charge of evaluating the applications EFSA/ EFSA/GMO/DE/2009/66 & EFSA/GMO/DE/2009/67
and
Comments submitted on the EFSAnet on mandate of the Biosafety Council

Mandate for the Group of Experts: mandate of the Biosafety Advisory Council (BAC) of 31 August 2009
Coordinator: Prof. dr. ir. Dirk Reheul
Experts: Pascal Cadot (Consultant), Rony Geers (KUL), André Huyghebaert (UGent), Peter Smet (Consultant), Jan Van Doorsselaere (KH Zuid-West Vlaanderen), Hadewijch Vanhooren (KUL)
Domains of expertise of experts involved: Genetics, molecular characterisation, human nutrition, animal nutrition, traceability of alimentary chain, analysis food/feed, substantial equivalence, toxicology in vitro and in vivo, general biochemistry, immunology, alimentary allergology, ecology, ecotoxicology, population genetics, plant-insect relations, nature conservation, herbicide tolerance.
Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman

INTRODUCTION

Dossier **EFSA/GMO/DE/2009/66** concerns an application of the company **Syngenta Crop Protection** for the marketing of the genetically modified **Bt11 x MIR162 x MIR604 x GA21 maize** for food and feed applications under Regulation (EC) 1829/2003.
Dossier **EFSA/GMO/DE/2009/67** concerns an application of the company **Syngenta Crop Protection** for the marketing of the genetically modified **Bt11 x MIR162 x GA21 maize** for food and feed applications under Regulation (EC) 1829/2003.

Both applications have been officially acknowledged by EFSA on 21 July 2009. They are submitted together in one dossier.
The scope of these applications is:
☒ GM plants for food use
☒ Food containing or consisting of GM plants
☒ Food produced from GM plants or containing ingredients produced from GM plants
☒ GM plants for feed use
☒ Feed produced from GM plants
☒ Import and processing (Part C of Directive 2001/18/EC)
☐ Seeds and plant propagating material for cultivation in European Union (Part C of Directive 2001/18/EC)

Depending on their expertise, the experts were asked to evaluate the genetically modified plant considered in the application on its 1) molecular, 2) environmental, 3) allergenicity, 4) toxicity and/or 5) food and feed aspects. It was expected that the expert should evaluate if the information provided in the application is sufficient in order to state that the marketing of the genetically modified plant for its intended uses, will not raise any problems for the environment or human or animal health. If information is lacking, the expert was asked to indicate which information should be provided and what the scientifically reasoning is behind this demand.

The comments are structured as in the "Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed" (EFSA Journal (2004), 99, 1-94). Items are left blank when no comments have been received either because the expert(s) focused on other related aspects, or because for this dossier the panel of experts who accepted to evaluate the dossier didn't have the needed expertise to review this part of the dossier.
It should be noted that all the comments received from the experts are considered in the evaluation of this dossier and in formulating the final advice of the Biosafety Advisory Council. Comments placed on the EFSAnet are indicated in grey.

List of comments received from the experts

GENERAL COMMENTS

Comments/Questions of the expert(s)

A. GENERAL INFORMATION

Comments/Questions of the expert(s)

Comment 1

No comments.

B. INFORMATION RELATING TO THE RECIPIENT OR (WHERE APPROPRIATE) PARENTAL PLANTS

Comments/Questions of the expert(s)

Comment 1

No comments.

C. INFORMATION RELATING TO THE GENETIC MODIFICATION

Comments/Questions of the expert(s)

Comment 1

No comments.

D. INFORMATION RELATING TO THE GM PLANT

D.1 DESCRIPTION OF THE TRAITS AND CHARACTERISTICS WHICH HAVE BEEN INTRODUCED OR MODIFIED

Comments/Questions of the expert(s)

Comment 1

No comments.

D.2. INFORMATION ON THE SEQUENCES ACTUALLY INSERTED OR DELETED

Comments/Questions of the expert(s)

Comment 1

Page 37

For GA21 maize, in order to increase the readability of the dossier it would be appropriated to refer to p50 in Appendix 2 or p42 in Appendix 3 when the insert is described. The phrase “six contiguous regions” should be replaced by “six copies at a single locus”.

Page 39:

Is it 100% sure that a mutation occurred in the vip3Aa gene? It is not mentioned whether all sequenced clones contain the mutation (Appendix 4). Has sequencing been performed on PCR product (because it is known that Pfu polymerase also generates mutations during PCR)?
However the aa substition is conservative and therefore it can be anticipitated that this will have no effect on Vip3Aa protein function and toxicity.

