Bioveiligheidsraad
Conseil de Biosécurité

25-03-2013

Secretariaat
Secrétariat

O./ref.: WIV-ISP/41/BAC/2013_0194

Title: Advice of the Belgian Biosafety Advisory Council on the application EFSA/GMO/NL/2009/70 from Monsanto under Regulation (EC) No. 1829/2003

Context

The application EFSA/GMO/NL/2009/70 was submitted by Monsanto on 29 May 2009 for the marketing of genetically modified maize MON87460 for food and feed uses, import and processing within the framework of Regulation (EC) No. 1829/2003[1]. Maize MON87460 carries a gene encoding for protein CspB allowing cells to maintain cellular function under various stress conditions. In maize it is expected to reduce yield loss caused by drought stress. This GM maize also carries a gene coding for neomycin phosphotransferase type II (NPTII) which was used as antibiotic resistance marker gene during product development. This gene confers resistance to kanamycin and related antibiotics.

The application was officially acknowledged by EFSA on 21 January 2010. On the same date 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). Seven 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 placed on the EFSAnet on 27 April 2010.

The opinion of the EFSA Scientific Panel on GMOs was adopted on 18 October 2012 (EFSA Journal 2012; 10(11):2936[2], and published together with the responses from the EFSA GMO Panel to comments submitted by the experts during the three-month consultation period.

On 19 November 2012 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 the comments formulated in their initial assessment of the dossier were not taken into account in the opinion of EFSA.
In addition on 11 March 2013 two of the experts were asked to give some precisions regarding the results of the 90-day rat feeding study provided in the application. The comments formulated by the experts together with the opinion of EFSA including the answers

[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).
[2] See http://www.efsa.europa.eu/en/efsajournal/pub/2936.htm

of the EFSA GMO Panel form the basis of the advice of the Biosafety Advisory Council given below.

Scientific evaluation

1. Environmental risk assessment

This GM maize contains the antibiotic resistance marker gene *npt*II. The EFSA scientific opinion contains a detailed evaluation of risk assessment of the presence of this ARM gene in particular with regards to the potential risk arising from their horizontal gene transfer (HGT) to bacteria. Considering the expected low frequency of gene transfer from MON 87460 to bacteria compared to that between bacteria, and based on the EFSA Statement on the use of this antibiotic resistance gene as marker gene in genetically modified plants[3], the contribution of HGT to the environmental prevalence of ARM genes is considered negligible.

According to the Biosafety Advisory Council no major risks were identified concerning the 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 is of the opinion that the composition of the GM maize MON87460 is compositionally equivalent to commercial maize.

The Biosafety Advisory Council also considers that, although not required by the OECD Document on compositional considerations for new varieties of maize (OECD, 2002), 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 and recommends the adaptation of the OECD consensus document accordingly.

3.2. Assessment of toxicity

The applicant provided different data to substantiate that the CspB protein is not toxic. Additionally, the applicant provided the results of a 90-day rat feeding study, even though the EFSA guidelines on the safety assessment of food and feed from GM crops do not require such a study since no biologically relevant differences were identified in the compositional, agronomic and phenotypic characteristics of maize MON 87460 (see point 3.1.). The animal trial design was based on OECD technical guideline 408 (for chemical toxicity testing in rodents during 90 days), which in this case was adapted to testing a whole food/feed product. The size of each treatment group consisted of 20 animals / gender / treatment.
Although *ex ante* the animal trials were properly designed, from a statistical point of view, *ex post* the number of animals/treatment was too small to detect statistically significant differences, if present.

[3] Statement on the safe use of the nptII antibiotic resistance marker gene in genetically modified plants by the Scientific Panel on genetically modified organisms (GMO) – 22/03/2007 . http://www.efsa.europa.eu/en/efsajournal/pub/742.htm

[4] As the application doesn't imply a cultivation of the GM crop in the EU, a full environmental assessment is not required in EFSA procedure and was not performed.

The toxicological assessment of NPTII protein has been previously done by the BAC in the frame of previous applications (see advices on applications EFSA/GMO/RX/MON863 (maize)[5] and EFSA/GMO/RX-MON1445 (cotton)[6].
Therefore, with regard to toxicity the Biosafety Advisory Council is of the opinion that the information provided is sufficient and does not raise safety concerns.

3.3. Assessment of allergenicity

The potential allergenicity of the newly expressed proteins, CspB and NPTII, has been assessed. The Biosafety Council considers CspB and NPTII proteins as unlikely to be allergenic.

