Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the notification (Reference C/NL/00/10) for the placing on the market of insect-tolerant genetically modified maize 1507, for import and processing, under Part C of Directive 2001/18/EC from Pioneer Hi-Bred International/Mycogen Seeds[1]

(Question No EFSA-Q-2004-011)

Opinion adopted on 24 September 2004

SUMMARY

This document provides an opinion of the Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) on 1507 maize, genetically modified to provide protection against specific lepidopteran pests. The maize also contains a gene providing tolerance to the herbicide glufosinate. The opinion is based on a question raised by the Commission related to an application for the placing on the market of 1507 maize under the environmental release Directive 2001/18/EC. The GMO Panel was asked to consider whether there is any scientific reason to believe that the placing on the market of 1507 maize, for import and processing, is likely to cause any adverse effects on human health and the environment (Notification C/NL/00/10). The question followed a scientific assessment which was initially made by the competent authorities of The Netherlands and subsequently evaluated by all other Member States. An assessment of the 1507 maize was requested by the Commission because of questions raised by several Member States following the evaluations at the national level. When this is the case, EU legislation requires that EFSA carries out a further assessment and provides an opinion.

In delivering its opinion the Panel considered the application, additional information provided by the applicant and the specific questions and concerns raised by the Member States. Further information from other applications for the placing on the market of 1507 maize under current regulatory procedures, i.e. notification C/ES/01/01 with the extended scope for cultivation and an application under the novel foods Regulation (EC) 258/97 which was transformed into application EFSA-GMO-NL-2004-02 for the authorisation of food products under Regulation (EC) No 1829/2003 on GM food and feed, were taken into account where appropriate. For formal reasons the assessment of the latter applications will result in separate opinions.

The 1507 maize was assessed with reference to its intended use and the appropriate principles described 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, 2004). The scientific assessment included examination of the DNA inserted into 1507 using particle bombardment and the nature and safety of the target proteins produced by the transgenic event with respect to toxicology and allergenicity. Furthermore, a comparative analysis of agronomic

[1] For citation purposes: Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the Notification (Reference C/NL/00/10) for the placing on the market of insect-tolerant genetically modified maize 1507, for import and processing, under Part C of Directive 2001/18/EC from Pioneer Hi-Bred International/Mycogen Seeds, *The EFSA Journal* (2004) 124, 1-18.

traits and composition was undertaken and the safety of the whole feed was evaluated. A nutritional and an environmental assessment, including monitoring plan, were both undertaken.

1507 maize has been developed for protection against specific lepidopteran pests such as the European corn borer (*Ostrinia nubilalis*) and *Sesamia* spp. and for tolerance to the herbicide glufosinate. The insect resistance was provided by expression of a truncated CRY1F protein from *Bacillus thuringiensis* ssp. *aizawai* and tolerance to the herbicide was conferred by a phosphinothricin-N-acetyltransferase (PAT) from *Streptomyces viridochromogenes*. Maize embryos were transformed by particle bombardment to transfer a restriction fragment containing these two genes. As a result of the genetic modification, the 1507 event contains an insert bearing both *cry*1F and *pat* genes, under the control of the maize ubiquitin and the 35S promoters, respectively.

Molecular analysis showed that 1507 maize contains one copy of the DNA fragment used for transformation and that this is present in a single insertion locus in the nuclear genome of the GM plant. The complete DNA sequence of the insert is provided. In addition to the intact genes, the insert in 1507 maize includes DNA sequences originating from the fragment used for transformation as well as maize chloroplast and nuclear genome sequences at both ends of the inserted sequence. Whilst these sequences may have resulted from the transformation process (insertional events) there were no indications that these additional fragments would result in the transcription of new RNA other than the mRNAs transcribed from the *cry*1F and *pat* genes. In the unlikely event that this does occur, bioinformatics analysis showed that any resulting peptides or proteins would have no homology to known toxins or allergens. Analysis of DNA sequences flanking both ends of the insert shows that they correspond to maize genomic DNA.

Analysis of kernel chemical composition from field trials in South America and Europe showed that 1507 was substantially equivalent to its non-GM comparator. Furthermore, appropriate animal feeding trials indicated that 1507 is nutritionally equivalent to its non-GM comparator.

The notification C/NL/00/10 only concerns import and processing. There is therefore no requirement for scientific information on possible environmental effects associated with the cultivation of the maize lines. The GMO Panel agrees that unintended environmental effects due to the establishment and spread of GM maize will not be different from that of traditionally bred maize. The monitoring plan provided by the applicant is in line with the intended uses for the GMO.

In conclusion, the Panel considers that the information available for 1507 maize addresses the outstanding questions raised by the Member States and considers that 1507 maize will not have an adverse effect on human and animal health or the environment in the context of its proposed use.

The GMO panel is of the opinion that a strict separation of the GMO seeds between food and feed chain uses is extremely unlikely. For this reason no single authorisation should be considered unless aspects of both food and feed safety are authorised.

Key words: GMO, maize, *Zea mays*, 1507, insect protection, CRY1F, PAT, feed safety, human health, environment, import, Regulation (EC) 258/97, Regulation (EC) 1829/2003, Directive 90/220/EEC, Directive 2001/18/EC.

TABLE OF CONTENTS

BACKGROUND

The Commission received the notification (Reference C/NL/00/10) for the placing on the market of insect-tolerant genetically modified maize 1507, for import and processing, under Part C of Directive 2001/18/EC (EC, 2001) from Pioneer Hi-Bred International/Mycogen Seeds, on 12 February 2003, following a positive assessment from the lead Member State (The Netherlands).

In accordance with the Directive 2001/18/EC, the notification was then transmitted to the competent authorities of other Member States, a number of which raised objections during the statutory 60-day period. The applicant provided the Member States with additional information in response to the objections raised during this 60-day period. The Member States had until 21 February 2004 to confirm or lift their objections. Where these objections are maintained, the Commission is required to consult the relevant Scientific Committees for opinion, now represented by the EFSA. Some Member States maintained specific objections.