Blastanalysis of the 5’ and 3’ flanking sequences:
This is not always clear. E.g. using the 5’ flanking sequence, significant hits were obtained with two large clones. One should expect that the 3’ flanking sequences would also show significant homology with these clones but apparently this is not the case. What is the reason for this?
Is the cyclophilin gene located on these two large clones?

D.3. INFORMATION ON THE EXPRESSION OF THE INSERT

Comments/Questions of the expert(s)

Comment 1

Is there an explanation for the discrepancy in results between the two studies (pages 47-48) in PMI protein content in pollen (5.07 event; 48.07 stack versus 4.62 event; 4.79 stack).

D.4. INFORMATION ON HOW THE GM PLANT DIFFERS FROM THE RECIPIENT PLANT IN: REPRODUCTION, DISSEMINATION, SURVIVABILITY

Comments/Questions of the expert(s)

D5. GENETIC STABILITY OF THE INSERT AND PHENOTYPIC STABILITY OF THE GM PLANT

Comments/Questions of the expert(s)

D.6. ANY CHANGE TO THE ABILITY OF THE GM PLANT TO TRANSFERR GENETIC MATERIAL TO OTHER ORGANISMS

Comments/Questions of the expert(s)

D.7. INFORMATION ON ANY TOXIC, ALLERGENIC OR OTHER HARMFUL EFFECTS ON HUMAN OR ANIMAL HEALTH ARISING FROM THE GM FOOD/FEED

D.7.1 Comparative assessment

Comments/Questions of the expert(s)

Comment 1

Why weren't conventional reference lines incorporated in this study?

Analytes determined in grain of both Bt11 x MIR162 x GA21 and Bt11 x MIR162 x MIR604 x GA21:

Proximates		**Minerals**	
moisture	X	calcium	X
protein	X	copper	X
fat	X	iron	X
ash	X	magnesium	X
carbohydrates	X	manganese	X
acid detergent fiber (ADF)	X	phosphorus	X
neutral detergent fiber (NDF)	X	potassium	X
total detergent fiber (TDF)	X	selenium	X
starch	X	sodium	X
		zinc	X
		total nitrogen	

Vitamins		Amino acids		Fatty acids		Secondary metabolites		Antinutrients	
A (β-carotene)	X	alanine	X	14:0 myristic		ferulic acid	X	phytic acid	X
B1 (thiamine)	X	arginine	X	15:0 pentadecanoic				Stachyose	
B2 (riboflavin)	X	asparagine		16:0 palmitic	X	furfural	X	raffinose	X
B3 (niacin)	X	aspartic acid	X	16:1 palmitoleic		inositol	X	trypsin inhibitor	X
B6 (pyridoxine)	X	cysteine	X	18:0 stearic	X	p-coumaric acid	X	gossypol	
B9 (folic acid)	X	glutamic acid	X	18:1 oleic	X			malvalic acid	
C (ascorbic acid)		glycine	X	18:2 linoleic	X			sterculic acid	
E (α-tocopherol)	X	histidine	X	18:3 linolenic	X			dihydrosterculic acid	
		isoleucine	X	20:0 arachidic					
		leucine	X	20:1 gadoleic					
		lysine	X	22:0 behenic					
		methionine	X	24:0 lignoceric					
		phenylalanine	X						
		proline	X						
		serine	X						
		threonine	X						
		tryptophan	X						
		tyrosine	X						
		valine	X						

Bt11 x MIR162 x GA21 (app 14)

Conclusion:
Statistic significant differences between Bt11 x MIR162 x GA21 grain and the nontransgenic grain occur, but the mean value is always within the range provided by the literature. The only exception is vitamin B2 in grain. The mean value for the nontransgenic control lies above the range found in the literature.

Bt11 x MIR162 x MIR604 x GA21 (app 13)

Conclusion:
Statistic significant differences between Bt11 x MIR162 x MIR604 x GA21 grain and the nontransgenic grain occur, but the mean value is always within the range provided by the literature. The only exception is vitamin B2 in grain. The mean values for Bt11 x MIR162 x MIR604 x GA21 grain and its nontransgenic control lie above the range found in the literature.

Since the deviation in vitamin B2 content is not consistently found in the stacked events (it occurs in Bt11 x MIR162 x MIR604 x GA21 but not in Bt11 x MIR162 x GA21), and it is present in the control lines, there seems to be no relation with the genetic manipulation.