3.4. Nutritional value

The Biosafety Advisory Council is of the opinion that the information provided is sufficient and shows the nutritional equivalence of the GM maize with its non-GM counterpart and conventional maize varieties.

4. Monitoring

As 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.
As the statistical power of the animal trials was not perfect, the Biosafety Advisory Council advises to be vigilant on the chronic toxicity and nutritional value.

Conclusion

Based on the scientific assessment of the dossier done by the Belgian experts, taking into account the opinion of EFSA, the answers of the EFSA GMO Panel to the questions raised by the Belgian experts, and considering the data presently available, the Biosafety Advisory Council is of the opinion that in the context of its proposed uses, maize MON 87460 is unlikely to pose any risk to human and animal health.

Given the scope of the application and the fact that the establishment of volunteer plants in Europe is very limited, the potential accidental release into the environment of maize MON 87460 during transport and processing is unlikely to pose any threat to the environment.

In addition, the Biosafety Advisory Council recommends following up any unanticipated allergenicity aspects of the GM plant in monitoring systems

p.o. Ph. Herman

Prof. D. Reheul
President of the Belgian Biosafety Advisory Council

Annex I: Full comments of experts in charge of evaluating application EFSA/GMO/NL/2009/70 and comments submitted on the EFSAnet (ref. BAC_2010_0332)

[5] Reference of document : BAC/2010/0531
[6] Reference of document : BAC/2012/0217

Bioveiligheidsraad
Conseil de Biosécurité

27-04-2010

Secretariaat
Secrétariat

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Compilation of comments of experts in charge of evaluating the application EFSA/GMO/NL/2009/70 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 12 February 2010
Coordinator: Françoise Vancutsem
Experts: Jacques Dommes (ULg), Rony Geers (KUL), Johan Grooten (UGent), André Huyghebaert (UGent), Peter Smet (Consultant), Bart Van Droogenbroeck (LVO), Hadewijch Vanhooren (KUL)
Domains of expertise of experts involved: Molecular characterisation, plant biology, breeding techniques, human nutrition, animal nutrition, analysis food/feed, substantial equivalence, traceability of alimentary chain, toxicology in vitro, in vivo, general biochemistry, Immunology, alimentary allergology, plant biology, abiotic stress resistance, ecology, GMO traceability, risk analysis, maize
Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman, Katia Pauwels

INTRODUCTION

Dossier **EFSA/GMO/NL/2009/70** concerns an application of the company **Monsanto** for the marketing authorisation of the genetically modified **maize MON 87460** for food and feed applications under Regulation (EC) 1829/2003.
The application has been officially acknowledged by EFSA on 25 January 2010.
The scope of the application 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)

Comment 1

The maize MON87460 GMO was assessed for safety from the viewpoint of substantial equivalence to its conventional counterpart except for two specific traits, namely the expression of the heterologous proteins CspB from *Bacillus subtilis* and NptII from *Escherichia coli*. On this basis, the assessment focused on both these specific traits in concordance with the European Commission Recommendation of July 29, 1997 (97/618/EC).

Comment 2

- This application concerns request for approval of a new trait, i.e. drought tolerance. Throughout the dossier it is pointed out that limited water availability has a higher impact on yield during certain maize growth stages (e.g. Pg 97, last paragraph: end off vegetative growth – flowering growth stages).
- To describe the different maize growth stages a code system is used. It would be informative to include a scheme depicting these different growth stages of maize, mentioning the different codes used in the sampling procedures applied in the dossier.
- The T-DNA inserted in MON87460 contains two loxP sites. “The loxP sites were inserted to facilitate the potential excision of the nptII cassette, specifically using CRE recombinase” (citation from Pg 36, second paragraph). The use of the Cre/Lox marker removal system is not explained in detail and only a single reference is given (Russell et al. 1992). Given that MON87460 is among the first dossiers that also includes such sequences (other example is LY058 = EFSA-GMO-NL-2006-31), a more detailed description and discussion on this topic, documented with update references seems relevant.

Additional comment from SBB

Dossier GMO/NL/2006/31 has been withdrawn by the applicant, Renessen Europe. There is no EFSA opinion on this dossier. It has however been evaluated by the Belgian experts and one expert noticed that “comprehensive information is given on … the strategy used to excise the selectable marker using the cre-lox system and subsequent breeding history.”

Additional comment from coordinator

There are many mistakes in the dossier: missing or incorrect references, missing figures (see comments under respective references).

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.

