Import and processing of genetically modified maize NK603xT25

COGEM advice CGM/101213-02

The present application by Monsanto Europe S.A. (file EFSA/GMO/NL/2010/80) concerns import and processing for use in feed and food of the genetically modified maize line NK603xT25. Cultivation is not part of this application.

Maize line NK603xT25 was produced by conventional crossbreeding of parental maize lines NK603 and T25. NK603xT25 expresses the genes cp4 epsps*,* cp4 epsps L214P *and* pat*. As a result, this maize line is tolerant to glyphosate and glufosinate ammonium containing herbicides.*

Maize line NK603xT25 has not been previously assessed by COGEM. COGEM did advise positively on cultivation of both parental maize lines.

The molecular analysis of the inserts present in this maize line has been updated. The presence of newly created open reading frames at the junction sites of the DNA introduced in maize NK603 and T25, and present in maize line NK603xT25, was analyzed. The amino acid sequence was deduced and analyzed in silico *for similarity to known toxins or allergens. No similarity was found. The molecular characterization of maize line NK603xT25 meets the criteria of COGEM.*

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 to closely related species since wild relatives of maize are not present in Europe.

In view of the above, COGEM is of the opinion that the risks for humans and the environment associated with import and processing of maize line NK603xT25 are negligible. A food/feed safety assessment is carried out by other organizations. Therefore, COGEM abstained from advice on the potential risks of incidental consumption.

Introduction

The present notification (EFSA/GMO/NL/2010/80) by Monsanto Europe S.A. concerns import and processing of maize line NK603xT25. This maize line was produced by conventional crossbreeding of the parental maize lines NK603 and T25. It expresses the genes *cp4 epsps*, *cp4 epsps L214P* and *pat*. As a result maize NK603xT25 is tolerant to glyphosate and glufosinate ammonium containing herbicides. Maize NK603xT25 has recently been authorized for cultivation, import and processing in Japan (2010).[1]

Maize line NK603xT25 has not been previously assessed by COGEM. Both parental maize lines were, however, previously assessed by COGEM.

In 2003, COGEM advised positively on import and processing of maize line NK603.[2] In 2004 and 2005, NK603 was authorized for import, food and feed purposes in the European Union.[3,4] In 2006, COGEM issued a positive advice on cultivation of maize NK603.[5] In her scientific opinion on cultivation of NK603, EFSA concluded that maize line NK603 is as safe as conventional maize.[6] A decision on the authorization for cultivation of maize NK603 in the European Union is currently pending.

Maize line T25 was authorized for cultivation in the European Union in 1998.[7] The predecessor of the EFSA, i.e. the Scientific Committee on Plants, assessed T25 maize and concluded that there is no evidence to indicate that T25 is likely to cause adverse effects on human or animal health and the environment.[8] An application for renewal of the authorization was filed in 2008. In light of this application, COGEM assessed maize line T25 and issued a positive advice on the renewal of the application for cultivation of this maize line.[9] An EFSA opinion on the renewal has not yet been published.

Aspects of the crop

Maize (*Zea mays* L.) is a member of the grass family *Poaceae.* Maize is cultivated as an agricultural crop, originating from Central America. Although insect pollination cannot be completely excluded, maize is predominantly wind pollinated.[10,11] According to literature, pollen viability varies between 30 minutes and 9 days.[11,12,13] In Europe, no wild relatives of maize are present and, therefore, hybridization with other species cannot occur.[21]

Throughout the world the appearance of volunteers is very rare. Seed kernels are the only survival structures of maize.[21] Due to the structure of the corn cob (ear on a stiff central cob enclosed in husks) natural dissemination of the kernels rarely occurs. Maize needs human intervention to disseminate its seed.[21] In addition, kernels exhibit poor dormancy resulting in a short persistence. Besides, maize can only survive within a narrow range of climatic conditions and, as maize is originally a subtropical crop, it is frost-sensitive.[14] Maize is very sensitive to weed competition.[22] During the long process of domestication, maize has lost the ability to survive in the wild.[21] 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

The genetically modified maize line NK603xT25 was produced by crossing of the two parental maize lines NK603 and T25 using traditional breeding methods. The molecular characterization of maize NK603 and T25 was previously evaluated by COGEM. COGEM concluded that the molecular characterization of both parental lines was adequate.[15,16]

Southern blot analysis showed that the inserts from NK603 and T25 are present in maize NK603xT25. An overview of the construction and of the inserted genetic elements of both parental lines is given below.

The sequences of the junctions between the NK603 and T25 inserts and maize genomic DNA and between the intended insert and fragments of the insert or chloroplast sequences were reanalyzed in the current application. The results from these analyses are described below.