Article 18(1) of Directive 2001/18/EC states that the period of time during which the Commission is awaiting the opinion of the Scientific Committee shall not exceed 90 days. The evaluation by EFSA started on 26 March 2004, after receipt of the complete background information (request from the Commission, dossier of the applicant and final objections maintained by the Member States). During the 90-day period, EFSA requested further clarifications from the applicant.

TERMS OF REFERENCE

EFSA was requested, under Article 29(1) and in accordance with Article 22(5)(c) of Regulation (EC) No 178/2002 (EC, 2002), to provide a scientific opinion as to whether there is any scientific reason to believe that the placing on the market of 1507 maize for import and processing is likely to cause any adverse effects on human health and the environment within the scope of Directive 2001/18/EC.

In particular, EFSA was requested to take account of the scientific objections raised by the competent authorities of Member States in this context.

EFSA was not requested to give an opinion on the non-scientific objections raised by competent authorities in their replies, in the context of the entry into force of forthcoming legislation or requests for further legislative/implementing measures.

ASSESSMENT

1. Introduction

GM maize 1507 was assessed with reference to its intended use and the appropriate principles described 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, 2004). In its evaluation the Panel focused in particular on the issues that were raised by Member States during the initial assessment of the applications introduced under Directive 2001/18/EC and Regulation (EC) 258/97. The assessment presented here is based on the information provided in all available applications related to GM maize 1507 submitted in the EU including additional information from the applicant in reply to Member States questions.

2. Molecular characterization

2.1. Issues raised by Member States

(1) PCR analysis was requested to demonstrate the continuity of the DNA on both sides of the insert in comparison to the recipient plant; (2) a question over the presence of the detected sequences on both sides of the insert giving rise to instabilities of the insert was raised; (3) a question over the existence of a secondary insertion site detectable by Southern analysis was raised; (4) the possibility that very high levels of CRY1F toxin accumulated in specific tissues not subjected to analysis and which might be missed in the analyses was presented.

2.2. Relevant background data

2.2.1. The transformation process and vector constructs

Embryogenic cells of Pioneer Hi-II maize were transformed using particle acceleration technology with tungsten particles coated with a purified linear fragment PHI8999A derived from plasmid PHP8999. For this purpose two restriction fragments of 6235 bp and 3269 bp were produced through *Pme*I-digestion of PHP8999. The larger fragment, named PHI8999A, was purified after agarose gel electrophoresis and the small fragment was discarded.

PHI8999A contains two adjacent plant gene expression cassettes. The first contains a truncated *cry*1F gene derived from the *Bacillus thuringiensis* spp*. aizawai* sequence (Chambers et al., 1991). The coding sequence is regulated by a maize ubiquitin promoter and a maize ubiquitin intron sequence introduced upstream of the *cry*1F sequence. The 3' terminator sequence used is from the *Agrobacterium tumefaciens* mannopine synthase gene. The second expression cassette contains the *pat* gene (OECD, 1999) which is regulated by a 35S CaMV promoter and terminator. The coding sequence of both genes has been optimised to achieve a high level of expression in maize.

2.2.2. Transgenic constructs in the genetically modified plant

The molecular characterisation and expression analysis of 1507 maize revealed that both intended genes, *cry*1F and *pat*, are intact within the transgenic event.

Southern analysis and PCR have been used to provide data on the insert over several generations of the maize event 1507. Only a single insertion locus (comprising a complex structure of different fragments) was detected. Southern analysis using a *cry*1F probe showed that the two observed bands arise from the known truncated *cry*1F fragment present at the 5'

end of the insert and from the *cry*1F gene inserted (and expressed) to produce the insect protection trait. The absence of vector backbone in the 1507 plants has been confirmed by Southern blotting using probes that cover the entire discarded 3269 bp fragment.

The insert of maize event 1507 has been entirely sequenced, including 3' and 5' adjacent maize genome sequences. From the sequence analysis it appears that the insert comprises one almost complete copy[2] of fragment PHI8999A without internal rearrangements. Both, *cry*1F and *pat*, gene cassettes are intact within the transgenic event. The DNA sequences of the genes in 1507 are identical to those in the original plasmid. The proteins produced in the plants are the ones intended, including a leucine residue (replacing a phenylalanine) at position 604 (of 605 amino acids in total) of CRY1F. This modification was introduced to create a specific restriction site for cloning purposes.

Analysis of the insert sequences adjacent to the nearly complete copy of fragment PHI8999A revealed DNA fragments that correspond to small segments from PHI8999A, including incomplete sequences from the *pat* and *cry*1F-genes, the maize ubiquitin promoter and the mannopine synthase terminator from *Agrobacterium*. Furthermore, different fragments of chloroplast DNA and a number of sequences with similarity to retrotransposons are also present in the border region of the insert.

PCR analyses indicated that the fragments in the flanking regions can also be found in the recipient line (Hi-II). No data documenting the intactness of the insertion site was shown. Therefore, a direct comparison of the insertion locus and the respective site in the recipient plant is not possible. Sequences found in the border regions showed a high degree of homology to retrotransposon-like sequences that are considered to be very abundant throughout the maize genome. The design of PCR primers to provide unequivocal evidence that sequences detected in the flanking regions of the 1507 insert are also to be found as continuous sequences in the recipient plant is in general technically difficult. Thus it cannot be assumed that DNA deletions have not occurred during the transformation process. However there is no indication that such a deletion produces any phenotypic effect in the transformed maize line (see Section 3.).

2.2.3. Information on the expression of the insert

Expression analysis of the CRY1F and the PAT proteins were carried out by Western analysis and ELISA. The tissues and plant samples examined were leaf, pollen, silk, stalk, whole plant and grain. The CRY1F protein was found in all tissues examined while the PAT protein could be detected only in leaf and whole plant.

