# Import and processing of genetically modified maize line MON87460

## COGEM advice CGM/100330-01

### Summary

*The present application of Monsanto Company (file EFSA/GMO/NL/2009/70) concerns the import and processing for use in feed and food of maize line MON87460. Cultivation is not part of this application.*

*Maize line MON87460 was obtained by Agrobacterium-mediated transformation of conventional maize and expresses cold shock protein B (CspB) from Bacillus subtilis and neomycin phosphoptransferase II (NptII) from Escherichia coli. CspB functions as a RNA-chaperone and facilitates adaptation to environmental stress in both bacteria and plants. In maize line MON87460 CspB provides a reduced yield loss under water-limited conditions. NptII confers resistance to kanamycin and neomycin and was used to facilitate the selection process of transformed plant cells.*

*The applicant showed by Southern blot analyses that one copy of the insert was integrated at a single integration locus in the genome of MON87460 and that the backbone of the plasmid used for transformation was absent in MON87460. Bioinformatic analysis spanning the 5' and 3' junctions showed that no endogenous maize open reading frames (ORFs) were disrupted. Nine ORFs encoding eight or more amino acids were identified. No sequence similarities between any known toxins or allergens were identified. In the opinion of COGEM, the molecular analysis of MON87460 has been adequately performed.*

*During its long domestication process, maize has lost its ability to survive in the wild. In the Netherlands, the appearance of maize volunteers is rare and establishment of volunteers in the wild has never been reported. There are no reasons to assume that the introduced traits will increase the potential of maize to establish feral populations. The introduced genes cannot spread since wild relatives of maize are not present in Europe.*

*Although the General Surveillance plan could be improved by a guarantee that operators will monitor for unanticipated effects, COGEM considers the current GS plan sufficient for import and processing.*

*In conclusion, COGEM is of the opinion that import and processing of maize line MON87460 poses a negligible risk to the environment and has no objections against an authorization for import and processing of MON87460. COGEM points out that a food/feed safety assessment is carried out by other organizations. Therefore, COGEM abstains from advice on the potential risks of incidental consumption. Noteworthy is that the presence of antibiotic resistance genes in GM crops is rejected by certain factions in society. In their perception the presence of antibiotic genes is a safety risk because in their view horizontal gene flow could lead to antibiotic resistant pathogenic bacteria.*

### Introduction

The present application (file EFSA/GMO/NL/2009/70) by Monsanto Europe S.A., concerns the import and processing of genetically modified (GM) maize line MON87460. MON87460 was developed to improve drought tolerance and to reduce yield loss under water limited conditions compared to conventional maize.

MON87460 maize was produced by *Agrobacterium*-mediated transformation of conventional maize and expresses cold shock protein B (CspB) from *Bacillus subtilis* and neomycin phosphoptransferase II (NptII) from Tn5 of *Escherichia coli*. CspB belongs to the family of cold shock proteins (CSPs) and is suggested to function as a 'RNA-chaperone.[1,2] 'RNA-chaperones' are known to facilitate adaptation to environmental stress in both bacteria (*e.g. B. subtilis*, *E. coli*)[1,2] and plants (*e.g. Arabidopsis*, wheat, rice).[3-5] NptII confers resistance to kanamycin and neomycin[6] and was used to facilitate the selection process of transformed plant cells.

## Previous COGEM advice

To date, COGEM has not advised on maize line MON87460. COGEM has never advised on the *cspB* gene and stress tolerant GM crops before.

In 1998 COGEM advised on the use of the antibiotic resistance marker gene neomycin phosphotransferase II (*npt*II) gene in GM crops.[7] The *nptII* gene was initially isolated from the transposon Tn5 that was present in the bacterium strain *Escherichia coli* K12. *NptII* is abundantly present in the soil microflora. The *nptII* gene confers resistance tot aminoglycoside antibiotics like kanamycin and neomycin. NPTII is widely used as selectable marker for plant transformation and in gene expression and regulation studies in different organisms. Based upon the fact that horizontal gene transfer is highly unlikely[22] and that *nptII* is abundantly present in soil and intestinal bacteria COGEM concluded that the presence of the *nptII* gene in GM crops poses negligible risks for human health and the environment. This opinion is supported by EFSA.[18] The *nptII* gene is also present in the recently in the EU for cultivation approved starch potato 'Amflora'. It has to be noticed that the presence of antibiotic resistance genes in GM crops is rejected by certain factions in society. In their perception the presence of antibiotic genes is a safety risk because in their view horizontal gene flow could lead to antibiotic resistant pathogenic bacteria.

