Notification 6786-01-0185 (B/DE/06/185)

Summary of the risk assessment of the German Competent Authority

regarding LMO

NK603, MON89034, MON88017, MON89034 x MON88017, MON89034 x NK603

(courtesy translation, only the German text is authentic)

III.1.2.1. Evaluation of changes in the genetically modified plants effected by nucleic acid sequence transfer

(a) The *epsps* gene

The gene for glyphosate-tolerant EPSPS from the *Agrobacterium* sp. strain CP4a in these genetically modified maize plants is constitutively expressed under the control of the CaMV 35S promoter and the *Act1* promoter from rice (*Oryza sativa*). The presence of introns in both transcription units leads to enhanced gene expression. The upstream position of the EPSPS chloroplast transit peptide derived from *Arabidopsis thaliana* (CTP2) results in the post-translational import of CP4 EPSPS into the chloroplasts. As a rule, the transit peptide is cleaved on import (processing).

The endogenous EPSPS, as well as the CP4 EPSPS inserted into the maize plants by means of transformation, act as catalysts in the chloroplast, effecting a reaction of the shikimat-3-phosphate with phosphoenolpyruvate to give 5-enolpyruvylshikimate-3-phosphate, an intermediate stage in the biosynthesis of aromatic amino acids and other aromatic substances of secondary plant metabolism. In contrast to the endogenous EPSPS, the CP4 EPSPS is not inhibited by glyphosate.

The additional expression of CP4 EPSPS in genetically modified maize catalyses the same reaction as the corresponding enzymes that occur naturally in maize and other cultivated crops. Since no adverse health effects have been attributed to the *Arabidopsis thaliana*-derived transit peptide EPSPS CTP2, or to any other currently known signal peptides, whether processed or unprocessed, it can be assumed that the same applies to transit peptide-enzyme compounds (in this case CP4 EPSPS). No evidence exists to suggest that newly formed EPSPS could have a toxic effect.

The mode of action of EPSPS inserted by means of transformation is not expected to pose risks to human or animal health or to the environment.

(b) The *cry3Bb1* gene

The *cry3Bb1* gene is derived from *Bacillus thuringiensis* ssp. *kumamotoensis* and codes for a coleopteran-specific protein toxin (Bt toxin). There is no evidence of enzymatic activity in the protein expressed in the genetically modified organism. It can therefore be assumed that

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apart from the formation of Bt toxin in the genetically modified plant, there will be no further impact on the plant metabolism.

The additional gene present in genetically modified maize plants, which codes for the synthetic variant of the Cry3Bb1 gene, is constitutively expressed under the control of the CaMV 35S promoter. The intron of the rice actin 1 gene enhances transcription efficiency. In the alkaline environment of the intestinal tract of insect larvae the so-called δ-endotoxin is solubilized. It then permeates the peritrophic membrane and binds to specific receptors in the epithelium of the midgut, altering the electrolyte permeability of the intestine and leading to a disturbance of the pH value of the digestive tract. The insect ceases to feed and dies. Receptors for δ-endotoxin do not exist in the digestive tract of mammals. In feeding studies attached to the application for placing on the market of MON863 maize (which expresses the same protein), there was no indication of any negative effects resulting from the Bt protein in rat, chicken and mouse feed.

Notably, the results of the toxicity study on rats recently led to divergent interpretations in regard to the safety of MON863 and the Cry3B1 protein. The toxicity study represents just one element of safety testing carried out on the basis of the documentation submitted by the applicant. A number of other comprehensive parameters were included in the safety evaluations conducted by the German regulatory authority, the Robert Koch Institute (RKI), and by the European Food Safety Authority (EFSA). The feeding study alone can not adequately confirm the safety of MON863, nor is it an appropriate tool for establishing potential risks associated with the use of MON863. Bt toxin's mode of action and spectrum of efficacy are known and have been well investigated in the past. MON863 and other forms of genetically modified Bt maize, and varieties derived thereof, have been on the market outside Europe for a number of years. To date no risks have been associated with its use in foodstuffs or animal feed, nor have risks been demonstrated during the course of the approval procedure. No evidence of a possible allergenic potential of Cry3Bb1 has been documented. Material harvested from the proposed release project is not intended for use in foodstuffs or animal feed.

In a two-year field study conducted in the United States the influence of a Cry3Bb1 protein-producing Bt maize plant on the microbial biomass and activity, and on the structure of the microbial community in the soil, was determined. No differences between the genetically modified maize and the control lines were established.