CRY1F Western analyses with protein extracts from different plant tissues revealed a double band (65 to 68 kDa) in the range of the predicted size of 66 kDa which corresponds to the microbially produced CRY1F protein control. The smaller band detected in the 1507 protein extract is assumed to be the result of a limited N-terminal processing of the full size 1507 CRY1F protein during the extraction process by a plant protease with trypsin-like specificity. This assumption is supported by results from N-terminal amino acid sequencing of the protein which revealed a putative trypsin cleavage site starting at amino acid 28 (of 605) of the CRY1F protein. As no further bands were detected by Western analysis there is no evidence that unintended CRY1F-fusion proteins are expressed in 1507 maize.

As additional information the applicant submitted tables including recalculated data of CRY1F ELISA experiments. The data are presented on a ng CRY1F protein/mg tissue dry weight basis

[2] Base pairs 1-10 at the 5´ end and base pairs 6197-6235 at the 3´ end are missing. Both missing parts represent polylinker regions of fragment PHI8999A.

and show that the expression values fall within the same order of magnitude for different years and locations. Maximum expression (on a tissue dry weight basis) was found in pollen (average 20.0 and maximum 29.3 ng CRY1F protein/mg tissue dry weight). The values for whole plant extracts ranged between 1.0 and 6.9 ng CRY1F protein/mg tissue dry weight and for kernels between 1.2 to 3.1 ng CRY1F protein/mg tissue dry weight. The expression of CRY1F was not influenced by the application of glufosinate.

Measurable expression levels of PAT protein were only found in leaves (<LOD[3] – 136.8 pg/µg TEP[4]) and whole plant extracts (<LOD – 38.0 pg/µg TEP) whereas the mean value for leaf was 42.0 pg/µg TEP and for whole plant below LOD. For kernels all measured data were below LOD. Western analysis of PAT protein in leaves revealed only two bands of the expected size (ca. 22 kDa and 43 kDa [putative homodimer]). This indicates that no partial PAT proteins or fusion proteins were present at detectable levels.

Bioinformatics analysis of the insert sequence indicates the presence, in addition to the two intended transcripts detected in the transgenic plant, of one further ORF of more than 300 bp length (ORF4: 630 bp) on fragment PHI8999A and a number of other ORFs (including ORF3 of 753 bp length) spanning the junctions between maize DNA and DNA originating from the transformation fragment. This raises the possibility that new putative fusion proteins could be produced. A detailed analysis of the potential gene expression is provided for the two sequences longer than 300 bp (ORF3 and ORF4). No transcript corresponding to ORF3 was detected by either Northern or RT-PCR analysis in experiments with mRNA from developing kernels. Northern analysis revealed no expression of ORF4 but a weak signal was detected using RT-PCR which also indicated that the detected mRNA originates from a read-through product of the *cry*1F gene. In the very unlikely event that a protein were expressed from ORF4 on the read-through mRNA by using an alternative translation start codon or indeed if any of the other ORF were transcribed and translated at a very low level, no adverse effects are expected as bioinformatics analysis revealed no significant homologies with known allergens. No known allergenic, toxic or coeliakie related proteins are encoded.

2.2.4. Inheritance and Stability of inserted DNA

The 1507 event was produced in the maize line Hi-II. The event was transferred to a Pioneer elite inbred line and the resulting plants backcrossed to the elite line for six generations. The Mendelian inheritance pattern of the traits was assessed together with the physical linkage of the target genes in resulting progeny. Southern blots and maintenance of the phenotype indicated genetic and phenotypic stability of the transgenic line and their progeny over several generations. No instability of the DNA sequences flanking the insert was observed.

2.2.5. Conclusion

GM maize line 1507 was generated through particle bombardment transformation of maize line Hi-II. Detailed molecular analysis of the insert and Mendelian inheritance of the trait indicated that one copy of fragment PHI8999A used for the transformation was inserted stably over several generations at a single locus in the maize nuclear genome. The inserted fragment is flanked by several fragments originating from the recipient maize plant chloroplast and nuclear genome and from fragment PHI8999A.

Evidence that the maize genomic DNA was contiguous with the flanking regions of the insert was not provided. The possibility of undetected deletions at the insertion site caused by the

[3] LOD = limit of detection

[4] TEP = total extractable protein

transformation process has been considered. The Panel is of the opinion that it is very unlikely that putative deletions or rearrangements at the insertion locus would result in undiscovered adverse effects. Firstly, a large proportion of the maize genome consists of non-coding sequences. Secondly, other elements of the overall risk assessment (see data provided in Section 3) show no indication of any unintended adverse effects. Thirdly, deleted components will in most cases be complemented in commercial hybrids.

In conclusion, the Panel is of the opinion that the transgenic insert in 1507 maize was sufficiently analyzed and described. None of the DNA stretches including the chloroplast DNA sequences detected in the insert region provide grounds for specific concern.

The intended expression of the PAT and CRY1F proteins was demonstrated and the expression levels were shown to be in the same range for different locations and growing seasons. The detection of a read-through mRNA comprising ORF4 sequences was shown. Bioinformatics assessment provided no indication that the development of allergenic or toxic products would arise in the very unlikely event that the read-through mRNA is translated to the respective protein.

Stability of the inserted DNA and of the expression of CRY1F and PAT proteins in the transgenic line was demonstrated. There were no indications of instabilities in expression.

3. Comparative Analysis

3.1. Issues raised by Member States

(1) Additional data on lignin content were requested, based upon literature data indicating that these levels would be increased in transgenic maize lines expressing *B. thuringiensis* insecticidal proteins; (2) it was questioned whether levels of CRY1F in tissues of 1507 maize were significantly different over the locations and years.

3.2. Relevant background data

3.2.1. Choice of comparator

1507 maize was compared with control hybrids that had not been genetically modified and that had background genetics representative of 1507 maize, except for the inserted genes (F_1-generation). Kernels obtained from maize plants grown during the field trials were used for analysis (F_2-generation).

3.2.2. Agronomic Traits

Extensive agronomic data were collected and confirmed the similarity of 1507 maize to its non-transgenic counterpart.