## Aspects of the crop

Maize (*Zea mays L.*) is a member of the grass family *Poaceae.* Maize is an annual plant and cultivated as an agricultural crop, originating from Central America. Although insect pollination cannot be completely excluded, maize is predominantly wind pollinated.[7,10] According to literature, pollen viability varies between 30 minutes and 9 days.[7-12] In Europe no wild relatives of maize are present and therefore hybridization with other species cannot occur.[13]

Throughout the world the appearance of volunteers is very rare. Seed kernels are the only survival structures of maize.[13] Due to the structure of the corncob (ear on a stiff central cob enclosed in husks) natural dissemination of the kernels rarely occurs. Maize needs human intervention to disseminate its seed.[13] In addition, kernels exhibit poor dormancy resulting in a short persistence. Besides, maize can only survive within a narrow range of climatic conditions and, originally a tropical crop, maize is naturally frost-sensitive.[15] Maize is very sensitive to weed competition. During the long process of domestication, maize has lost the ability to survive in the wild.[13]

Establishment of maize plants in the wild has never been observed in the Netherlands and COGEM is not aware of any reports of wild maize plants elsewhere in Europe.

**Molecular characterization**

MON87460 maize was produced by *Agrobacterium*-mediated transformation of conventional maize using the binary plasmid vector PV-ZMAP595. This vector contained a single T-DNA with two expression cassettes: a *cspB* expression cassette, which contains the coding sequence for CspB from *B. subtilis* and a *nptII* cassette from Tn5 of *E. coli*, which confers resistance to kanamycin and neomycin. The CspB protein is identical to the native CspB protein produced in *B. subtilis* with the exception of one amino acid change in the second position from leucine to valine to facilitate the assembly of the plasmid vector for plant transformation. An overview of the genetic elements of the T-DNA is given below:

- B-right border region. DNA from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA
- P-*Ract1*. Promoter and leader from the rice actin gene, *act1*, of *Oryza sativa*
- I-*Ract1*. Intron from the rice actin gene, *act1*, of *O. sativa*
- Intervening sequence used in DNA cloning
- CS-*cspB*. Codon modified coding sequence of the *cspB* gene from *B. subtilis* encoding CspB
- Intervening sequence used in DNA cloning
- T-*tr7*. 3' non translated sequence of *transcript 7* gene from *A. tumefaciens* that directs polyadenylation
- Intervening sequence used in DNA cloning
- *loxP*. Sequence from *Bacteriophage P1* for the recombination site recognized by Cre recombinase
- Intervening sequence used in DNA cloning
- P-*35S*. Promoter for the 35S rRNA of the *Cauliflower mosaic virus*
- Intervening sequence used in DNA cloning
- CS-*nptII*. Coding sequence from Tn5 of *E. coli* encoding neomycin and kanamycin resistance
- Intervening sequence used in DNA cloning
- T-*nos*. 3' non-translated sequence of the nopaline synthetase (*nos*) gene from *A. tumefaciens* which terminates and directs polyadenylation
- Intervening sequence used in DNA cloning
- *loxP*. Sequence from *Bacteriophage P1* for the recombination site recognized by Cre recombinase
- Intervening sequence used in DNA cloning
- B-Left Border region. DNA from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA

*Properties of the genes introduced in MON87460*

The T-DNA introduced in MON87460 contains a *cspB* and an *nptII* expression cassette.

CspB is derived from *B. subtilis*. It belongs to the family of CSPs, a group of small proteins that contains a highly conserved RNA-binding sequence identified as cold shock domain (CSD), and is suggested to function as a ‘RNA-chaperone’.[1,2] ‘RNA-chaperones’ are known to facilitate adaptation to environmental stress in both bacteria (*e.g. B. subtilis*, *E. coli*) and plants (*e.g. Arabidopsis*, wheat, rice).[1-5] CSPs recognize single stranded polynucleotides without apparent sequence specificity and facilitate the initiation of translation by destabilizing nonproductive secondary structures in mRNA under extreme conditions such as chemical stress, water-limited conditions, and high or low temperature.[1,2,16] CSD-containing proteins accumulate in actively growing tissues.[3,5] In MON87460 the expression of the *cspB* gene is under control of the rice actin promoter which enables constitutive expression of the protein.