Comment 2

Maize Bt11 x MIR162 x MIR604 x GA21 will be referred as maize 66
Maize Bt11 x MIR162 x GA21 will be referred as maize 67
The approach used is quite similar to previous applications. Stacked maize 66 and 67 were compared with relevant control maize, not genetically modified. Commercial varieties are also included in the comparison.

D.7.2 Production of material for comparative assessment

Comments/Questions of the expert(s)

Comment 1

No furthers comments on the locations, growing seasons, geographical spreading and replicates.
The nutritional composition of whole grain kernels and maize forage derived from transgenic and isogenic maize was compared. This is in line with previous applications.

D.7.3 Selection of material and compounds for analysis

Comments/Questions of the expert(s)

Comment 1

The OECD guidelines were followed with respect to the selection of compounds. As it was the case in previous dossiers proximates, amino acids, fatty acids, minerals, vitamins, anti-nutrients and secondary metabolites were assessed in grain and proximates and selected minerals in forage.
The applicant concludes that there are no biologically significant changes in nutritive value and that the proposed maize 67 and 68 is compositionally equivalent to conventional maize.
Upon analysis however some differences were found. The applicant discusses the results and concludes that the results obtained are within limits of natural variations.
I agree with the conclusions.

D.7.4 Agronomic traits

Comments/Questions of the expert(s)

Comment 1

No particular comments.

Additional comment from the coordinator

In the trials used to assess agronomic performances(AP) and compositional analysis (CA) of MIR162 different hybrids were used; the design was OK. In both AP and CA trials the transgenic material was correctly compared to nearly isogenic non-transgenic material, but the construction of the hybrids differed !
Concerning the complete stack Bt11 x MIR162 x MIR604 x GA21 identical hybrids were used both for AP and CA. However the appendices promise a view on how the hybrids were constructed in appendix A but appendix A does not provide this information.

Annex 9: Agronomic equivalence trials were conducted using two MIR162-derived yellow dent field maize hybrids. Near-isogenic nontransgenic commercial varieties were used as controls. The material identification numbers and pedigree of each hybrid are shown in the table below.

Hybrid	2005 Trials		2006 Trials	
	Material ID	Pedigree	Material ID	Pedigree
Control	04MG045535	NP2222/NP2010	05MG065560	NP2673/NP2171
MIR162	04MG043975	NP2010/NP2222	05MG054840	NP2673/NP2171

Annex 12. Compositional analysis

At each location, one hybrid pair, composed of a MIR162 maize hybrid and the corresponding nontransgenic hybrid, was grown in a randomized complete block design, with three replicates for each genotype, as shown in Appendix A.
The hybrid pair for this study was identified as follows:

Hybrid Code	Genotype	Description
E3 (+)	NP2276(MIR162)/NP2391	MIR162 hybrid
E2 (-)	NP2276/NP2391	Nontransgenic, near-isogenic hybrid

Both the MIR162 and the nontransgenic hybrids were treated with conventional pesticides as needed. Plants were self-pollinated by hand and the developing ears were bagged to avoid crosspollination.

D.7.5 Product specification

Comments/Questions of the expert(s)

D.7.6 Effect of processing

Comments/Questions of the expert(s)

Comment 1

The applicant refers to previous applications where processing according to dry and wet milling were studied. Key nutrients were analysed. As the transgenic maize is nutritionally equivalent to conventional maize no particular effects on processing are to be expected.
I agree with this conclusion.

D.7.7 Anticipated intake/extent of use

Comments/Questions of the expert(s)

Comment 1

The proposed maize will replace some of the conventional maize. As no particular differences in composition have been demonstrated, no effects are to be expected.

D.7.8 Toxicology

Comments/Questions of the expert(s)

Comment 1

<u>Table 1:</u> Mean protein concentration of Vip3Aa20 and PMI in MIR162 maize hybrids on a dry-weight basis (µg/g DW)