3.2.3. Compositional analysis

Compositional analysis was performed on whole plants collected from field trials and on maize tissues including ears with kernels. These field trials occurred during 3 seasons and at different locations (6 locations in Chile (1998-1999), 3 locations in France and Italy (1999), and 6 locations in France, Italy and Bulgaria (2000). Maize plants in Chilean field trials were treated with glufosinate, while those in the European field trials were split in treated and untreated groups.

The proximate and mineral analyses (fat, protein, acid detergent fibre, neutral detergent fibre, ash, carbohydrate, phosphorus, and calcium) of forage from maize line 1507 (glufosinate-treated and non-treated) were comparable to forage from the non-transformed version of the hybrid and within typical ranges reported in literature for commercial maize hybrids. Statistically significant differences were occasionally observed in some GM plants, for example increased overall levels of carbohydrates and decreased levels of fat in forage of maize line 1507 (both sprayed and non-sprayed) in the 2000 season. However, there were no differences that were consistently observed over years and at each location.

The compositional analysis of kernels of 1507 maize hybrid and its control included proximate analyses (as for forage above), fatty acid composition [palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)], amino acids (12 essential and 6 non-essential amino acids), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), vitamins (vitamin B1, vitamin B2, folic acid, and total tocopherols), secondary metabolites (inositol, raffinose, furfural, *p*-coumaric acid, and ferulic acid), and anti-nutrients (phytic acid and trypsin inhibitor). Kernels from the 2000 season were additionally analysed for crude fibre, arachidic acid, provitamin A, and vitamin E.

In summary, the analysis of nutrient composition of kernels from maize line 1507 (glufosinate-treated and non-treated) occasionally revealed statistically significant differences in some compounds. For example, kernels of 1507 maize contained higher overall levels of potassium, linoleic acid, linolenic acid, and tocopherols, as well as lower levels of fat, manganese, stearic acid, oleic acid, cysteine, methionine, and vitamin B1, than control kernels in the 1998-1999 season. The levels of protein, amino acids (Ala, Asp, Glu, Gly, His, Leu, Phe, Pro, Ser, Thr, Tyr, and Val), and potassium were increased, while the level of vitamin B2 was decreased, in kernels of 1507 maize (both sprayed and non-sprayed) compared to control kernels in 1999. In the 2000 season, ash, amino acids (Ala, Phe, Tyr), and potassium were increased, while manganese was decreased in kernels of maize line 1507 (both sprayed and non-sprayed) compared to controls. However, across locations and between years there were no consistent statistically significant differences. All analytical data were either very close to or within the ranges published in the literature.

It has been suggested that lignin levels might be increased in transgenic maize lines expressing *B. thuringiensis* insecticidal proteins (Saxena and Stotzky, 2001). However, a recent broader and more extensive study on lignin content in Bt-maize does not support this conclusion (Jung and Sheaffer, 2004).

3.2.4. Conclusion

Based on these results of compositional analysis, it is concluded that forage and kernels of 1507 maize are compositionally equivalent to those of conventional maize, except for the presence of CRY1F protein in 1507 maize. These data apply to samples from South America (Chile) and Europe (France, Italy, and Bulgaria). Given the fact that South America (*e.g.* Argentina) has a significant export of maize kernels to the EU, these geographical areas are representative for areas of maize cultivation and export.

4. Food/Feed Safety Assessment

4.1. Issues raised by Member States

(1) Bioinformatic analysis was requested to compare the conformations of MR872 (microbially produced, trypsinized Bt-toxin) and the plant-expressed CRY1F protein; (2) it was argued that the CRY1F produced by plants might differ from the Bt-toxin produced by bacteria, e.g. with regard to posttranslational modifications besides glycosylation; (3) further animal feeding studies,

including tests on ruminants, laying hens, pigs, fish, and crustaceans, with whole products, including forage, derived from 1507 maize were requested; (4) additional toxicological testing comprising various tests, including chronic testing was requested.

4.2. Relevant background data

4.2.1. Product description and intended use

The notification (C/NL/00/10) covers import and processing of 1507 maize including the placing on the market of feed products derived thereof as any other conventional maize. The food uses of 1507 maize are covered by another application[5]. Maize kernels are a rich source of carbohydrate, while starch production produces by-products, such as maize gluten and maize gluten feed, which are used as animal feed.

Maize kernel products are used in various animal feeds, including cattle, swine, poultry, and in fish feed.

4.2.2. Stability during processing

Experimental fish feed containing 38.7 % maize meal was prepared in order to test the stability of CRY1F during processing. The CRY1F level in transgenic maize kernels was 2.2-3.5 ng/mg tissue dry weight prior to processing. The production of fish feed included an extrusion step, exposing feed ingredients to high pressure and temperature. CRY1F was not detectable in the final product, as established through an insect bioassay and immuno-assay (ELISA – LOD = 0.04 ng/mg tissue dry weight).

In addition, the thermostability of recombinant CRY1F protein produced by *Pseudomonas fluorescens* at elevated temperatures was assessed by heating solutions of 1.3 ppm CRY1F in phosphate buffer pH 7.5 at 60, 75, or 90 ºC for 30 minutes. Aliquots were taken from these solutions and added to feed used in a bioassay for insecticidal activity on tobacco budworm (*Heliothis virescens*). It was thus observed that the CRY1F proteins heated at 75 and 90ºC had lost their insecticidal activity.