NptII confers resistance to kanamycin and neomycin[6], and was used to facilitate the selection process of transformed plant cells. Expression of the *nptII* gene enables the MON87460 maize cells to survive in the presence of aminoglycosides (*e.g.* kanamycin). Aminoglycosides disrupt protein synthesis by binding to the 30S ribosomal subunit. This causes misreading of mRNA and results in non-functional, misfolded proteins leading to cell death.[17] The NptII protein modifies the aminoglycoside molecule and subsequently cannot bind to the 30S ribosomal subunit, protein synthesis is not longer disrupted and the maize cells will survive.[6]

*Molecular analysis MON87460*

The applicant showed by Southern blot analyses that one copy of the T-DNA containing the *cspB* and *nptII* expression cassette was integrated at a single integration locus in the genome of MON87460. Hybridization analyses showed that the insert is stable over several generations. Furthermore, the applicant demonstrated by hybridization analysis that the backbone of plasmid PV-ZMAP595 was absent in the genome of MON87460.

Results obtained by PCR amplification and DNA sequence analyses showed that the integrated T-DNA was not intact. From the Left Border region 194 bp was missing, the complete Right Border region was absent and 733 bp of the *P-ractI* element flanking the Right Border was missing. Sequence comparison between the pre-insertion site and the MON87460 flanking regions indicated a 22 bp deletion of maize genomic DNA at the insertion site. Bioinformatic analysis by BLASTn and BLASTx searches (GenBank databases, June 2009) showed that no endogenous maize ORFs were disrupted by the T-DNA insertion.

DNA sequences spanning the 5’ and 3’ junctions of the MON87460 insertion site and the genomic DNA were analyzed from stop codon to stop codon. Nine open reading frames encoding eight or more amino acids were identified. Bioinformatic analyses were performed by alignment searches in allergen, toxin and protein databases (AD_2009, TOX_2009 and PRT_2009, respectively). No sequence similarities were demonstrated between any known toxins or allergens and the nine putative polypeptides.

In conclusion, COGEM is of the opinion that the molecular characterization of MON87460 has been adequately performed and meets the criteria laid down by COGEM.[20]

## Environmental risk assessment

The current application of the GM maize line MON87460 concerns import and processing and does not include cultivation. MON87460 is a drought tolerant maize which expresses the c*spB* and *nptII* genes.

In case of incidental spillage, maize kernels may be released into the environment. However, volunteers are rarely found in Europe and establishment of feral populations has never occurred.[13] Maize has lost the ability to survive in the wild during the long process of domestication.[13]

The biological characteristics of maize determine its disability to establish feral populations under the European climatic conditions. In Europe no wild relatives of maize are present and therefore hybridization with other species cannot occur.[13] An overview of some of the characteristics of maize which determine the absence of invasiveness and persistence is given below:

- due to the structure of the corn crop (seed shed enclosed in husks) kernels remain on the cob after ripening[13]
- kernels exhibit poor dormancy[13]
- maize is naturally frost-sensitive[15]
- maize is sensitive to weed competition[13]

Field trial data for MON 87460 obtained in the USA and Chile demonstrated that under water-limited conditions the expression of the CspB protein in MON87460 maize results in reduced yield loss compared to conventional maize. The applicant states that the major component contributing to the improved yield is the increased number of kernels per ear. According to the applicant, field trial data showed that no other biologically significant phenotypic differences were observed regarding germination, survivability or biological fitness of MON87460 compared to conventional maize. Moreover, the applicant states that no effect on persistence or invasiveness of MON87460 maize was observed. COGEM points out that the studies regarding environmental stress tolerance on GM maize MON87460 in comparison with conventional maize show some deficiencies. Data from US and Chilean field studies are not representative for the European and Dutch situation. Additionally, the number of experiments and, therefore, the statistical power was minimal.

Besides field trials, greenhouse and growth chamber studies were performed. According to the applicant, exposure to abiotic stress (drought, cold, heat, and salt) imposed during early vegetative growth stages, did not show significant differences between MON87460 and conventional maize. COGEM is of the opinion that experimental studies on exposure to low temperatures are relevant for the Dutch situation. In COGEM’s opinion the applicant sufficiently showed in greenhouse and growth chamber studies that sensitivity to cold of GM MON87460 maize was not affected in comparison with conventional maize.