Insert from parental maize line NK603

The genetically modified maize line NK603 was produced by particle bombardment. A restriction fragment of plasmid PV-ZMGT32, containing two *cp4 epsps* expression cassettes was inserted into maize line NK603.

The following elements were introduced in NK603:

- P-ract1/I-ract1, promoter, transcription start site and intron derived from the rice actin 1 gene from *Oryzae sativa*; intron promotes transcription

- *ctp2*, sequence from *Arabidopsis thaliana*; encoding a chloroplast transit peptide
- *cp4 epsps*, gene derived from *Agrobacterium tumefaciens* CP4; encoding 5 enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS)
- Nos 3', terminator from the nopaline synthase gene from *A. tumefaciens*; stops transcription
- P-E35S, constitutive promoter derived from *Cauliflower mosaic virus* (CaMV)
- *hsp70*, intron derived from the *hsp70* gene from *Z. mays*; stabilises transcription
- *ctp2*, sequence derived from *A. thaliana*; encoding a chloroplast transit peptide
- *cp4 epsps L214P*, gene derived from *A. tumefaciens* CP4; encoding CP4 EPSPS
- Nos 3', terminator from the nopaline synthase gene from *A. tumefaciens*; stops transcription
- fragment of P-ract1, containing part of the enhancer region of the rice actin promoter
- chloroplast genomic DNA

Parental maize line NK603 contains one insert with a single copy of the two *cp4 epsps* expression cassettes. At the 3' end of the insert a fragment (217 basepairs) of the expression cassette is present. This fragment contains part of the ract-1 promoter (167 basepairs), but the sequences needed for promoter activity are not present. Adjacent to this fragment 305 basepairs of chloroplast genomic DNA are present. The chloroplast genomic DNA was probably introduced during the transformation process.

Thus, introduction of two expression cassettes created four junctions that were not present in the original maize line: a junction between maize genomic DNA and the 5' end of the insert; a junction between the *nos* terminator of the *cp4 epsps L214P* gene and the ract-1 promoter fragment; a junction between the ract-1 promoter fragment and chloroplast genomic DNA; and a junction between the chloroplast genomic DNA and the maize genomic DNA. These four junctions were reanalyzed in 2010. Putative open reading frames newly created at the four junction sites were investigated. The junction sequences were translated from stop codon to stop codon in all six reading frames. The deduced amino acid sequences were analyzed *in silico* for sequence similarity to known proteins, toxins or allergens. Some putative polypeptides (minimum size eight amino acids) showed sequence similarity to known proteins, e.g. a RNA polymerase, ribosomal proteins or hypothetical proteins, but no similarity to known toxins or allergens was found.

Insert from parental maize line T25

Maize line T25 was generated by introducing pUC/Ac plasmid DNA by polyethylene-glycol mediated protoplast transformation.

The following elements were introduced into T25:

- *bla*, part of the 5' sequence of the *β-lactamase* gene derived from *Escherichia coli.* A functional *β-lactamase* gene would confer resistance to ampicillin
- cvs, part of the sequence of the pUC/Ac plasmid needed for the maintenance of the plasmid in *E. coli*
- T-35S terminator, terminator derived from CaMV; stops transcription
- *pat*, synthetic gene encoding phosphinothricin acetyl transferase (PAT), originally derived from *Streptomyces viridochromogenes*.
- P-35S promoter, constitutive promoter derived from CaMV

- cvs, part of the sequence of the pUC/Ac plasmid needed for the maintenance of the plasmid in *E. coli* including the origin of replication
- *bla*, part of the sequence of the *β-lactamase* gene derived from *Escherichia coli.* A functional *β-lactamase* gene would confer resistance to ampicillin
- P-35S promoter, part of the constitutive promoter derived from CaMV

Parental maize line T25 contains one insert with a single copy of the *pat* expression cassette. The insert contains the *pat* expression cassette, but also part of the backbone of the pUC/Ac plasmid. Approximately 75% of the β-lactamase gene is present**,** but β-lactamase is not produced. In addition, at the 3' end of the insert a duplication of a fragment of the 35S promoter is present.

Introduction of the *pat* gene in T25 thus created three junctions that were not present in the original maize line: two junctions between the maize genomic DNA and the insert; and one junction between the partial β-lactamase gene and the fragment of the duplicated 35S promoter. In view of the current application, these junctions were reanalyzed in 2010 to assess the presence of potential newly created open reading frames leading to the production of proteins. An open reading frame was defined as a region between a start and a stop codon or as a region between two translation stop codons (minimum size three amino acids). The predicted potential newly created open reading frames were reanalyzed for similarity to sequences of known toxins and allergens. The amino acid sequences of some open reading frames showed identities to known proteins (maize proteins, CaMV promoter peptides, fragments of ampicillin resistance proteins), but no significant sequence similarities to known toxins or allergens were found. In addition, using several bioinformatics methods the applicant analyzed whether the identified potential newly created open reading frames would lead to the expression of newly created proteins. From this analysis the applicant concluded that it is highly unlikely that the predicted potential newly created open reading frames are expressed.