The mode of action of the Cry3Bb1 protein inserted by means of transformation is not expected to result in risks to human or animal health. In view of the selective mechanisms of action of Bt toxins due, amongst other things, to receptor-specific binding in the intestinal tract of sensitive insects, no adverse effects on the environment are expected.

(c) The *cry1A.105* gene

The *Cry1A.105* gene codes for a lepidoteran-specific protein toxin (Bt toxin). This protein is a modified *Bacillus thuringiensis* Cry1A protein with an amino acid sequence which is 90%, 93.6% and 76.7% identical to Cry1Ab, Cry1Ac and Cry1F, respectively. The protein is organized in four domains of the following origin: Domains I and II are derived from Cry1Ab and Cry1Ac, respectively and are 100% identical in this region. Domain III is derived from Cry1F. The C-terminal domain essentially stems from Cry1Ac. There is no evidence of enzymatic activity in the protein expressed in the genetically modified organism. Therefore, it may be

assumed that apart from the formation of Bt toxin in the genetically modified plant no other effects on the plant metabolism will occur.

The additional gene present in genetically modified maize plants which codes for the Cry1A.105 is constitutively expressed under the control of the CaMV *35S* promoter. Transcription efficiency is increased by the rice actin gene intron *Ract 1* as well as the 5' untranslated region of the gene for the wheat chlorophyll a/b-binding protein (L-Cab). The mode of action in the intestinal tract of insect larvae is the same as described under b).

In studies on the toxicity and allergenicity of Cry1A.105, a description of which is given in the 2006 U.S. Environmental Protection Agency (EPA) report, no evidence of possible adverse effects of the Bt protein was found. In acute toxicity studies Cry1A.105 derived from bacterial culture was administered to mice via gavage. The studies showed that, in terms of body weight, weight gain and the results of pathology, there were no variations between the study group and two control groups. The amino acid sequence displayed no similarities to known toxic proteins. The allergenic potential of Cry1A.105 was also examined. In studies to date *Bacillus thuringiensis* has not appeared as a source of allergenic proteins. A bioinformatic comparison using a search matrix based on 8 amino acids in an unbroken oligomer revealed no similarities to, or homologies with, known allergenic proteins. Furthermore, the level of concentration of Cry1A.105 expressed in the tested maize tissue is below that found in potent food allergens. Cry1A.105 is degraded in synthetic digestive juices and is not glycosylated in maize. Against this background the allergenic potential of Cry1A.105 is deemed negligible. Material harvested from the proposed release project is not intended for use in foodstuffs or animal feed.

The mode of action of Cry1A.105 proteins inserted by transformation is not expected to have a negative impact on human or animal health, or on the environment.

(d) The *cry2Ab2* gene

The *cry2Ab2* gene is a synthetically optimized version of the gene isolated from *Bacillus thuringiensis* subsp. *kurstaki* and only differs from the bacterial protein by a single amino acid.

The mode of action in the intestinal tract of insect larvae is the same as described in b). In studies that accompanied the application for placing on the market of cotton MON15985, it was demonstrated that the bacteria-derived protein Cry2Ab2, which is identical to the protein produced in the genetically modified plants, was fully degraded in *in vitro* experiments with simulated intestinal fluid. In application experiments on insects the products of digestion had no further insecticidal effects. Bioinformatic database analysis did not reveal any similarities with known animal or human toxins. In toxicity studies in mice even the maximum dosage of Cry2Ab2 protein did not reveal any adverse effects on the test animals.

The additional gene contained in genetically modified maize plants which codes for the Cry2Ab2 protein is expressed constitutively under the control of the FMV promoter of the figwort mosaic virus. The intron of the *hsp*70 gene from maize enhances transcription efficiency. The upstream position of the chloroplast transit peptide of the maize-derived ribulose 1,5 biophosphat-carboxylase (TS-*SSU*-CTP) results in the post-translational import of Cry2Ab2 into the chloroplasts and is generally cleaved (processing) following import. Since no potential health risks have been attributed to this transit peptide, nor to any other currently

known signal peptides, whether processed or unprocessed, it can be assumed that the same applies to the transit peptide-Cry protein compound.

Material harvested from the proposed deliberate release experiment is not intended for use in foodstuffs or animal feed.