		Mean µg Vip3Aa20/g DW range		Mean µg PMI/g DW range	
Tissue	**Hybrid & Location**	**Anthesis**	**Seed Maturity**	**Anthesis**	**Seed Maturity**
Leaves	MIR162-A BMI	109.76 104.68 – 118.79	95.37 77.25 – 118.60	6.77 5.71 – 8.44	4.73 3.77 – 6.23
	MIR162-B YNE	105.72 97.10 – 118.80	148.21 136.85 – 159.66	9.31 6.98 – 12.11	4.80 4.20 – 5.93
Roots	MIR162-A BMI	27.42 26.30 – 29.38	25.79 22.57 – 27.48	2.91 2.10 – 4.35	2.21 1.62 – 2.57
	MIR162-B YNE	29.26 28.72 – 30.20	14.79 9.87 – 21.83	2.89 2.09 – 3.58	1.11 0.95 – 1.50
Kernels	MIR162-A BMI	N/A	45.72 41.10 – 50.50	N/A	1.70 1.58 – 1.93
	MIR162-B YNE	N/A	41.40 40.47 – 42.32	N/A	1.23 1.01 – 1.81
Pollen	MIR162-A BMI	42.72 41.45 – 43.36	N/A	3.22 3.02 – 3.40	N/A
	MIR162-B YNE	51.53 48.60 – 53.52	N/A	5.17 3.59 – 6.03	N/A
Whole Plants	MIR162-A BMI	65.17 61.68 – 69.52	59.83 55.06 – 63.14	5.06 4.75 – 5.28	3.53 3.21 – 4.03
	MIR162-B YNE	70.05 68.04 – 72.63	38.24 34.84 – 42.14	5.60 5.45 – 5.72	2.12 1.62 – 2.73

Table 2: Mean protein concentration (and ranges) of Cry1Ab and PAT (in Bt11 maize), Vip3Aa20and PMI (in MIR162 maize) and mEPSPS (in GA21 maize) in tissues of individual-event and stacked Bt11 x MIR162 x GA21 maize hybrids on a dry-weight basis (µg/g DW)

Tissue type (Stage)	Hybrid	Cry1Ab	PAT	Vip3Aa20	PMI	mEPSPS
Leaves (Anthesis)	Event	33.7 30.22—40.12	0.657 0.51—0.81	133.2 112.36—154.79	6.74 6.03—7.41	34.8 30.39—40.44
	Stack	32.7 21.86—40.39	0.629 0.48—0.97	139.9 116.71—187.47	7.44 6.39—8.76	34.1 28.86—39.59
Roots (Anthesis)	Event	12.8 10.33—15.53	0.580 0.35—0.79	32.0 9.54—51.60	2.03 1.45—3.65	14.8 11.81—18.98
	Stack	11.9 9.17—14.45	0.403 0.31—0.47	28.4 19.07—38.45	2.15 1.54—3.18	12.8 10.68—15.44
Pollen (Anthesis)	Event	0.0636 0.06—0.07	<LOD-<LOD <LOD-<LOD	107.6 103.72—111.18	4.62 4.25—5.30	102.4 65.79—116.33
	Stack	0.0858 0.05—0.15	<LOD-<LOD <LOD-<LOD	157.0 138.53—173.94	4.79 4.41—5.30	131.8 103.15—144.19
Kernels (Physiological Maturity)	Event	6.91 4.35—10.67	<LOD-<LOD <LOD-<LOD	83.8 56.41—108.27	1.84 1.11—2.58	6.57 5.35—8.76
	Stack	4.79 4.85—10.64	<LOD-<LOD <LOD-<LOD	83.8 59.18—102.10	1.77 1.21—2.61	6.76 3.53—8.57
Whole Plant (Anthesis)	Event	19.6 16.15—22.72	0.872 0.66—1.08	80.4 64.92—102.64	3.94 3.56—4.42	46.7 35.65—62.96
	Stack	17.8 13.91—20.42	0.751 0.65—0.86	79.0 67.56—86.51	3.94 3.52—4.22	46.4 36.64—54.73

N/A – indicate instances where proteins were not analysed

Table 3: Mean protein concentration (and ranges) of Cry1Ab and PAT (in Bt11 maize), Vip3Aa20and PMI (in MIR162 maize), mCry3A, and MIR604 PMI (in MIR604 maize) and mEPSPS (in GA21 maize) in tissues of individual-event and stacked Bt11 x MIR162 x MIR604 x GA21 maize hybrids on a dry-weight basis (µg/g DW)