In conclusion, putative polypeptides at the junction sites of the DNA introduced in maize NK603 and T25, and present in maize line NK603xT25, did not possess any similarity to known toxins or allergens.

Properties of the introduced genes

Maize line NK603xT25 expresses the *cp4 epsps* and *cp4 epsps L214P* genes, which encode CP4 EPSPS proteins. EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) is an enzyme involved in the biosynthesis of aromatic amino acids. Glyphosate inhibits EPSPS, resulting in a lack of amino acids essential for growth and development of plants.[17] Maize line NK603xT25 expresses CP4 EPSPS proteins, which are not inhibited by glyphosate[18] and is therefore tolerant to glyphosate containing herbicides.

Maize line NK603xT25 is also tolerant to herbicides containing glufosinate ammonium. In non-transgenic plants glufosinate ammonium inhibits the activity of glutamine synthetase, an enzyme necessary for the production of glutamine and for ammonia detoxification.[19] The application of glufosinate ammonium leads to reduced glutamine and increased ammonia levels in non-transgenic plants.[19] Photosynthesis is inhibited and eventually the plant dies.[20] Maize line NK603xT25 expresses the *pat* gene which encodes phosphinothricin-N-acetyl transferase (PAT). This protein acetylates L-phosphinothricin, the active isomer of glufosinate

ammonium. The resulting compound N-acetyl-L-phosphinothricin does not inhibit the activity of glutamine synthetase.[19] As a result NK603xT25 is tolerant to L-phosphinothricin and thus to herbicides containing glufosinate ammonium.

Environmental risk assessment

During the long process of domestication, maize has lost the ability to survive in the wild.[21] Maize needs human intervention to disseminate its seed.[21] Maize kernels exhibit poor dormancy resulting in a short persistence. Maize is very sensitive to weed competition and cannot persist as a weed.[21,22] Furthermore, maize is naturally frost sensitive and can only survive within a narrow range of climatic conditions. In the Netherlands, volunteers are rarely found and establishment of maize plants in the wild has never been observed. In Europe, no wild relatives of maize are present and, therefore, hybridization with other species cannot occur.[21]

Maize line NK603xT25 expresses the *cp4 epsps, cp4 epsps L214P* and *pat* genes. As a result, NK603xT25 is tolerant to glyphosate and glufosinate ammonium containing herbicides. The current application concerns import and processing. In case of spillage maize kernels may be released into the environment. Maize kernels can only survive within a narrow range of climatic conditions. The introduced traits do not increase the ability of maize kernels to survive in the environment. In addition, the applicant carried out an agronomic assessment for NK603xT25. This assessment does not give any indication to assume that NK603xT25 has an increased survivability compared to conventional maize lines.

Tolerance to glyphosate and glufosinate ammonium containing herbicides will only provide a selective advantage when these herbicides are used. Glyphosate and glufosinate ammonium containing herbicides are normally only applied in an agricultural environment. Therefore, tolerance to these herbicides will not provide a selective advantage in case of spillage of NK603xT25.

In view of the above, there are no reasons to assume that maize NK603xT25 has an increased potential for the establishment of feral populations in case of incidental spillage.

Since 2008, COGEM abstains from advices on the potential risks of incidental consumption in case a food/feed safety assessment is already carried out by other organizations. This application is submitted under Regulation (EC) 1829/2003, therefore a food/feed safety 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 safety 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

General surveillance has been introduced to be able to observe unexpected adverse effects of genetically modified crops on the environment. The setting or population in which these effects might occur is either not, or hardly predictable. In addition, since the effects are unexpected, no hypothesis on the nature of the effects is present. Usually, in case of import and processing of genetically modified maize general surveillance is supposedly carried out by operators involved in the handling and use of viable maize.

Recently, COGEM formulated criteria on general surveillance plans. In COGEM's view applications for import and cultivation of gm-crops have to meet these criteria.[23] COGEM concluded that the general surveillance plans could be improved by a guarantee that operators will monitor for unanticipated effects. In the present general surveillance plan on NK603xT25 maize the authorization holder states that the operators have agreed to provide information relevant to the monitoring of NK603xT25 maize to the authorization holder. More important, it is stated that the authorization holder will be able to give evidence that the operators collect this information.

COGEM is content that her recommendation for improvement of the general surveillance plan has been followed and considers the general surveillance plan sufficient for import and processing of NK603xT25 maize.