4.2.3. Toxicology

4.2.3.1. CRY1F and PAT Proteins used for safety assessment

Given the low expression levels of CRY1F in 1507 maize, the applicant decided to use a trypsinized microbial analogue, MR872, of the truncated CRY1F protein expressed in maize line 1507 for safety testing. To this end, a fusion protein consisting of the non-truncated CRY1F (N-terminal) linked to CRY1Ab (C-terminal) was produced by recombinant *Pseudomonas fluorescens*. Trypsin cleavage sites in CRY1F are located between residues 28-29, 31-32, and 612-613. Enzymatic cleavage with trypsin of the fusion protein yielded a 'core' protein, MR872, identical to the truncated CRY1F protein expressed in 1507 maize, except for i) phenylalanine (Phe) at position 604 instead of leucine (Leu) and ii) a C-terminal extension of trypsinized MR872 with seven amino acid residues (606-612, Ala-Glu-Tyr-Asp-Leu-Glu-Arg). With regard to the conformation of CRY1F, it is considered unlikely that the substitution at position 604 would lead to conformational changes because both Phe and Leu are amino acids with hydrophobic side chains. The extension of the trypsinized MR872 protein with seven amino acids at the C-

[5] A separate application under Novel Food Regulation (EC) No 258/97 has been filed – recently transformed into an application under Regulation (EC) 1829/2003, file no EFSA-GMO-NL-2004-02.

terminus of domain III is also present in native CRY1F from *B. thuringiensis*, as well as in other CRY proteins. Comparison of the crystal structure of CRY1Aa containing this extension (Grochulski et al., 1995) with that of CRY3A lacking this extension (Li et al., 1991) does not indicate differences in the overall structure of domain III. It is therefore unlikely that this extension would affect the functional, toxicological, or allergenic properties of the protein.

Both bacterially produced CRY1F and plant-expressed CRY1F isolated from leaves and kernels of 1507 maize displayed a prominent 65 kDa band on Western blots, which corresponds to the N-terminally processed form of plant-expressed Cry1F as mentioned in section 2.2.3. Glycosylation was analysed after SDS PAGE using a commercial staining kit. The results demonstrate that the plant-expressed CRY1F is not glycosylated. Moreover, MALDI-TOF mass spectrometry was performed on trypsin-digests of the recombinant CRY1F proteins produced by transgenic *P. fluorescens* and 1507 maize and separated by electrophoresis. Fragments were observed in the spectra of both types of CRY1F protein that concurred with the predicted masses of peptides derived from trypsin digestion, covering 34-39 percent of the total protein sequence (605 amino acids) encoded by the *cry*1F transgene in 1507 maize in various experiments. Data provided by the applicant on insect bioassays with recombinant CRY1F show no notable differences between preparations of this protein isolated from transgenic maize event 1360 (modified with CRY1F) and *P. fluorescens*.

Taking into account all the evidence provided, the Panel is of the opinion that the trypsinized MR872 analogue is an appropriate substitute of the CRY1F protein expressed in 1507 maize for safety testing.

Bacterially produced recombinant PAT showed the same electrophoretic mobility as PAT expressed in 1507 maize during Western blotting. As noted above, levels of PAT were not quantifiable in kernels of 1507 maize.

4.2.3.2. Safety of expressed novel proteins in 1507 maize

Acute oral toxicity

An acute oral study was performed in albino mice dosed with 576 mg truncated CRY1F/kg bodyweight (5050 mg/kg test material containing 11.4 % CRY1F). No effects related to the administration of CRY1F were noted on bodyweight, gross necropsy, and mortality 14 days after the administration, except for one incidental finding out of 10 of lack of body weight gain between days 7 and 14.

For PAT, a study was performed, in which mice received 5000 mg PAT/kg bodyweight (equals 6000 mg test material/kg). After two weeks, no effects on bodyweight and gross pathology were noted.

Degradation in simulated digestive fluids

The trypsin-resistant core of the microbially produced CRY1F protein was rapidly degraded (<1 minute) in simulated gastric fluid at a CRY1F/pepsin molar ratio of 188:1 and 1:22. In the SDS PAGE gels of the incubation mixture, a 10-kDa band was visible that was relatively stable during the length of the experiments. This was probably a contamination of the microbial CRY1F-preparation, as it was not detected in Western analysis with anti-CRY1F immune sera.

In simulated intestinal fluid (pancreatin), the trypsin-resistant CRY1F core protein proved stable over the entire exposure of 120 minutes.

For degradation of the PAT protein, reference is made to previous studies in which PAT was degraded within 5 seconds in simulated gastric fluid.

4.2.3.3. Glufosinate residues

Since the safety of residues of glufosinate applied to 1507 maize has to be demonstrated for market approval under a different Directive (91/414/EEC), pesticide safety is not within the remits of this opinion.

4.2.4. Safety of the whole GM food/feed

Subchronic oral toxicity

A 90-day oral toxicity study has been performed on rats in five groups (12 animals/sex/group) fed diets containing 1507 maize (11 and 33 %), a non transgenic control line with comparable genetic background (11 and 33 %), and another non transgenic maize line as reference (33 %). The diets were analysed for nutrients, antinutrients, mycotoxins, pesticides, heavy metals, transgenic DNA, and CRY1F (insect bioassay). Kernels used in this study were obtained from 1507 maize plants which had not been treated with glufosinate. The measurements on animals included feed consumption, body weight, clinical pathology (serum, blood, urine), and anatomical pathology (organ weights, histopathology).

A statistically significant increase in feed consumption was observed in male rats fed 33% 1507 maize compared to rats fed control maize, but not to those fed the reference maize (27.5 ± 2.6, 25.7 ± 1.7, and 27.3 ± 1.7 g per day, respectively). This effect is therefore not considered to pose concerns over the safety and nutritional value of 1507 maize.

A number of histopathological changes were observed, in particular inflammation of liver, nephropathy, and cardiomyopathy (kidney and heart damage) in animals of both sexes. To a lesser degree, inflammations of prostate in males and pancreas in females, fatty change in liver of females, and atrophy of pancreas in males were observed. These effects were not linked to the test-substance, since their incidences were not substantially elevated in the animals fed 1507 maize compared to control animals. This study, on the basis of presented results, is considered satisfactory and does not raise concerns over the safety of 1507 maize.

4.2.5. Allergenicity

The strategies in assessing the allergenic risk concentrate on characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons and whether the transformation may have altered the allergenic properties of the modified food. A weight of evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (EFSA, 2004; CAC, 2003).

Potential homology of the gene products with known allergens

The PAT protein has been previously evaluated for its safety in the frame of other applications for the placing on the market of PAT-expressing GM crops. The potential allergenicity of the transgenic CRY1F protein and of the theoretical expression products of ORF4 (within PHI8999A copy on the insert), and 24 ORFs (including ORF3) coding for putative fusion proteins in the regions adjacent to the PHI8999A copy on the insert were considered in this dossier.