The mode of action of the Cry2Ab2 protein inserted by transformation is not expected to have a negative impact on human or animal health, or on the environment.

The proteins Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS are all expressed in the hybrid MON89034 x MON88017: Cry2Ab2 and CP4 EPSPS in the chloroplasts, Cry1A.105 and Cry3Bb1 in the cytoplasma. In the hybrid MON89034 x NK603, the proteins Cry1A.105, Cry2Ab2 and CP4 EPSPS are expressed in the same cell compartments. Protein interaction *in planta* is not anticipated since metabolic activity of the Cry proteins is unlikely and the enzymatic activity of CP4 EPSPS is clearly limited. Furthermore, since these proteins are fully degraded in the intestinal juices of mammals, the joint expression of Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS in the hybrid is not likely to pose any threat to human or animal health.

Overall, based on the selective action mechanism of Bt toxins due to, inter alia, receptor-specific binding in the intestinal tract of sensitive insects, and the limited enzymatic activity of CP4 EPSPS, no harmful effects on the environment are expected to result from the release of the maize plants.

(e) DNA fragments located outside the target sequences

Agrobacteria-mediated transformation normally only involves the integration into the plant genome of DNA located between the border regions. However, the transfer of DNA fragments outside the border regions has also been reported.

The following are contained outside the border regions of the plasmid vector PV-ZMIR39 used in the transformation of MON88107:

- the *aadA* gene derived from *Escherichia coli* transposon Tn7, under the control of its own promoter, which is only functional in bacteria;
- the ColE1 origin of replication required for plasmid pBR322 replication in *E. coli*;
- the origin of replication (oriV) of the RK2 plasmid for replication in *Agrobacterium tumefaciens*;
- the coding region *rop* which maintains the plasmid copy number.

According to information provided by the applicant the results of the Southern blot analyses show that plasmid components located outside the T-DNA regions were not transferred to the MON88017 maize line. Corresponding Southern blot data were submitted along with the application for release of the hybrid MON88017xMON810 (application number 6786-01-0169, statement of the Central Commission for Biological Safety (*Zentrale Kommission für die Biologische Sicherheit*) of March 14, 2006). Further assessment of the elements can therefore be waived.

The following are contained outside the T-DNA I border region of the plasmid vector PV-ZMIR245 used for the transformation of MON89034:

- the *aadA* gene derived from *Escherichia coli* transposon Tn7, under the control of its own promoter, which is only functional in bacteria;
- the ColE1 origin of replication required for pBR322 plasmid replication in *E. coli*;
- the origin of replication (*oriV*) of the RK2 plasmid for replication in *Agrobacterium tumefaciens*;
- the coding region *rop* which maintains the plasmid copy number.

Furthermore, within its T-DNA II border region it contains:

- the *npt*II gene, which codes for aminoglycoside 3'-phosphotransferase II derived from *Escherichia coli* transposon Tn5, under the control of the promoter and the 5' UTR of the cauliflower mosaic virus (CaMV) and the terminator of the nopaline-synthase gene (*nos*) from *Agrobacterium tumefaciens.* This gene acts as a selectable marker, conferring resistance on a number of aminoglycoside antibiotics.

The results of the Southern blot analysis submitted with the application indicate that plasmid components both outside the T-DNA I region and within the T-DNA II region were not transferred to the MON89034 gene. Further assessment of the elements can therefore be waived.

The plasmid vector PV-ZMGT32 used for the transformation of NK603 contains the following outside the target sequence:

- the bacterial origin of replication (*ori*)
- the gene for neomycin phosphotransferase type II (nptII) from the *Escherichia coli* transposon Tn5.

Prior to transformation the plasmid vector PV-ZMGT32 was digested with the restriction endonuclease *Mlu*I to separate the gene cassettes required for plant transformation from the rest of the plasmid DNA. Portions of the vector located outside the *Mlu*I fragment were not transferred to the genome. Further assessment of the elements can therefore be waived.

(f) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering methods are expressed at different levels, depending on the site of integration on the chromosome and on the integration site environment ("position effect"). Under field conditions the level of expression may be influenced by environmental factors, for instance, by temperature. In this particular case this could mean that the characteristics of the genetically modified maize are not modified to the same degree in the open field as under climate-controlled or greenhouse conditions. Potential risks to the environment or to human and animal health are not expected. Insertion of the foreign gene may affect the expression or regulation of the plant's inherent genes, either at or near the site of insertion. Such events may alter plant metabolic pathways. During the course of work on the genetically modified plants to date no observations were made that would suggest such an occurrence.