In summary, expression of CspB is able to reduce yield loss under water limited conditions in GM MON87460 maize compared to conventional maize. It cannot be excluded that other physiological processes have been affected. However, establishment of feral populations of maize in Europe is determined by several factors. Only a limited number of these factors can be affected by the introduction of CspB. For instance, the anatomy of the corncob is unaltered and kernels remain on the cob after ripening. If occasionally volunteers will occur, establishment of feral populations of GM MON87460 maize is highly unlikely.

MON87460 maize also harbors the *nptII* gene, which induces antibiotic resistance. COGEM advised on the use of antibiotic resistance marker genes in GM crops before. In 2009 EFSA released a statement on the use of antibiotic resistance genes as marker genes in GM plants.[18] Both COGEM and EFSA concluded that for the presence of the *nptII* gene in GM crops the risk on adverse effects on the human health and the environment is negligible. Furthermore, an interaction between CspB and NptII is not expected by COGEM since the proteins have different mechanisms of action.

In conclusion, in COGEM's opinion there are no reasons to assume that in case of incidental spillage the expression of the introduced traits increases the potential of MON87460 to establish feral populations in the Dutch climatic conditions.

Since 2008 COGEM abstains from giving advices on the potential risks of incidental consumption in case a food/feed assessment is already carried out by other organizations.[19] This application is submitted under Regulation (EC) 1829/2003, therefore a food/feed assessment is carried out by EFSA. Other organizations who advice the competent authorities can perform an additional assessment on food safety although this is not obligatory. In the Netherlands a food and/or feed assessment for Regulation (EC) 1829/2003 applications is carried out by RIKILT. Regarding the risks for food and feed, the outcome of the assessment by other organizations (EFSA, RIKILT) was not known at the moment of the completion of this advice.

## General surveillance plan

General surveillance (GS) has been introduced to be able to observe unexpected adverse effects of GM crops on the environment. The setting or population in which these effects might occur is either not, or hardly predictable.

The GS plan states that unanticipated adverse effects will be monitored by existing monitoring systems which include the authorization holder and operators involved in the handling and use of viable MON87460 maize. Recently, COGEM formulated criteria on which GS plans concerning Dutch applications for import and cultivation of GM crops have to comply.[21] Although the GS plan could be improved by a guarantee that operators will monitor for unanticipated effects, COGEM considers the current GS plan sufficient for import and processing of MON87460 maize.

## Advice

Maize has lost the ability to survive in the wild. In the Netherlands, volunteers are rare and establishment of maize plants in the wild has never been observed. In addition, introgression of genes into closely related species cannot occur, as wild relatives of maize are not present in Europe.

The current application of GM maize MON87460 concerns import and processing and does not include cultivation. GM maize MON87640 expresses proteins CspB and NptII. CspB is known to facilitate adaptation to environmental stress in both bacteria and plants. NptII confers resistance to the antibiotics kanamycin and neomycin, and was used to facilitate the selection process of transformed plant cells.

According to the applicant the traits introduced in MON87460 resulted in a maize line with increased tolerance to drought. It cannot be excluded that a limited number of other physiological processes have been affected. In COGEM's opinion the studies regarding environmental stress tolerance on germination, survivability, fitness, persistence and invasiveness show some deficiencies. However, for the ability to establish feral populations, multiple of the plant's characteristics have to be affected. Among other characteristics important for developing invasivity, maize lacks several ones such as dispersal mechanisms, the ability to survive in highly competitive plant communities without human intervention and dormancy mechanisms that allow long term survival in the soil. Besides, maize is frost-sensitive. In conclusion, establishment of feral populations of GM MON87460 is negligible.

In 1998 COGEM concluded that for the presence of the *nptII* gene in GM crops the risk on adverse effects on the human health and the environment is negligible. According to COGEM's opinion the molecular analysis of MON87460 has been adequately performed. Although the GS plan could be improved by a guarantee that operators will monitor for unanticipated effects, COGEM considers the current GS plan sufficient for import and processing of MON87460 maize.

In summary, COGEM is of the opinion that import and processing of maize line MON87460 poses a negligible risk to the environment and has no objections against an authorization for import and processing of MON87460.

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