Comment 2

Technical dossier, Part I, Pg 29, first paragraph: It is mentioned that the *Agrobacterium* strain used for transformation contains a disarmed Ti plasmid. However, it is not explained in detail how this was done, only the brief statement 'due to deletion' was included in the sentence. Perhaps a more detailed explanation can be included for reasons of completeness of the provided information.

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

Just a little mistake in the text concerning "1.1. Characterization of the CspB protein and its function", bottom of page 39: reference is made to figure 37, but it should be read figure 5 instead.

Comment 2

- Technical dossier, Part I, Pg 33: For all genetic elements used in the transformation vector a reference is given, however only for one element also (*aadA)* the GenBank accession code is provided. For reasons of completeness this could also be done for the other genetic elements of the vector.
- Technical dossier, Part I, Pg 37, Fig. 3: Suggestion linked to previous remark: include GenBank accession code of original CspB protein in legend of Fig. 3 and indicate difference with codon optimized CspB L2V sequence by indicating V in bold. The legend could use the suggested name CspB L2V instead of CspB as such to stress the codon optimization.
- Technical dossier, Part I, Pg 39, one but last paragraph: The text mentions that the Csp protein in MON87640 consists of 66 amino acids while both Fig. 3 and Fig. 5 (= Fig. 37) show 67 amino acids. Is this just a typing error?
- Technical dossier, Part I, Pg 39, last paragraph: This paragraph refers to Fig. 37 to illustrate the presence of RNP motifs in the AA sequence. However, Fig. 5 is identical to Fig. 37 and incorporated in the document on pg. 40, while Fig. 37 is found on pg. 273 (Section 7.8 Toxicology).
- Technical dossier, Part I, Pg 43, Figure 6, part B: The legend describes that increasing amounts of BSA are used while only one amount (50 µg) is shown on the figure.

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

Comments/Questions of the expert(s)

Comment 1

No comments.

Comment 2

- No comments. The southern blot data included in the Technical dossier illustrate that MON87460 contains only one insert, a 5' truncated version of the expected T-DNA with intact copies of the *cspB* and *npt*II expression cassettes. No additional elements from the transformation vector PV-ZMAP595, linked or unlinked to the insert, were detected in the genome of MON 87460. The absence of backbone sequence from plasmid PV-ZMAP595 was also demonstrated.
- Technical dossier, Part I, Pg 79 – Bioinformatic analyses of MON87460 flanking sequences. BLASTn and BLASTx evaluations indicate that it is unlikely that the inserted T-DNA disrupted endogenous maize genes within the genomic DNA flanking the insertion. Nothing is mentioned however about an analysis to evaluate the presence and functionality of possible novel chimaeric ORF, as requested by "Guidelines for Molecular Characterization of Genetically Modified Higher Plants to be Placed on the Market" from WIV-SBB, Final version Feb 18, 2003[1].

[1] Available at the following address : http://www.biosafety.be/gmcropff/EN/TP/partC/GuideMGC_PartB_C.htm

D.3. INFORMATION ON THE EXPRESSION OF THE INSERT

Comments/Questions of the expert(s)

Comment 1

No comments.

Comment 2

Technical dossier, Part I, Pg 93 Table 8 & 12 vs. Table 10 – A two-fold difference in accumulation level of the CspB protein was detected in the pollen samples collected at the US trial sites + Chilean QUI site versus the Chilean CT, CL and LUM sites. What could be the possible explanation? This is not discussed in this section.

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

Comments/Questions of the expert(s)

Comment 1

Technical dossier, Part I, Pg 104, Fig. 28. This figure is missing.
No further comments: data provided illustrates that no significant differences where found between GM plant and recipient plant for all characters evaluated.

Additional comment from coordinator
Technical dossier, Part I, Pg 103, Fig. 27. This figure is missing.

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

Comments/Questions of the expert(s)

Comment 1

No comments.

Comment 2

- Technical dossier, Part I, Pg 151: Generational stability of the insert. The T-DNA inserted in MON87460 has two *loxP* sites. The *loxP* sites were inserted to facilitate the potential excision of the *npt*II cassette, specifically using CRE recombinase (cfr. Pg 36, second paragraph). From the Southern blot shown in Fig. 155 it is clear that the intact *npt*II cassette is present in all generations. Indirectly it can be concluded that no non-specific endogeneous recombinase activity could be detected causing an unexpected removal of the *npt*II cassette (during the breeding

process). This aspect is not discussed in detail in the text and perhaps disserves some attention underlining the safe use of *loxP* sequences. In addition some scientific references (also elsewhere in the dossier) should be included illustrating the safe use of the *Cre*/*Lox* system in GM plants.