The amino acid sequence of the CRY1F protein has been compared to the sequences of allergenic proteins compiled in an allergen database[6]. This comparison focused on two types of identity between CRY1F and allergens: (1) short linear stretches; relevant minimum size is eight contiguous amino acids and (2) overall identity of 80-amino-acid peptides of CRY1F (min. 35% identity relevant).

For both types of comparison, the FastA algorithm was applied, with appropriate settings. No outcomes were equal to or exceeded the minimum relevant size. The length of the longest identical short linear stretch, for example, was six amino acids.

In addition, comparison of the CRY1F sequence against a general protein database yielded predominantly homologies with other CRY-proteins (e.g. CRY1Ab with 52.4% identity over a 614 residue alignment overlap), except for 3 proteins from *Methanosarcina acetivorans*, *Saccharomyces cerevisiae,* and *Sinorhizobium meliloti*. These 3 proteins are not known to be toxic and therefore this result does not indicate any homology of the CRY1F with toxic proteins

Three different linear six-amino-acid stretches were found to be shared by CRY1F with allergenic proteins (Der p 7 from house dust mite, beta-1,3-glucanase-like protein from olive, and Can f 3 from dog dander). The EFSA panel is aware of studies that show that using a threshold of 6 amino acids for identical stretches between a given protein and allergens yields a high number of false positives, i.e. this threshold makes the comparison unspecific. Using a newly developed methodology (Soeria-Atmadja et al., 2004), the Swedish National Food Authority found that for CRY1F, many 6-amino acid identities with non-allergenic proteins existed (data not published). Kleter and Peijnenburg (2002) further found that many transgenic proteins shared identical 6- and 7- amino acid stretches with allergens. For the identical sequences that CRY1F shared with allergens (same as found by the applicant) these authors found no indications that they were part of IgE-epitopes. Therefore it is unlikely that these identical stretches within CRY1F would induce allergic reactions.

In addition, the highest degree of identity of 80-residue fragments of CRY1F was 33.8% identities (27 residues) with a pollen allergen (Syr v I) from *Syringa vulgaris* and with related olive pollen allergens.

Because the minimum relevant matches are eight-amino-acid linear sequences and 35 % identity of 80-residue fragments, respectively, the search has yielded no outcomes that raise safety concerns for CRY1F.

The same methodology to search for short identical and larger similar stretches of homology to the proteins listed in the allergen database has been applied to assess the hypothetical peptides derived from ORF4 (within copy of PHI8999A on the insert) and the 24 ORFs (including ORF3) coding for putative fusion proteins in the regions adjacent to the PHI8999A copy on the insert. In addition, the ORF3- and ORF4-sequences were compared to the sequences of a general protein database.

For ORF 4, the length of the longest identical short linear stretch, for example, was six amino acids shared with allergenic proteins from durum wheat (glutenin) and wheat (gamma-gliadin). An 80-residue fragment of ORF4 shared twenty-two identical residues (27.5 %) with major hazel pollen allergen Cor a 1. In a comparison of ORF4 to general protein sequences, the protein from ORF VI of Cauliflower Mosaic Virus, followed by proteins from Carnation Etched Ring virus and *Plasmodium falciparum,* were most identical to the ORF4 sequence.

[6] Update March 2002 comprises 2033 entries compiled from published lists supplemented through a search of public domain protein databases (applied update: March 2002).

ORF3 shared two identical linear sequences of six amino acids with the allergen Gly m IA from soybean and with the allergens gamma-gliadin and alpha/beta-gliadin from wheat. In addition, an 80-residue fragment of ORF3 shared eighteen identical residues (22.5 %) with the allergenic barley alpha amylase/trypsin inhibitor precursor and also with Sin a I allergen from white mustard. The highest scoring identities of the sequence of ORF3 with general protein sequences in a public database were those with chloroplast RNA polymerases of various plant organisms and with phosphinothricin acetyltransferase enzymes. Some of the other 23 ORFs in the flanking regions shared 6-amino acid identities with allergens. However, none of these ORFs shared relevant homologies with allergens consisting of identical linear sequences of 8 amino acids minimum or 35%-identities of 80-aa subsequences. In the comparison of these ORFs with a general protein database, none of the sequences sharing the most relevant identities with the ORFs were known to be toxic.

The degradation of gene products during processing at high temperature and in simulated digestive fluids, which is also relevant for the assessment of potential allergenicity, has been discussed in section 4.2.2. and section 4.2.3.

Allergenicity of the whole plant

Allergenicity of the whole crop could be increased as an unintended effect of the random insertion of the transgene in the genome of the recipient, for example through qualitative or quantitative modifications of the pattern of expression of endogenous proteins. This issue does not appear relevant to the Panel since maize is not considered a major allergenic food and possible over expression of any endogenous protein that is not known to be allergenic would be unlikely to alter the overall allergenicity of the whole plant.

4.2.6. Nutritional assessment of GM food/feed

A 42-day feeding study was carried out with broilers to investigate nutritional equivalency. Diets contained on average 55 % dry matter (DM) maize kernels from either the transgenic hybrid maize 1507, the control hybrid maize Mycogen 7250, and four commercial maize hybrids. Each diet was fed to 35 animals (divided into 7 replicates of 5 animals). No statistically significant differences were observed for mortality, body weight, body weight gain, and feed conversion between the different maize lines.

Twenty lactating dairy cows were used in a single cross-over design in which there was 2 x 28-day feeding periods. The aim was to compare the effect of using maize silage and maize kernels derived from transgenic 1507 maize on feed intake and milk production when compared with maize silage and maize kernels derived from a non-GM control variety.