N/A – indicate instances where proteins were not analysed Tissue type (Stage)	Hybrid	Cry1Ab	PAT	Vip3Aa20	mCry3A	PMI Measured with the PMI Reference Protein	MIR604 PMI Measured with the MIR604 PMI Reference Protein	mEPSPS
Leaves (Anthesis)	Event	30.7 25.78—35.16	0.596 0.50—0.82	182.4 136.91-279.52	37.0 30.72—46.44	7.72 6.54—9.20	5.03 3.77—6.13	49.6 40.33—59.68
	Stack	29.7 22.00—38.64	0.603 0.46—0.76	187.2 132.81-259.25	31.0 24.37—47.44	16.27 11.82—19.83	9.95 7.48—14.01	43.3 26.52—57.66
Roots (Anthesis)	Event	11.5 9.56—13.27	0.905 0.50—1.14	52.1 36.03—65.91	22.6 16.40—25.40	2.58 1.94—3.33	2.41 1.71—3.08	15.8 10.32—23.5
	Stack	11.3 7.33—13.53	0.739 0.58—0.99	53.1 38.65—65.80	25.4 16.96—31.63	5.37 4.27—6.82	4.08 2.98—5.67	13.4 8.82—17.85
Pollen (Anthesis)	Event	0.0764 0.07—0.09	<LOD-<LOD <LOD-<LOD	97.2 82.19—117.58	<LOD-<LOD <LOQ-<LOQ	5.07 4.72—5.24	43.32 37.39-47.77	86.1 64.01—113.66
	Stack	0.0801 0.06—0.12	<LOD-<LOD <LOD-<LOD	85.4 74.64—95.92	<LOD-<LOD <LOQ-<LOQ	48.07 46.14-49.76	50.40 41.00-58.21	89.9 73.12—129.61
Kernels (Physiological Maturity)	Event	1.782 1.19—2.31	<LOD-<LOD <LOQ-<LOQ	123.8 54.25—165.94	0.717 0.19—1.11	2.48 1.08—3.16	2.33 1.55—2.99	5.34 3.62—7.61
	Stack	1.573 1.15—2.52	<LOD-<LOD <LOQ- <LOQ	140.1 89.74—164.69	0.620 0.40—0.77	5.18 ± 1.27 3.38—6.54	4.74 1.19—5.94	5.92 2.99—7.69
Whole Plant (Anthesis)	Event	15.93 13.63—18.42	0.912 0.71—1.26	73.0 56.66—87.55	18.1 14.54—21.54	3.87 3.02—4.62	4.37 3.51—5.54	23.1 19.58—25.64
	Stack	15.21 13.65—17.12	0.873 0.75—1.03	72.6 57.05—87.52	16.2 12.72—21.00	8.54 7.52—9.53	7.20 6.16—8.78	21.9 19.27—25.31

The concentrations of Cry1Ab, PAT, Vip3Aa20, mcry3A and mEPSPS were, in general, statistically similar in the Bt11 x MIR162 x MIR604 x GA21 hybrid and the corresponding individual-event hybrids. Although some statistically significant differences were seen, these differences were minimal and not consistent across the growing season. As expected, "total PMI" concentrations were consistently higher in the tissues of the Bt11 x MIR162 x MIR604 x GA21 hybrid as compared to PMI concentrations in the MIR162 maize hybrid or MIR604 PMI concentrations in the MIR604 maize hybrid, reflecting the presence of both PMI and MIR604 PMI in the Bt11 x MIR162 x MIR604 x GA21 hybrid.

Comment 2

The number of animals was too small in order to be able a statistically significant difference in the reported trials with mice (2) and rats.

Comment 3

Bt maize was investigated thoroughly and received approval for import, and food and feed use in the EU. GA21 maize was assessed by EFSA and received a positive opinion in 2007, leading to approval for import and all uses in 2008. MIR604 maize was also assessed by EFSA and received recently a positive opinion (2 July 2009).
MIR162 maize has not yet been assessed previously. The toxicity risk assessment is mostly focused on this event and on the stacked events.

D. 7.8.1 Safety assessment of newly expressed proteins

Comments/Questions of the expert(s)

Comment 1

Potential adverse effects to human and animal health arising from Cry1Ab, PAT, mCry3A, MIR604 PMI and mEPSPS were previously assessed as part of the risk assessments conducted to support the Bt11, MIR604 and GA21 applications.

a) Degradation of the Vip3Aa20 protein in simulated gastric fluid (app 22; Stacy, 2007).

Vip3Aa20 from two sources, Event MIR162 transgenic maize and recombinant *Escherichia coli,* was readily degraded in SGF. No intact Vip3Aa20 (molecular weight *ca.* 89 kDa) from either source was detected following incubation in SGF for one minute, as assessed by Western blot analysis. An immunoreactive fragment of *ca.* 60 kDa was detected in the plant-expressed sample following incubation in SGF for one minute. This protein fragment most likely represents a breakdown product of Vip3Aa20 due to pepsin action but was no longer detectable after 2 minutes of incubation in SGF for the plant-expressed protein.
The data presented in this report support the conclusion that Vip3Aa20 expressed in transgenic maize plants will be readily digested under typical mammalian gastric conditions.

b) Degradation of the Vip3Aa20 protein in simulated intestinal fluid (author).