- Technical dossier, Part I, Pg 157: The Invader® assay was used to select a homozygous plants. The principle of the assay is described briefly, but the result of the assay is not documented nor discussed.
- Technical dossier, Part I, Pg 157 and Table 44, Pg 158: It is mentioned that the segregation patterns reported in Table 44 are based on PCR-based assays. The header of Table 44 mentions that the table shows data of "segregation patterns of cspB between generations of Mon8746" and here a reference is mentioned. No further explanation is given. More detailed info would be welcome here.

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

Comments/Questions of the expert(s)

Comment 1

No comments.

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

The assessment is based upon a compositional analysis of forage and grain of maize MON87460 in comparison with reference maize with a similar genetic background.
Samples are derived from maize grown under similar conditions at six locations within the US. Field design, sampling and statistical analysis are described in detail.
Samples were also obtained from tests in Chile. Maize MON87460 and a reference control were grown under two different irrigation systems in order to detect any effect of drought stress.

Comment 2

Statistical analysis of the compositional data showed that there were no significant differences ($p>0.05$) for 407 of the 434 comparisons made between the mean component values of MON 87460 and the control. Of the 27 statistically significant differences (three from the combined-site analysis and 24 from the individual site analyses), all mean component values of the test and control substances were within the 99% tolerance interval established from the commercial references.

D.7.2 Production of material for comparative assessment

Comments/Questions of the expert(s)

Comment 1

See D.7.1

D.7.3 Selection of material and compounds for analysis

Comments/Questions of the expert(s)

Comment 1

For the US samples, 68 nutrients were selected for grains, and 9 for forage. For grain, nutrients were selected according to the OECD consensus documents. In addition to proximates and fibre, relevant amino acids, fatty acids, minerals and vitamins are included.

In most cases no significant differences were found. If any significance is observed values obtained are compared with literature values for commercial maize grains. It is concluded that the values obtained are within the natural variability mentioned in literature.

As anti-nutrients phytic acid and raffinose were analysed and as key secondary metabolites ferulic acid, p-coumaric acid and furfural. A similar approach was followed in the interpretation of these results: a statistical analysis and a comparison with literature data, in case any significance is detected.

The applicant concludes that maize MON87460 obtained in the US trials is compositionally equivalent to commercial maize .

A similar approach was followed for the Chilean samples. Particular constituents potentially relevant for drought stress are also analysed: sucrose, glucose, fructose, sorbitol, mannitol, glycerol, free proline, glycine betaine, choline, salicylic acid and abscissic acid.
Samples obtained by the two irrigation systems were analysed separately.

For samples obtained under well watered conditions no significant difference was found for most combinations. Is any significance was detected results were within the range of natural variability as mentioned in literature.

In case of water limited growing conditions analysis revealed no significant difference, or at least no values outside natural variability for nutrients. A similar conclusion is made for anti-nutrients and secondary metabolites.
The same conclusion applies for particular compounds analyzed to detect effects of drought stress.

The applicant concludes that there is no evidence for biologically relevant changes between maize MON87460 and conventional maize.

Comments:

I agree with the conclusion of the applicant. The file contains convincing evidence that maize MON87460 is compositionally equivalent to commercial maize.

Nutrients, anti-nutrients and secondary metabolites were selected according to up to date knowledge. The applicants refers to the OECD document to motivate the selection of compounds.

I repeat my previous comment that this OECD document needs to be updated at least in the field of fibre particularly in view of the ongoing discussions on the role of fibre constituents in human nutrition, among others the definition of dietary fibre and the definition of whole grain products.

I would like to make a comment about mycotoxins. Maize is known to be quite sensitive to mould development under adverse growing, harvesting or storage conditions. Only in a few cases, I found a reference to the mycotoxin problem, at least in the paragraphs related to my field of expertise. In this file maize MON87460 is proposed with particular properties related to drought stress. Humidity plays an important role in the development of fungi, in addition to other factors.

My question: is there any information on the resistance to mould development and mycotoxin formation of maize MON87460 in comparison to commercial maize?

Compositional equivalence is always studied, in this type of applications, in terms of nutrient, anti-nutrients and particular secondary metabolites. Taking into account the importance of mycotoxins in human and animal health, these range of compounds definitely need more attention in future applications.

D.7.4 Agronomic traits

Comments/Questions of the expert(s)

Comment 1

No comments.