Diets contained on average 43.0 % DM maize silage and 22.1 % concentrate of which 70.2 % was in the form of ground maize. Other feed ingredients included alfalfa hay, soybean meal, and cotton seeds. The diet composition was analysed for proximates, minerals (Ca, P, Mg, K), mycotoxins and silage fermentation products and found to be similar for both treatment groups. CRY1F was detected in transgenic maize kernels and silage. PAT was not detectable in kernels, and ranged from not detectable to slightly above the detection threshold in forage, of 1507 maize.

The following measurements were made: (1) Physical (weekly): body weight, condition, temperature, pulse, feed intake; (2) Milk production (daily); (3) Milk composition (weekly): protein, fat, dry matter, lactose, urea N, somatic cell count, CRY1F; (4) Blood analysis (prior to and at the end of both trials): chemical and haematological

One cow was positive for the presence of CRY1F in milk prior to and during both treatments, which can therefore be considered a false positive ELISA-reaction.

In conclusion, results showed no significant differences between dietary treatments and indicate nutritional equivalence between the transgenic 1507 maize and the non-GM control.

4.2.7. Conclusion

The transgenic CRY1F protein showed no adverse effects in an acute oral mouse study. In addition, CRY1F displayed instability towards conditions that prevailed during the production of fish feed including heating and was rapidly degraded in simulated gastric fluid.

The sequence of the transgenic CRY1F did not show any significant similarity with the sequences of known allergens. Neither did the hypothetical peptide sequences corresponding to 24 ORFs that are present on the insert in 1507 maize as well as ORF4 on fragment PHI8999A show significant similarity to allergens or toxins.

With regard to animal studies with the whole product, no oral toxicity of 1507 maize was observed in a 90-day rat study. In addition, nutritional data comprising target animal feeding studies with the whole maize kernel on broilers and dairy cows indicate that 1507 maize is nutritionally equivalent to other conventional maize varieties. These animal studies therefore further support the findings of the compositional analysis of no effect beyond the intended introduction of the PAT and CRY1F proteins.

The Panel is of the opinion that there is no need for additional toxicity testing.

5. Environmental Risk Assessment and Monitoring Plan

5.1. Issues raised by Member States

(1) Concerns were expressed that the environmental risk assessment did not adequately address the environmental exposure; (2) the provided monitoring plan was considered to be insufficient.

5.2. Relevant background data

The notification C/NL/00/10 for 1507 maize is for import and processing only, and thus the scope of the monitoring plan provided by the applicant is in line with the intended uses for the GMO. Maize is highly domesticated and not generally able to survive in the environment without cultivation. Maize plants are not winter hardy, they have lost their ability to release seeds from the cob and they do not occur outside cultivated land in Europe, despite cultivation for many years. In addition, there are no cross compatible wild relatives in Europe, and gene flow via pollen is largely restricted to neighbouring crops. Maize is a hybrid crop and thus imported seeds will be a segregated F2 generation and not as fit as the F1. Field experiments carried out in France, Italy, Bulgaria and in South America demonstrated that 1507 maize has no altered survival, multiplication or dissemination characteristics. The Panel agrees with the assessment that the likelihood of unintended environmental effects due to the establishment and spread of 1507 maize will be no different to that of traditionally bred maize.

There is an issue that gene products, particularly CRY proteins might enter the environment either from the gastro-intestinal tracts of animals or through horizontal gene flow to bacteria. Data supplied by the applicant and other literature suggests that most protein would be denatured by enzymatic activity in the gastro-intestinal tract so that little CRY toxin would

survive to pass out in faeces. There would subsequently be further degradation of proteins in the manure due to microbial processes. Thus amounts of CRY proteins being distributed onto land in manure would be very low minimizing the possibility for exposure of potentially sensitive non-target organisms.

The Panel considered possible differences between the plant expressed and the microbially derived CRY1F protein regarding potential effects on non-target organisms. Equivalence tests showed that the activity and structure of CRY1F proteins derived from plant and microbe are comparable. Furthermore, the amino acid sequence of the biologically active core, immunoreactivity, glycosylation and biological activity were comparable between plant-expressed and microbially-produced protein.

The objectives of a monitoring plan according to Annex VII of Directive 2001/18/EC are to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment are correct and to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment which were not anticipated in the environmental risk assessment. The scope of the monitoring plan provided by the applicant is in line with the intended uses for the GMO since the environmental risk assessment did not cover cultivation.

5.3. Conclusion

The 1507 maize is being assessed for import only and thus there is no requirement for scientific information on environmental effects associated with cultivation. Maize is highly domesticated and not able to survive in the environment without cultivation. The Panel agrees that unintended environmental effects due to the adventitious establishment and spread of GM maize will be no different to that of traditionally bred maize. The scope of the monitoring plan provided by the applicant is in line with the intended uses for the GMO since the environmental risk assessment did not cover cultivation. The Panel advises that appropriate management systems should be in place to restrict seeds of 1507 maize entering cultivation, as the latter requires specific approval under Directive 2001/18/EC.

CONCLUSIONS AND RECOMMENDATIONS

Maize line 1507 has been developed for protection against lepidopteran pests by expressing the CRY1F Protein and for tolerance to glufosinate by the introduction of a *pat* gene from *Streptomyces viridochromogenes.* The GMO Panel has assessed information provided on molecular inserts within the transgenic event, on the safety of the proteins expressed and on the potential for risks associated with any changes to the nutritional, toxicological and allergenic properties of 1507 maize. Analysis of the chemical composition of the maize and field trial data were also used to assess the potential for changes to safety, nutritional as well as agronomic parameters. No data has emerged to indicate that maize line 1507 is any less safe than its non-GM comparators. The EFSA GMO Panel is therefore of the opinion that there is no evidence to indicate that the placing on the market of maize line 1507 and derived products is likely to cause adverse effects on human and animal health and the environment in the context of its proposed use.

The GMO panel is of the opinion that a strict separation of the GMO seeds between food and feed chain uses is extremely unlikely. For this reason no single authorisation should be considered unless aspects of both food and feed safety are authorised.