Why was this study not performed ?.

c) Vip3Aa20: Acute Oral Toxicity Study in Mice (app 24; Draper, 2007).

Groups of five male and five female Alpk:APfCD-1 mice were dosed orally by gavage with 0 mg (control) or 1250 mg Vip3Aa20 protein/kg body weight (1488 mg MIR162VIP3A-0106 test substance/kg body weight) on a single day (as two fractions dosed 2 hours apart on day 1) using corn oil as the control substance and vehicle. Vip3Aa20 was the primary component of the test substance MIR162VIP3A-0106 (84% purity).

A dose of 1250 mg Vip3Aa20/kg body weight (equivalent to 1488 mg MIR162VIP3A-0106 test substance/kg body weight) administered orally was non-toxic to mice.

d) Vip3Aa20: Assessment of Amino Acid Sequence Homology with Known Toxins (app 20; Harper and Burroughs, 2009)

The BLASTP program was used to search the NCBI Entrez Protein Database to determine whether Vip3Aa20 had significant amino acid sequence similarity to known toxins. Of 57 protein sequences identified as having significant sequence similarity to Vip3Aa20, none were proteins known to be toxins other than insect-specific vegetative insecticidal proteins.

e) Degradation of the Phosphomannose Isomerase Protein protein in simulated gastric fluid (Privalle, 1999).

PMI was rapidly degraded in SGF such that no intact PMI was detected upon immediate sampling of the reaction mixture.
In order to demonstrate a time course of PMI degradation, the pepsin concentration in the SGF was reduced to 0.0001 times the standard concentration in a separate experiment. Under these conditions, both PMI protein and enzymatic activity were undetectable after 10 min at 37°C. These data indicate that PMI expressed in transgenic plants will likely be readily digested as conventional dietary protein under typical mammalian gastric conditions.

f) Degradation of the Phosphomannose Isomerase Protein protein in simulated intestinal fluid (Privalle, 1999).

PMI was rapidly degraded in SIF and no intact PMI was detected after 2 min of incubation at 37°C.

g) Phosphomannose Isomerase Protein: Acute Oral Toxicity Study in Mice (app 25; Kuhn, 1999).

Groups of seven male and six female mice were dosed orally by gavage with 3030 mg PMI protein/kg body weight in two doses, administered one hour apart. Groups of control males and females were also included. 0.5% w/v aqueous carboxymethylcellulose was used as the control substance and vehicle. Clinical observations and body weight were measured throughout the study. Fourteen days after dosing, the animals were sacrificed and subjected to an examination *post mortem*. Selected organs were weighed. There were no treatment related effects of the PMI protein, therefore the acute oral LD_{50} as well as the no adverse effect level in mice was determined to be greater than 3030 mg PMI protein/kg body weight.

h) Phosphomannose Isomerase Protein: Assessment of Amino Acid Sequence Homology with Known Toxins (app 21: Harper, 2009)

The BLASTP program was used to search the NCBI Entrez Protein database to determine whether PMI had significant amino acid sequence similarity to known toxins. Of 580 sequences identified as having significant sequence similarity to PMI, none were proteins known to be toxins.

Comment 2

Proteins to be assessed: Vip3Aa20 protein and PMI protein (MIR162 maize).
No further comments.
The newly expressed proteins have been assessed well. There is no significant amino acid homology to known mammalian protein toxins and these proteins are readily degraded in *in vitro* digestibility assays. The Vip3Aa20 protein and PMI protein showed no acute toxicity in the single dose acute oral toxicity study in the mouse. A number of the tests were performed with Vip3Aa20 and PMI proteins produced by *E. coli*. The structural, biochemical and functional equivalence of the microbial substitute to the plant expressed proteins were clearly demonstrated.

D.7.8.2 Testing of new constituents other than proteins

Comments/Questions of the expert(s)

Comment 1

No further comments.

D.7.8.3 Information on natural food and feed constituents

Comments/Questions of the expert(s)

Comment 1

MIR162 maize
Only maize from one growing season (USA, 2005) was tested. Unfortunately no other controls than the corresponding non-transgenic, near-isogenic hybrid were used (no commercial control).

Forage
Statistically significant different (Stat. Sign. Diff.): Neutral Detergent Fiber (NDF) between genotypes. Average levels were within the ranges for conventional maize hybrids published by ILSI (2006)
Grain
Stat. Sign. Diff.: proximates ash, NDF, starch; minerals calcium, iron, phosphorus; vitamins A, B6, E; linoleic and linolenic fatty acids; secondary metabolites ferulic acid and ρ-coumeric acid. Average levels were within the ranges for conventional maize hybrids published by ILSI (2006) and OECD (2002).