Mobile genetic elements (transposable elements), which when transposed into the genome can exert effects on existing plant genes at the target site, occur naturally in plants. Inactivation of genes or alterations in gene regulation also take place in a range of other naturally occurring processes such as point mutations, deletions or translocations and are traditionally used in plant breeding. Therefore, even in non-genetically modified plants such events may

at any time influence plant metabolic pathways. In this respect the properties of the genetically modified plants planned for release here do not differ fundamentally from non-genetically modified plants.

According to present knowledge it is not possible to make a reliable prediction about the possible allergenic action of a protein on the basis of the amino acid sequence. However, in the numerous releases of plants that express the *epsps* gene under the control of non-tissue specific promoters, no evidence of increased plant allergenicity has been recorded. Likewise, there is no evidence of increased allergenicity with regard to the Bt protein expressed in the plants.

The genetically modified maize referred to in the proposed release is not intended for use in foodstuffs or animal feed.

III.1.2.2. Evaluation of the capactiy of the genetically modified plants to persist or establish on open land

Maize plants and maize seeds are not hardy. Maize can not persist in Central European climate conditions. The genetic material inserted into the maize plant confers resistance to certain coleopteran and lepidopteran insects and imparts glyphosate herbicide tolerance to the plant. It can be assumed that the persistence characteristics have not been altered.

Genetically modified maize may reach grain maturity during the vegetation period. The establishment of volunteer maize has not been observed in the flora of Central Europe, even in the case of grain maize, which is harvested when fully mature. Should genetically modified maize plants accumulate in the experimental area after the end of the release period, they would be subsequently recorded and destroyed in the course of the required cultivation interval and post-trial monitoring, as set down in provisions II.10 [of the decision on this application]. These measures help to ensure the spatial and temporal limitation of the release project.

To dispose of both the genetically modified maize plants and the non-genetically modified maize plants after conclusion of the proposed trials, the plants will be hackled and worked into the ground where they degrade. Maize grain will be ground down and incorporated into the soil to decompose. Even if some of the maize grain is not broken down by hackling or grinding it can be assumed that no persistent plants would develop under open-field conditions. If the residue from hackling or grinding were to be integrated into the composting process on the release area or put through the fermentation process in a biogas plant the same result can be expected.

The non-genetically modified maize plants of the border rows and maize grown within the 200 m isolation zone should be disposed of in the same manner as the genetically modified maize plants.

III.1.2.3. Assessment of the possibility of the pollen transfer of genes inserted into the genetically modified plants to other plants

Due to the lack of a crossing partner in the flora of Central Europe, the transfer of the genes inserted into the genetically modified maize plants to other plant species can be ruled out.

Therefore, the focus here is solely on the possibility of pollen transfer from the genetically modified maize plants to other maize plants.

Maize pollen is normally spread by wind, whereby the maximum pollen drift distance is in no way identical to the maximum outcrossing distance. Sensitivity of the pollen to weather conditions such as heat, humidity and UV radiation, all of which quickly cause sex cells in the pollen grain to die off, is responsible. This explains why the recorded pollen drift distance is normally much greater than the recorded outcrossing distance. Another important factor for outcrossing is the size of the pollen donor and receiver population areas. For example, the smaller the pollen donor area, the shorter the outcrossing distance. The flowering period of the receiving population also plays a role. The longer the female flowers are receptive, the higher the rate of outcrossing, given simultaneous flowering. For this reason, outcrossing data from the earlier literature is often not pertinent, since modern high-yield varieties have a narrower cross-pollination time frame than older maize varieties. In more recent international studies outcrossing is rarely found within a 200 m range of areas which are approximately 10 times larger than proposed here (0,5 ha). In the production of hybrid maize seeds, seed legislation stipulates – in the absence of other isolation measures - a minimum separation distance of 200 m to other maize fields to adequately minimize cross-breeding with pollen of other varieties.

Provision II.8 [of the decision on this application] requires the observance of an isolation distance of 200 m between plants in the release area and other commercial maize stock. In addition, the applicant plans to sow 3 m wide border rows of non-genetically modified maize around the release plot. These measures will ensure that the risk of pollen transfer to other maize populations is adequately addressed.