Advice

COGEM has been asked to advice on import and processing of maize line NK603xT25. This maize line was produced by conventional crossbreeding of parental maize lines NK603 and T25.

The molecular analysis of the inserts present in this maize line has been updated. Putative polypeptides at the junction sites of the DNA introduced in maize NK603 and T25, and present in maize line NK603xT25, did not possess any similarity to known toxins or allergens. The molecular characterization of maize line NK603xT25 meets the criteria of COGEM.

Maize has lost the ability to survive in the wild. In addition, maize needs human intervention to disseminate its seed. In the Netherlands, volunteers are rare and establishment of maize plants in the wild has never been observed. There is no reason to assume that expression of the *cp4 epsps, cp4 epsps L214P* and *pat* genes increase the potential of maize to establish feral populations in case of incidental spillage. In addition, an agronomic assessment of NK603xT25 did not give any indication to assume that NK603xT25 has an increased survivability compared to conventional maize lines. Introgression of the introduced genes into closely related species cannot occur, as wild relatives of maize are not present in Europe.

In view of the above, COGEM is of the opinion that the risks for humans and the environment associated with import and processing of maize line NK603xT25 are negligible. A food/feed safety assessment is carried out by other organizations. Therefore, COGEM abstained from advice on the potential risks of incidental consumption.

References

1. Center for Environmental Risk Assessment GM Crop Database. www.cera-gmc.org/index.php?action=gm_crop_database (18 oktober 2010)

2. COGEM (2003). Markttoelating 'NK603 maize tolerant to glyphosate'. Advies CGM/030319-08

3. European Commission (2005). Commission Decision authorising the placing on the market of foods and food ingredients from gm maize line NK603. Official Journal of the European Union 21.6.2005 L158/20-22

4. European Commission (2004). Commission Decision concerning the placing on the market of a maize product (NK603) genetically modified for glyphosate tolerance. Official Journal of the European Union 18.9.2004 L295/35-37

5. COGEM (2006). Cultivation of herbicide tolerant maize line NK603. Advies CGM/060704-01

6. EFSA GMO Panel (2009). Scientific opinion on applications for the placing on the market of gm maize NK603 for cultivation, food and feed uses, import and processing and for renewal of the authorisation of maize NK603 as existing products. The EFSA Journal 1137: 1-50
7. European Commission (1998). Commission decision concerning the placing on the market of gm maize T25. Official Journal of the European Communities 5.5.98 L131/30-31
8. Scientific Committee on Plants (1998). Opinion on the Scientific Committee on plants regarding 'submission for placing on the market of glufosinate tolerant corns T25' by the Agrevo Company. February 10th 1998
9. COGEM (2008). Renewal application cultivation of maize T25. Advies CGM/080806-02
10. Hin CJA (2001). Rapport Landbouwkundige risico's van uitkruising van GGO-gewassen. Centrum voor Landbouw en Milieu (CLM)
11. Treau R & Emberlin J (2000). Pollen dispersal in the crops Maize (*Zea mays*), Oil seed rape (*Brassica napus* ssp. *Oleifera*), Potatoes (*Solanum tuberosum*), Sugar beet (*Beta vulgaris* ssp. *vulgaris*) and Wheat (*Triticum aestivum*)- Evidence from publications. Soil Association (= leading organization for organic certification UK)
12. Coe EHJR, Neuffer MG & Hoisington DA (1988). The genetics of Corn. pp. 81-258. In: Sprangue GF, Dudley JW, Editors. Corn and Corn Improvement, Third Edition. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, Wisconsin. 986 pp
13. Luna VS, Figueroa MJ, Baltazar MB *et al.* (2001). Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Science 41: 1551-1557
14. Miedema P (1982). The effects of low temperature on *Zea mays*. Advances in Agronomy 35: 93-128
15. COGEM (2009). Cultivation of maize line MON89034xNK603. Advies CGM/091208-01
16. COGEM (2008). Renewal application cultivation of maize T25. Advies CGM/080806-02
17. Green JM (2007). Review of glyphosate and ALS-inhibiting herbicide crop resistsance and resistant weed management. Weed technology 21: 547-558
18. Funke T, Han H, Healy-Fried ML *et al.* (2006). Molecular basis for the herbicide resistance of Roundup Ready crops. Proceedings of the National Academy of Sciences of the United States of America: 103: 13010-13015
19. OECD (1999). Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide
20. OECD (2002). Module II: Phosphinothricin
21. OECD (2003). Consensus document on the biology of *Zea mays* subsp. *mays* (Maize)
22. Crop Protection Compendium (2007). *Zea mays* (maize). CD-ROM edition, © Cab International 2007, Nosworthy way, Wallingford, UK
23. COGEM (2010). General Surveillance. Signalering CGM/100226-01