Import and processing of maize Bt11 x MIR604

COGEM advice CGM/080521-03

Summary

The present application by Syngenta Seeds S.A.S. (file EFSA/GMO/UK/2007/50) concerns import and processing for use in feed and food of the genetically modified maize line Bt11 x MIR604. Cultivation is not part of this application.

Maize line Bt11 x MIR604 was obtained by conventional cross-breeding of the two parental lines. Previously, COGEM issued positive advices on import and processing, and on cultivation of maize line Bt11. COGEM also advised positively on import and processing of maize line MIR604.

The hybrid maize line Bt11 x MIR604 contains the cry1Ab and mcry3A gene conferring resistance to respectively certain lepidopteran and coleopteran insects. In addition, this maize line contains the pat gene, resulting in tolerance to glufosinate ammonium containing herbicides. Furthermore, Bt11 x MIR604 expresses the pmi gene allowing the plant to utilize mannose as a sole carbon source.

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 genes introduced in Bt11 x MIR604 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 incidental spillage of Bt11 x MIR604 poses negligible risks to the environment. Therefore, COGEM considers the risks associated with import and processing of maize line Bt11 x MIR604 negligible. Additionally, COGEM questions some aspects of the general surveillance plan and gives a few comments on the revised information regarding the molecular characterization.

Introduction

The present application by Syngenta S.A.S., file EFSA/GMO/UK/2007/50 concerns the import and processing of maize line Bt11 x MIR604 for use in feed and food. Maize line Bt11 x MIR604 was obtained by conventional cross-breeding of the two parental lines Bt11 and MIR604. The hybrid maize line Bt11 x MIR604 contains the *cry1Ab, mcry3A, pat* and *pmi* genes, which are constitutively expressed. As a result Bt11 x MIR604 is resistant to certain lepidopteran and coleopteran insects and tolerant to glufosinate ammonium containing herbicides. Additionally, the *pmi* gene is used as a selectable marker because it enables the plant to use mannose as a sole carbon source.

Previous COGEM advices

In 1997, COGEM issued a positive advice on import and processing of maize line Bt11 (1). In addition, a positive advice on cultivation of this maize line has been issued in 2005 (2). COGEM has also advised on the import of maize line MIR604 and concluded that the ecological risks associated with the import and processing of this maize line are negligible (3).

Aspects of the crop

Maize (*Zea mays*) is a member of the *Poaceae* family (grasses). Maize was domesticated in Central America and is nowadays cultivated throughout the world (4). In Europe, hybridization with other species cannot occur as wild relatives of maize are not present in Europe (4). The appearance of volunteers is very rare under Dutch conditions. Grains exhibit no germination dormancy, resulting in a short persistence. Establishment of maize plants in the wild has never been observed in the Netherlands. Besides, observations outside the Netherlands indicate that feral maize populations do not occur in Europe.

Molecular characterization

Maize line Bt11 x MIR604 was produced by traditional cross-breeding of the two genetically modified parental maize lines Bt11 and MIR604. The molecular characterization of these parental lines is discussed below.

Parental maize line Bt11:

Bt11 maize was generated by transformation of *Z. mays* protoplasts using a *Not*I restriction fragment which contains *cry1Ab* and *pat* gene cassettes. Besides these gene cassettes a 1.1 kb fragment of vector sequence is present upstream of the *cry1Ab* gene cassette. This fragment contains the ColE1 *ori*, the origin of replication that permits replication of plasmids in *Escherichia coli*, but which is not functional in plants. Maize line Bt11 contains a single DNA insertion with one copy of the *Not*I restriction fragment.