III.1.2.4. Assessment of the possibility of transfer of the inserted foreign genes from the genetically modified plants to soil micro-organisms by horizontal gene transfer

(a) Gene expression cassettes of the *epsps* gene, the cry3Bb1 gene, the *cry1A.105* gene and the *cry2Ab2* gene

The transferred sequences are stably integrated into the genome of the recipient organism. There is no evidence of the transfer of genetic information from plants or its expression in micro-organisms taking place under natural conditions. However, studies on the transformation ability of soil bacteria under natural conditions suggest that the transfer of plant genetic material to soil bacteria is theoretically possible, although it is assumed that a gene transfer of this type would constitute an extremely rare event.

Insofar as we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy is actually possible, it could be concluded that the occurrence of an exchange of heterologous genetic material does not in itself represent a safety criterium, since in every case a possible uptake of any form of heterologous genetic material, including plant DNA, can result from such an exchange.

The genetically modified plants contain copies of the *CP4 epsps* gene, the *cry3Bb1* gene, the *cry1A.105* gene and the *cry2Ab2* gene, whereby the coding region of the epsps gene is N-terminally fused with the plant transit peptide sequences. Such transit peptide sequences have no function in bacteria.

The expression of glyphosate-tolerant EPSP synthases occurs naturally in soil micro-organisms. Bacteria with corresponding resistance are commonly found in the environment.

The *cry3Bb1* and *cry2Ab2* genes are derived from *Bacillus thuringiensis,* a ubiquitous soil bacterium. Even in the case of transfer of these genes from the genetically modified plants to soil micro-organisms, there would be no increase in the overall frequency of these genes in the environment. The likelihood of adverse ecological consequences resulting from this type of gene transfer is minimal.

The synthetic *cry1A.105* gene is made up of gene fragments which code for the different domains of the proteins Cry1Ab, Cry1Ac and Cry1F. Since they are derived from ubiquitous soil bacteria (*Bacillus thuringiensis*), the genes of these proteins are widespread in the environment. The mode of action of CRY1A.105 does not differ from that of other lepidopteran-specific Cry proteins. Even in the unlikely event of a transfer of the *cry1A.105* gene to (soil) micro-organisms the resulting genetic constellation would not have to be evaluated any differently than in the case of the uptake of the corresponding natural *cry* gene or *cry* gene fragments. The uptake of *cry1A.105* through horizontal gene transfer does not appear to confer a selective advantage on the recipient micro-organisms.

(b) Further DNA fragments located outside the T-DNA region

Attached to the present application are the results of studies which reliably document that fragments located outside the T-DNA or target sequences were not transferred to the plant genome. In the unlikely event that this should occur, the transfer of these sequences is not expected to result in a significant increase in the overall frequency of the corresponding DNA sequences, since these are derived from common, naturally occurring micro-organisms.

(c) Regulation sequences

The transfer of regulation sequences used in the construct is also unlikely to result in a significant increase in the overall frequency of the corresponding DNA sequences. These regulation sequences are derived from *Agrobacterium tumefaciens, Oryza sativa, Triticum aestivum, Zea mays, Arabidopsis thaliana,* CaMV and FMV, all of which are commonly found in plants and soil.

This type of gene transfer is not expected to have any ecological consequences.

III.1.2.5. The use of Agrobacteria to produce the genetically modified plants

An Agrobacterium-mediated binary transformation system was used to produce the genetically modified parental lines MON89034 and MON88017. Each Agrobacterium strain contains a “deactivated” helper plasmid, from which the T-DNA region has been deleted, and a plasmid whose T-DNA can be integrated into the plant genome.

This Agrobacterium strain does not produce plant tumours. It is not known whether the genetically modified maize was tested for the absence of Agrobacteria. However, in view of generative breeding over a number of generations it can be assumed that the plants do not contain any more genetically modified Agrobacteria.

Even if a small number of Agrobacteria were to remain in the genetically modified plant material, there would be no risk involved. In this case, the possibility of Agrobacteria-mediated transfer of transgenes to other plants needs to be considered. Such a transfer, if it were to take place, would have no further impact, since following the transformation of a plant cell by the modified Agrobacteria it would then have to spontaneously regenerate to produce a fertile plant so that the transgenes could be passed on to the next generation. This is not expected to happen under natural conditions.

Furthermore, the possible transfer of Agrobacteria-derived transgenes to other bacteria in the environment by horizontal gene transfer should be considered.

Possible effects have already been discussed under III.1.2.4.