D.7.5 Product specification

Comments/Questions of the expert(s)

Comment 1

No comments.

D.7.6 Effect of processing

Comments/Questions of the expert(s)

Comment 1

Due to the compositional equivalence of maize MON87460 to commercial maize the applicant is not expecting any significant effect on processing.
A major part of maize is used as an animal feed after milling of the whole grain or as forage.

In addition two basic techniques are used for processing of maize grains: wet and dry milling. Maize kernels are separated into fractions with a particular composition and use.
Both processes are reviewed and the composition and use of the fractions discussed.

Wet milling fractions are: starch, syrups, ethanol, maize oil, maize germ meal, maize gluten feed, condensed steep water and maize gluten meal.
Dry milling converts maize into fractions like grits, meal, flour, hominy feed, germ and bran. Processes are different if degermination is applied or not.

Comment:
I basically agree with the applicant's conclusion that due to the compositional equivalence of maize MON87460 to commercial maize, processing will not be affected.

D.7.7 Anticipated intake/extent of use

Comments/Questions of the expert(s)

Comment 1

This section is well-documented. Most conservative intake calculations were made, providing conservative high end exposure scenarios.
Protein levels were taken **from the CBI** Mozaffar and Silvanovich study (2008a): field trail US 2006 season, grown under normal agronomic practices. This study provided the highest protein levels (Chile 2006-2007: lower protein levels, grown under well-watered and water-limited conditions).
For the estimation of the animal dietary intake, the mean protein levels used were expressed on a dry weight basis, however for the human dietary intake mean protein levels were expressed on a fresh weight basis. What is the reasoning for using fresh weight figures? (However this has no consequences on the outcome of the Margin of Exposure)
No further questions.

D.7.8 Toxicology

D. 7.8.1 Safety assessment of newly expressed proteins

Comments/Questions of the expert(s)

Comment 1

CspB protein

The MON87460 protein is nearly identical to the protein present in *Bacillus subtilis* except for a single amino acid substitution at position 2 (from leucine to valine) necessary for cloning purposes. CspB protein is present in MON87460 grain at a mean concentration of 0.072, 0.048 and 0.038 µg/g dry weight under normal agronomic, well-watered and water-limited conditions, respectively.

The MON87460 CspB protein was characterised by N-terminal sequencing by Edman degradation chemistry, Tryptic peptide mapping followed by MALDI-TOF MS analysis, Western blot analysis (immunoreactivity), SDS-PAGE (molecular weight), functional dsDNA-destabilisation (melting) assay. The equivalence between the *E.coli*- and MON87460-produced proteins was established by SDS-PAGE (molecular weight), Western blot analysis (immunoreactivity), Glycosylation analysis, and functional dsDNA-destabilisation (melting) assay.

A bioinformatics analysis demonstrated that the CspB protein does not show structural similarity to known toxins or other biologically active proteins that could cause adverse effects. The acute oral toxicity study with CD-1 mice demonstrated that the CspB protein is not acutely toxic and does not cause any adverse effects. No treatment-related effects were observed on survival, clinical observations, body weight gain, food consumption or gross pathology (NOAEL = 4.7 mg/kg bw, only dose tested). No rationale was provided why this low dose was chosen, however it is three to four orders of magnitude above the conservative estimates for expected human exposure to CspB from consumption of MON87460. Additionally, the CspB protein was shown to be rapidly and completely digested following sequential *in vitro* digestion in simulated gastric fluid and simulated intestinal fluid assays (The transiently stable 2.5 kDa fragment which was observed between the 30 s and 30 min time points in SGF, was digested within 30 s by SIF).

Furthermore, the CspB protein in MON 87460 is similar to several bacterial Csps (yogurt) and their plant homologues (rice, wheat) that are ubiquitous in the human diet and directly consumed in common foods establishing a history of safe exposure.

NptII protein

The absence of toxic potential associated to the NptII protein has been previously established. An extensive database exists regarding its safety. The level of NptII protein in MON87460 grain was below the limit of quantification (LOQ).

The NptII protein extracted from MON87460 leaves was characterised and compared to the *E. coli*-produced NptII reference protein standard (western blot: immunoreactivity, molecular weight) confirming the identity and establishing the equivalence.