Bt11xMIR162xGA21 maize (growing season USA, 2006)

Unfortunately no other controls (commercially maize hybrids) than the corresponding non-transgenic, near-isogenic hybrid were used in the comparison.

Forage

Stat. Sign. Diff.: carbohydrates, phosphorus (genotypes). Average levels were within the ranges for conventional maize hybrids published by ILSI (2007) and OECD (2002).

Grain

Stat. Sign. Diff.: **protein**; vitamin B1, B3, B6; **many of the amino acids**; stearic, oleic fatty acids; phytic acid. Average levels were within the ranges for conventional maize hybrids published by ILSI (2007) and OECD (2002), except for vitamin B2 (slightly higher).

Bt11xMIR162xMIR604xGA21 maize (growing season, USA, 2006)

Unfortunately no other controls (commercially maize hybrids) than the corresponding non-transgenic, near-isogenic hybrid were used in the comparison.

Forage

Stat. Sign. Diff.: carbohydrates (location-by-genotype interaction). Average levels were within the ranges for conventional maize hybrids published by ILSI (2007) and OECD (2002).

Grain

Stat. Sign. Diff.: NDF; copper, potassium; vitamins B1, B6; stearic, oleic and linoleic fatty acids. Average levels were within the ranges for conventional maize hybrids published by ILSI (2007) and OECD (2002) except for vitamin B2 (slightly higher).

Conclusion (after the risk assessment of the appendices 12, 13, 14, 15): The statistical significant differences are not biologically relevant. The compositional studies performed confirmed that the stacked Bt11xMIR162xGA21 maize and Bt11xMIR162xMIR604xGA21 maize are not different in composition to conventional maize.

D.7.8.4 Testing of the whole GM food/feed

Comments/Questions of the expert(s)

Comment 1

a) MIR162: 44-day feeding study in broiler chickens (app 29; Brake, 2007).

Results of the broiler feeding study showed that neither the MIR162 grain, nor the non-transgenic, near-isogenic control grain fed broiler chickens demonstrated any adverse effects associated with consumption of poultry diets containing Vip3Aa20 or PMI compared to broiler chickens consuming diets made with commercially available grain containing no Vip3Aa20 or PMI. All diets supported rapid broiler chicken growth at low mortality rates and excellent feed conversion ratios without significant impact on overall carcass yield or quality. The study showed that the transgenic maize had no deleterious effects on broiler chickens.

b) MIR162: 90-Day rat feeding study (app 28: Barnes and Milburn, 2006).

Groups of twelve male and twelve female Alpk:APfSD (Wistar-derived) rats were fed diets incorporating Event MIR162 transgenic maize (corn) grain at 10.0% or 41.5% w/w, for at least 90 consecutive days.

There were no differences between groups of animals fed diets containing Event MIR162 positive transgenic maize grain or nontransgenic control maize grain in body weight, food consumption, clinical condition (including ophthalmoscopy and functional observation battery), clinical pathology, organ weights or histopathology that were considered to be attributable to the inclusion of the Event MIR162 positive transgenic maize grain in CT1 diet.

c) Bt11 x MIR162 x MIR604 x GA21: 49-day feeding study in broiler chickens (app 30: Brake, 2008).
Three sources of maize grain were used to prepare poultry diets. Grain from Bt11 × MIR162 × MIR604 × GA21 transgenic maize plants was used to prepare diets designated as 'Bt11 × MIR162 × MIR604 × GA21'; grain from nontransgenic, near-isogenic maize plants was used to prepare diets designated as 'nontransgenic'; and a commercially available source of North Carolina maize grain grown during the 2006 season was used to prepare diets designated as 'NC 2007.'

This study demonstrated that diets prepared with Bt11 × MIR162 × MIR604 × GA21 transgenic maize grain did not have any effect on performance of broiler chickens when compared with diets prepared with nontransgenic, near-isogenic maize grain or a commercially available source of maize grain. Poultry diets prepared with Bt11 × MIR162 × MIR604 × GA21 maize grain supported rapid broiler chicken growth at low mortality rates, with very good feed conversion ratios, and without affecting carcass yield. There were no observed deleterious effects associated with broilers' consumption of transgenic maize grain when compared with consumption of control maize grain.

d) Bt11 x MIR162 x MIR604 x GA21: 90-Day rat feeding study ().

Not performed. No further testing is needed.