DOCUMENTATION PROVIDED TO EFSA

1. Note to Mr. Podger, dated 23 January 2004 with ref. C4/HM/KT/sfD(2004) 440072, from Mr. J. Delbeke – advance copy of a request to EFSA concerning notification C/NL/00/10 (1507 maize).

2. Note to Mr. Koëter, dated 26 February 2004 with ref. C4 KT/ D(04) 440238, from Mr. J. Delbeke – transmission of Member State objections concerning notification C/NL/00/10 (1507 maize).

3. Initial comments and final objections from Member States with regard to notification C/NL/00/10 (1507 maize).

4. Meeting record between the competent authorities, applicant and Commission, on 2 February 2004, where the objections were discussed.

5. Note to Mr. Koëter, dated 4 March 2004 with ref. C4HM/KT/sf/ D(04) 440273, from Mr. J. Delbeke – late response from France concerning notification C/NL/00/10 (1507 maize).

6. Note to Mr. Koëter, dated 12 March 2004 with ref. C4/KT/sf/D(04) 440299, from Mr. J. Delbeke – additional responses from Austria and Italy concerning notification C/NL/00/10 (1507 maize).

7. Submission from Pioneer/Mycogen Seeds (26 March 2004) to EFSA regarding the scientific review by EFSA of the Application for consent to place on the market insect-tolerant genetically modified maize 1507, for import and processing, under Part C of Directive 2001/18/EC, containing:

    a) a letter from Pioneer/Mycogen Seeds to the competent authority of The Netherlands concerning submission of the notification,
    b) the summary of the notification,
    c) the assessment report of the notification carried out by the competent authority of The Netherlands,
    d) the notification submitted by Pioneer/Mycogen Seeds,
    e) additional information submitted by Pioneer/Mycogen Seeds in response to comments and objections raised by the competent authorities of Member States.

8. Letter from EFSA to applicant with request for clarification/additional information (ref. SR/ (2004) 436, 17 June 2004).

9. Additional information submitted by Pioneer/Mycogen Seeds on 25 June 2004 in response to EFSAs request for further information.

10. The following applications dossiers concerning 1507 maize including assessment reports, the respective Member States comments/objections and additional information submitted by Pioneer/Mycogen Seeds were considered where appropriate:

    a) Notification (C/ES/01/01) to market products containing genetically modified organisms in accordance with Directive 2001/18/EC submitted by Pioneer/Mycogen Seeds to EFSA on 19 May 2004.
    b) Application for placing on the market of novel foods and novel food ingredients containing genetically modified organisms in accordance with Regulation (EC) 258/97 submitted by Pioneer/Mycogen Seeds to EFSA on 26 March 2004.

c) Transformed application (EFSA-GMO-NL-2004-02) for authorisation of food products of 1507 maize in accordance with Regulation (EC) 1829/2003, submitted by the Dutch authorities to EFSA on 10 June 2004.

REFERENCES

CAC, 2003. Codex principles and guidelines on foods derived from biotechnology. Joint FAO/WHO Food Standards Programme, Food and Agriculture Organisation, Rome. ftp://ftp.fao.org/codex/standard/en/CodexTextsBiotechFoods.pdf

Chambers, J.A., Jelen, A., Gilbert, M.P., Jany, C.S., Johnson, T.B. and Gawron-Burke, C., 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* sbsp. *aizawai*. J. Bacter., 173(13), 3966-3976.

EC, 1991. Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. Official Journal of the European Communities, L230, 1-32. http://europa.eu.int/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=31991L0414&model=guichett

EC, 2001. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities, L106, 1-39. http://europa.eu.int/eur-lex/pri/en/oj/dat/2001/l_106/l_10620010417en00010038.pdf

EC, 2002. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ, L31, 1-24. http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/l_031/l_03120020201en00010024.pdf

EFSA, 2004. Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. The EFSA Journal, 99, 1-94. http://www.efsa.eu.int/science/gmo/gmo_guidance/660_en.html

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.L., Brousseau, R., Cygler, M., 1995. Bacillus thuringiensis CryIA(a) Insecticidal Toxin: Crystal Structure and Channel Formation. J. Mol. Biol. 254(3), 447-64.

Jung, H.G. and Sheaffer, C.C., 2004. Influence of Bt Transgenes on Cell Wall Lignification and Digestibility of Maize Stover for Silage. Crop Science, 44, 1781-1789.

Kleter, G.A., Peijnenburg, A.A.C.M., 2002. Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens. BMC Structural Biology, 2, 8. http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=139984&action=stream&blobtype=pdf

Li, J.D., Carroll, J., Ellar, D.J., 1991. Crystal structure of insecticidal delta-endotoxin from Bacillus thuringiensis at 2.5 A resolution. Nature, 353, 815-21.

OECD, 1999. Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. Series on Harmonization of Regulatory Oversight in Biotechnology No. 11. Organisation for Economic Co-operation and Development, Paris. http://www.olis.oecd.org/olis/1999doc.nsf/LinkTo/env-jm-mono(99)13

Saxena, D. and Stotzky, G., 2001. Bt corn has a higher lignin content than non-Bt corn. Am. J. Bot., 88, 1704-1706.

Soeria-Atmadja, D., Zorzet, A., Gustafsson, M, Hammerling, U., 2004. Statistical evaluation of local alignment features for prediction of allergenicity using supervised classification algorithms. Int. Arch. Allergy Immunol., 133, 101-112.

SCIENTIFIC PANEL MEMBERS

Hans Christer Andersson, Detlef Bartsch, Hans-Joerg Buhk, Howard Davies, Marc De Loose, Michael Gasson, Niels Hendriksen, Sirpa Kärenlampi, Ilona Kryspin-Sørensen, Harry Kuiper, Marco Nuti, Fergal O'Gara, Pere Puigdomenech, George Sakellaris, Joachim Schiemann, Willem Seinen, Angela Sessitsch, Jeremy Sweet, Jan Dirk van Elsas and Jean-Michel Wal.

ACKNOWLEDGEMENT

The GMO Panel wishes to thank Gijs Kleter and Richard Phipps for their contributions to the draft opinion.