An up to date bioinformatics analysis demonstrated that the NptII protein does not show structural similarity to known toxins or other biologically active proteins that could cause adverse effects. The acute oral toxicity study with CD-1 mice (older published study: Fuchs et al, 1993b) demonstrated that the NptII protein is not acutely toxic and does not cause any adverse effects. No treatment-related effects were observed on survival, clinical observations, body weight gain, food consumption or gross pathology (NOAEL = 5000 mg/kg bw, highest dose tested). In the same publication it was shown that

the NptII protein was rapidly degraded in simulated gastric and intestinal fluids with respective half-lives of less than 10 seconds and between 2 and 5 minutes.
Furthermore, the NptII protein is ubiquitous in E. coli, and normally present in the human gastrointestinal tract.

Concerning antibiotic resistance:
The GMO Panel reiterated its earlier conclusions (EFSA, 2004) that the use of the nptII gene as selective marker in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment. The GMO Panel also confirmed earlier safety assessments of GM plants and derived food/feed comprising the nptII gene (EFSA, 2007).

In conclusion, the data set indicates that the MON87460-produced CspB protein and NptII protein are safe for food/feed use

Comment 2

Mean concentrations in different MON 87460 tissues of both CSPB and NPTII protein have been determined and expressed on a dry weight basis.
Based on these results estimated protein intake was calculated. Taken together with the results of the acute toxicity testing, margins of exposure were determined to be approx. 10000 for CSPB and 10^8 for NPTII.

a) Degradation of the CSPB protein in simulated gastric fluid (**from CBI :** Kapadia *et al.*, 2008).

A fragment with an apparent molecular weight of ~2.5 kDa was observed between the 30 s and 30 min digestion time points. No protein fragments were visible at the 60 min digestion time point (SDS-PAGE).
Western blot analysis demonstrated that the CspB protein was digested below the LOD within 30 s of incubation in SGF.
After digestion in SGF for 2 min, followed by treatment with SIF, the fragment of ~2.5 kDa was rapidly digested (< 30 s).

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

Cfr. a) of this section.

c) CSPB: Acute Oral Toxicity Study inMice (**from CBI :** CRO-2007-182, 2008).

There were no adverse effects of CSPB when administered to mice by single oral gavage at a dose of 4.70 mg/kg body weight. Therefore, based on the results of this study, the no-observed-adverse-effect level (NOAEL) of CSPB is 4.70 mg/kg body weight.

d) CSPB: Assessment of Amino Acid Sequence Homology with Known Toxins (**from CBI :** Tu, 2009; Tu and Silvanovich, 2009a; Tu and Silvanovich, 2009b)

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the CspB and NptII proteins and any known toxic or other biologically active proteins that would be harmful to human or animal.

e) Degradation of the NPTII protein in simulated gastric fluid (Fuchs *et al.*, 1993b).

The metabolic fate of the protein was evaluated in simulated gastric (pepsin, pH = 1.0) and intestinal (pancreatine, pH = 7.5) fluids. Both western blot analysis and enzymatic assays confirmed that NptII readily degrades in simulated gastric and intestinal fluids with respective half-lives of less than 10 seconds and between 2 and 5 minutes.

f) Degradation of the NPTII protein in simulated intestinal fluid ()

Cfr. e) of this section.

g) NPTII: Acute Oral Toxicity Study in Mice (**from CBI :** MSL 11940).

There were no treatment related adverse findings in any of the groups administered NPTII protein by oral gavage at dosages up to the highest dosage of NPTII tested, 5000 mg/kg, which is considered to be the no-effect level.

h) nptII: Assessment of Amino Acid Sequence Homology with Known Toxins ()

cfr. d) of this section.

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

Compositional analyses were performed on forage and grain collected from MON87460 which was grown in 2 different field trails under normal agronomic conditions (USA 2006 season, 6 fields) and under well-watered and water-limited conditions (Chile 2006-2007 season, 4 fields but 1 excluded). No consistent alterations in the level of the studied components were found between sites/growing seasons/water conditions. Furthermore, the differences were generally small and fell within the interval of natural variation calculated from the occurrence of these constituents in conventional maize varieties.
Though, a more targeted compositional screening was triggered by the type of modification: supplementary data was included on additional secondary metabolites considered to be associated

with stress tolerance (Chile study). Again no consistent differences were found across sites in the levels of additional secondary metabolites.

In conclusion, compositional analysis of drought-tolerant MON87460 maize, which was grown under normal agronomic, well-watered and water-limited conditions, showed the equivalence to conventional maize grown under the same conditions. For all analysed components in forage and grain from MON87460, there were no compositional differences of biological significance compared to the conventional control maize.