Comment 2

MIR162 maize
A 90-day rat feeding study and a 44-day broiler feeding study were performed. No major comments on these studies.
No further comments.

Bt11xMIR162xGA21 maize
No feeding study available. This approach is consistent with the EFSA guidance: In addition to the compositional analysis with Bt11xMIR162xGA21 maize, the wholesomeness and safety of the stacked product was confirmed in the broiler feeding study with the higher level stack Bt11xMIR162xMIR604xGA21 maize.

Bt11xMIR162xMIR604xGA21 maize
A 49-day broiler feeding study was performed. No major comments on this study.
No further comments.

D.7.9 Allergenicity

Comments/Questions of the expert(s)

Comment 1

Assessment of the allergenicity of the newly expressed proteins.
According to the data currently available, Cry1Ab, mCry3A, PAT, Vip3Aa and mEPSPS are unlikely to be allergenic.
About MIR604 PMI, in a previous dossier (EFSA/GMO/UK/2007/50, maize Bt11xMIR604), the applicant described possible cross-reactivity with a moderately important latex allergen, Hev b 13, the homology being between 29.6% and 36.2%, depending on the comparative method. This was not considered a significant allergen homology as per the guidelines set by the Codex Alimentarius Commission (2003)."
Although such PMI-Hev b 13 homology is not found in the present dossier, the reviewer still finds that 29.6% of homology represents enough amino-acids to construct several cross-reactive epitopes with Hev b 13. Therefore, it is relevant to evaluate the reactivity of PMI on patients allergic to Hev b 13 by using in vivo (skin tests) and/or in vitro (IgE binding) techniques.

Assessment of the allergenicity of the whole GM plants or crops.
The applicant did not assess the allergenicity of the two whole GM plants. By so doing, the applicant follows the EFSA GMO panel who consider that assessment of the allergenicity of the whole plant is not necessary if this plant is not listed in the official allergen list available in the frame of the EU regulations regarding labeling of food. Maize is not listed.
Nevertheless, the reviewer feels that, due to the introduction of the new traits as described in the application, over-expression of endogenous proteins, among them possibly the maize allergens already described, may occur. Therefore, it seems relevant to analyze whether the expression levels of known maize allergens is increased in the genetically modified maize grains or to analyze whether the overall allergenicity of the modified maize has increased, compared to a natural counterpart. Patient IgE binding to maize grain extract or titration of known major allergens of maize should be carried out.

D.7.10 Nutritional assessment of GM food/feed

Comments/Questions of the expert(s)

Comment 1

The number of pens was too small in both broiler trials in order to be able to find a statistically significant difference with respect to feed conversion ratio. The large variability with respect to dry air temperature during both trials might have interfered with the performance results.

D.7.11 Post-market monitoring of GM food/feed

Comments/Questions of the expert(s)

D.8. MECHANISM OF INTERACTION BETWEEN THE GM PLANT AND TARGET ORGANISMS (IF APPLICABLE)

Comments/Questions of the expert(s)

D.9. POTENTIAL CHANGES IN THE INTERACTIONS BETWEEN THE GM PLANT WITH THE BIOTIC ENVIRONMENT RESULTING FROM THE GENETIC MODIFICATION

D.9.1. Persistence and invasiveness

Comments/Questions of the expert(s)

D.9.2 Selective advantage or disadvantage

Comments/Questions of the expert(s)

D.9.3 Potential for gene transfer

Comments/Questions of the expert(s)

D.9.4 Interactions between the GM plant and target organism

Comments/Questions of the expert(s)

D.9.5 Interactions of the GM plant with non-target organism

Comments/Questions of the expert(s)

D.9.6 Effects on human health

Comments/Questions of the expert(s)

D.9.7 Effects on animal health

Comments/Questions of the expert(s)

D.9.8 Effects on biogeochemical processes

Comments/Questions of the expert(s)

D.9.9 Impacts of the specific cultivation, management and harvesting techniques

Comments/Questions of the expert(s)

D.10. POTENTIAL INTERACTIONS WITH THE ABIOTIC ENVIRONMENT

Comments/Questions of the expert(s)

D.11. ENVIRONMENTAL MONITORING PLAN

D.11.1 General

Comments/Questions of the expert(s)

D.11.2 Interplay between environmental risk assessment and monitoring

Comments/Questions of the expert(s)

D.11.3 Case-specific GM plant monitoring

Comments/Questions of the expert(s)

D.11.4 General surveillance of the impact of the GM plant

Comments/Questions of the expert(s)

D.11.5 Reporting the results of monitoring

Comments/Questions of the expert(s)

References

None