The *cry1Ab* gene cassette consists of the following elements:

- 35S promoter, derived from *Cauliflower mosaic virus* (CaMV)
- IVS6-ADH1 intron, intervening intron sequence 6 derived from the alcohol dehydrogenase 1 (*adh1*) gene of maize
- truncated *cry1Ab* gene, derived from *Bacillus thuringiensis* var. *kurstaki* HD-1, truncated at the 3' end and modified to enhance expression in plants. The Cry1Ab protein confers resistance to certain lepidopteran insects
- NOS terminator, derived from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens*

The *pat* gene cassette consists of the following elements:

- 35S promoter, derived from *CaMV*
- IVS2-ADH1 intron, intervening intron sequence 2 derived from the alcohol dehydrogenase 1 (*adh1*) gene of maize
- *pat* gene, derived from *Streptomyces viridochromogenes* strain Tu494 and codon-optimized to enhance expression in maize. The *pat* gene encodes phosphinothricin acetyl transferase which confers resistance to glufosinate ammonium containing herbicides
- NOS terminator, derived from the nopaline synthase (*nos*) gene of *A. tumefaciens*

The regions that flank the insert in maize line Bt11 are homologous to the *Z. mays* 180 bp knob-associated tandem repeat. Knobs are components of the maize heterochromatin and therefore strongly repressed.

Parental maize line MIR604:

MIR604 maize was produced by *Agrobacterium* mediated transformation. A modified version of the gene *cry3A (mcry3A)* is introduced conferring resistance to the western corn rootworm (*Diabrotica virgifera virgifera*) and the northern corn rootworm (*D. longicornis barberi*). Furthermore, the plant is able to use mannose as a sole carbon source by insertion of the *pmi* gene.

Mcry3A expression cassette:

- MTL promoter, derived from the *Zea Mays* metallothionein-like-gene; provides root-preferential expression
- *Mcry3A* gene, from *B. thuringiensis* subsp. t*enebrionis*; confers resistance to lopeopteran insects
- NOS terminator, derived from the *nopaline synthase* (*nos*) gene of *A. tumefaciens*

Selectable marker expression cassette:

- ZmUbiInt promoter, derived from the *Z. mays* polyubiquitin gene, provides constitutive expression in monocots
- *Pmi* gene, from *E. coli*; catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate
- NOS terminator, derived from the *nopaline synthase* (*nos*) gene of *A. tumefaciens*

Properties of the introduced genes conferring insect resistance

Maize line Bt11 was genetically modified by amongst others the insertion of the *cry1Ab* gene. The *cry1Ab* gene encodes a δ-endotoxin specific for certain lepidopteran

insects, e.g. the European corn borer (*Ostrinia nubilalis*). The δ-endotoxins are solubilized in the midgut of susceptible insects and are activated by midgut proteases to release a toxin fragment. The toxin fragment binds to specific receptors on the epithelial surface of the midgut. Subsequently, pores are formed in the membranes of the gut cells of the insect, enabling midgut bacteria to enter the body cavity, which leads to septicemia and death (5).

Hybrid maize line Bt11 x MIR604 contains a second insect resistance gene called *mcry3A*. Additional changes were made to the gene *cry3A* to enhance the activity of the expressed protein against certain coleopteran pests, particularly the western and the northern corn rootworm. The gene *cry3A* is derived from *B. thuringiensis* (subsp. *tenebrionis*). By inserting the gene, plants will produce δ-endotoxins (Bt-toxins).

Properties of the introduced genes conferring herbicide tolerance

Maize line Bt11 x MIR604 expresses the *pat* gene conferring tolerance to glufosinate ammonium containing herbicides. The *pat* gene encodes phosphinothricin-N-acetyl transferase (PAT). This protein acetylates L-phosphinothricin, the active isomer of 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. The application of glufosinate ammonium leads to reduced glutamine and increased ammonia levels in non-transgenic plants (6). Photosynthesis is inhibited and eventually the plant dies (7). In maize line Bt11 the PAT 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 (6). As a result maize line Bt11 x MIR604 is tolerant to L-phosphinothricin and thus to glufosinate ammonium containing herbicides.