D.7.8.4 Testing of the whole GM food/feed

Comments/Questions of the expert(s)

Comment 1

The trials with rats and mice have one common shortcoming, i.e. the power of the statistical analysis is too low in order to able detected significant differences, if present, because he number of animals per treatment is too small.

Comment 2

The compositional analysis was already extended with a more targeted screening on additional secondary metabolites considered to be associated with stress tolerance. However, stress-associated metabolism is very complex and not well understood yet.
In my opinion this type of modification also triggers the testing of the MON87460 grain both in the 42-day feeding study in broilers AND in the 90-day oral toxicity study in rats. Both tests were performed in this application.

90-day oral toxicity study in the rat

The study included 3 groups: 33% conventional control grain, 11% MON87460 grain, 33% MON87460 grain. 33% MON87460 grain in the diet is the equivalent to 23.6 g/kg bw/d for male broilers and 28.2 g/kg bw/d for females broilers, which is 2 orders of magnitude above the estimated human consumption of maize. Historical control data was available. The few statistically significant differences observed were not dose-dependent, fell within the historical control range, could not be confirmed by histology or other linked changes. In conclusion, there were no MON87460 grain-related deaths, clinical observations, effects on body weight, food consumption, clinical pathology parameters, organ weight alterations, macroscopic or microscopic findings. The administration of MON89460 grain did not indicate any adverse effects in the 90-day feeding study in rats.

42-day feeding study in broilers

A 42-day feeding study with broilers was conducted with diets containing maize grain from MON87460, the conventional control, and six commercial reference grains. There were no biologically relevant differences in broiler performance, carcass yields, or meat composition between broilers fed diets containing MON87460 and those fed diets containing the conventional control grain. This study did not indicate any toxic effects and any unanticipated or pleiotropic effects.

No further comments.

Comment 3

a) 42-day feeding study in broiler chickens ().
Not performed. No further testing needed.

Additional comment from the SBB:
The 42-day feeding study in broiler chicken has been performed: see point 7.10.2 Nutritional assessment in the Technical dossier and comment above from another expert.

b) 90-Day rat feeding study (WI-2007-064, 2008).

The study included three groups of Sprague-Dawley (Crl:CD®[SD]) rats with each group consisting of 20 males and 20 females. One group was administered a diet containing approximately 11% (w/w) of corn grain from MON 87460, supplemented with approximately 22% (w/w) of conventional control corn grain. The second group was fed a diet formulated to contain approximately 33% (w/w) of corn grain from MON 87460.
A concurrent control group received conventional control corn grain formulated into the diet at approximately 33% (w/w).

There were no test substance-related deaths or clinical observations. There were no test substance-related effects on body weights, food consumption or clinical pathology parameters. There were no test substance-related organ weight alterations or macroscopic or microscopic findings noted.
In conclusion, administration of grain from MON 87460 to rats for at least 90 consecutive days at concentrations up to 33% (w/w) in the diet (equivalent to 23.6 g/kg body weight/day for males and 28.2 g/kg body weight/day for females) had no effects on the growth or health of rats.

D.7.9 Allergenicity

Comments/Questions of the expert(s)

Comment 1

It is now well established that provided antigens are formulated in combination with potent adjuvants or when antigen exposure coincides with a condition of acute immune (hyper)reactivity in genetically predisposed individuals, immune tolerance of the organism to any environmental food or airborne antigen can be overcome, resulting in allergic sensitisation and disease. Notwithstanding such extreme conditions, environmental antigens differ dramatically in their capacity to elicit allergic sensitisation under conventional conditions. As common traits, allergenic antigens share physicochemical properties such as high solubility and resistance to proteolysis but also the presence of enzymatic activity, especially protease and lipase activity (Mills et al, Crit Rev Food Sci Nutr 44:379-407, 2004; Shakib et al, Trends Immunol 29: 633-642, 2008). Accordingly, the present assessment of allergenic potential of the heterologous CspB and NptII proteins expressed in the MON87460 maize GMO was performed taking into account these criteria in addition to the source of the protein, amino acid sequence similarity to known allergens and allergen peptides, and susceptibility to digestive proteolysis.

Allergenic potential of CspB:

- Protein function: The protein is characterized in literature as a small protein containing a RNA-binding sequence and exerting a melting or unwinding activity on polynucleotides such as single stranded and double stranded RNA. On this basis, the protein is classified as a RNA chaperone. No other (enzymatic) activities have been reported. Evidence is provided that the CspB protein expressed in MON87640 does not differ in structure and function from other family members. Furthermore, similarly to the bacterial protein, the MON87640 protein is not glycosylated, thus excluding the occurrence of potentially allergenic post-translational changes in glycosylation. → **low risk.**

For Efsanet:

- Protein function: The protein is characterized in literature as a small protein containing a RNA-binding sequence and exerting a melting or unwinding activity on polynucleotides such as single stranded and double stranded RNA. → **low risk.**
- Source: *Bacillus subtilis* (the source of the gene) is a GRAS organism for use in food, thus implying that the protein is from a non-allergenic source. No cases of allergies to *Bacillus subtilis* have been reported. → **low risk.**

For Efsanet:

Source: *Bacillus subtilis* . → **low risk.**

- Expression: The protein is present in grain although reportedly at low levels. Dose-response characteristics of allergen exposures are especially difficult to predict and the underlying molecular mechanisms still elusive. This means that the presence of a protein rather than its expression level is to be considered. → **potential risk.**
- Sequence similarity: A bioinformatics screen was performed based on the FASTA sequence alignment tool and the updated allergen database (AD_2009[51]). This was supplemented with a sliding eight-amino acid window search (ALLERGENSEARCH). No positive hits relevant from an allergenic assessment perspective were obtained. → **low risk.**

For Efsanet:

Sequence similarity: A bioinformatics screen was performed → **low risk.**

- Susceptibility to (digestive) proteolysis: Sequential exposure to acid pH/pepsin and neutral pH/pancreatin revealed rapid degradation of the protein into a smaller fragment (acid pH/pepsin) and the further degradation of this fragment under neutral pH/pancreatin. The results reported are convincing but a few questions remain. First, the source of the recombinant CspB used in these assays is not specified nor whether the wild type or the MON87640 mutant (L2V) was used. Second, these assays were performed with free CspB, not bound to RNA. While it is likely that in the maize plant free CspB will be present, also CspB bound to RNA should occur. Quoting from the report 'In the absence of polynucleic acids, the CspB protein has a very low thermodynamic stability and is susceptible to rapid proteolytic degradation' (page 39), it seems crucial to determine the sensitivity to proteolysis also of the RNA complexed CspB in order to fully judge this characteristic. → **awaiting further info and data.**

Conclusion: Monsanto performed a thorough analysis of characteristics associated with allergenicity. The results are satisfactory but before reaching a final conclusion additional data on the susceptibility to proteolysis are necessary.

Allergenic potential of NptII:

- Protein function: NptII confers resistance to kanamycin. → low risk
- Source: A non-virulent strain of E.coli served as donor of the NptII-encoding gene. → low risk
- Expression: NptII protein was barely detectable or below detection limits in MON87460 grain, thus indicating extremely low levels of exposure upon intake. → reduced risk
- Sequence similarity: Using the same bioinformatics tools as mentioned above, the NptII protein sequence was compared to the updated allergen database, revealing no relevant amino acid sequence similarities. → low risk
- Susceptibility to (digestive) proteolysis: Using the same experimental setup as mentioned above, a rapid proteolytic degradation of NptII was observed with half-lives of less than 10 seconds (acid pH/pepsin) and between 2 to 5 minutes (neutral pH/pancreatin). → low risk

For Efsanet:

Allergenic potential of NptII:

Protein function: → low risk

Source: A non-virulent strain of E.coli → low risk

Expression: → reduced risk

Sequence similarity: → low risk

Susceptibility to (digestive) proteolysis: → low risk

Conclusion: Monsanto performed a thorough analysis of characteristics associated with allergenicity. The results do not point to a significant risk for allergenicity of the NptII protein in MON87460 maize grain when used as feed or food.

D.7.10 Nutritional assessment of GM food/feed

Comments/Questions of the expert(s)

Comment 1

Four studies with animals were reported, one on the performance of broilers, one on health aspects of rats, and two on health aspects of mice. An important drawback in the broiler trial is, that data were collected on pen level instead of animal level, so that the most important information on variability within the data is lost. Nevertheless, the number of pens is sufficient to detect significant differences if present, but that could have been different, if data were collected at animal level. Another important aspect is the mortality rate, which is much higher than within farming conditions.

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)

NOT APPLICABLE

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

NOT APPLICABLE

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

Mills EN, Jenkins JA, Alcocer MJ, Shewry PR. 2004. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. Crit Rev Food Sci Nutr 44: 379-407.

Shakib F, Ghaemmaghami AM, Sewell HF. 2008. The molecular basis of allergenicity. Trends Immunol 29: 633-642.