Properties of the introduced selectable marker

Maize line MIR604 was genetically modified with the gene *pmi* (manA) encoding for the enzyme phosphomannose isomerase (PMI). As a result of the gene insertion, plants are capable of using mannose as a sole carbon source. Mannose is phosphorylated to mannose-6-phosphate (M6P) which can be converted to fructose-6-phosphate with the help of PMI. In non-gm plants lacking PMI, conversion of M6P will not occur. M6P will accumulate, block glycolysis, and inhibit plant growth. Consequently, the insertion of *pmi* has led to the introduction of a selection system in MIR604. Mannose is used as the selective agent and is applied to cell cultures to select transformed cells. It is not used as a selective agent in mature plants.

Molecular analysis

In the past, COGEM assessed the molecular analysis of both parental maize lines Bt11 and MIR604.

The molecular analysis of maize line Bt11 was assessed and considered adequate in a previous advice (2). Recently, the applicant provided revised information regarding the sequence data of maize line Bt11 in the renewal application for continued import and processing of this maize line. An update on the molecular characterization revealed 8 base pair changes on the Bt11 sequence. Five of the aberrant nucleotides are located in the intergenic region of the insert and two of the nucleotides are present in the maize genomic region that flanks the 3'end of the insert. In addition, a different nucleotide was identified in both terminators of the insert. This nucleotide change was identical for both terminators. Four of the aberrant nucleotides are located in non-coding regions. The applicant states that the Cry1Ab proteins are expressed correctly and concludes that the nucleotide changes in the terminator do not have an effect on its function.

COGEM is of the opinion that there are no reasons to assume that the observed changes in nucleotides will lead to different characteristics of the maize plants. Therefore, the observed differences will not change the outcome of previous environmental risk assessments.

The molecular characterization of maize line MIR604 was first assessed in 2005 and considered adequate (3). The integration patterns of the introduced genes in the parental lines remain stable and unchanged in the upcoming generations. Furthermore, a calculation based on the detection limit of the southern blot system used and the probe and genome size indicated that no backbone sequence is present in maize variety MIR604. The information provided by the applicant in 2005 showed that no novel ORF's potentially encoding new proteins or fusion proteins are present.

The current application of hybrid maize line Bt11 x MIR604 included revised information on the molecular characterization of MIR604. The applicant revised the sequence data of MIR604 and determined 16 base pair changes compared to the original characterization. The changes in the MIR604 sequence include 2 base pairs in the 5' flanking sequence and 14 base pairs in the 3' flanking sequence. The revised 3' flanking sequence revealed a new putative ORF of 258 base pairs starting in the last 13 base pairs of the NOS terminator and extending 175 base pairs into the 3' flanking region. The exact cause of these deviations (technical/analytical flaws or occurrence of mutations) remains unclear to COGEM. Besides, due to unclear presentation of the data, it took the advisers of COGEM a needless amount of time to interpret the data. Therefore COGEM urges the applicant to present the data in a clear manner.

According to the applicant, the putative protein encoded by this ORF does not show any significant homology with known toxins or allergens. However, the applicant defines an ORF as a region that initiates with an ATG codon and ends with any of the three stop codons TAA, TAG or TGA. COGEM notes that translation may initiate with other codons or that an ORF, which does not start with an ATG, becomes part of a longer ORF by the process of pre-mRNA splicing. Besides, only ORFs longer than 50 amino acids are analyzed. COGEM is of the opinion that also ORFs

that could initiate with other start codons should be examined. Furthermore, potential ORFs shorter than 50 amino acids should be included in the analysis as well.

However, recently COGEM abstains from advices on the potential risks of incidental consumption in case a food/feed assessment is already carried out by other organizations. If an application is submitted under Regulation (EC) 1829/2003 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 and RIVM. If an application is submitted under Directive 2001/18/EC a food/feed assessment is not carried out by EFSA and then COGEM advices on the potential risks of incidental consumption. Since this application is submitted under Regulation (EC) 1829/2003, a food/feed assessment is carried out by EFSA, RIKILT and RIVM. Therefore, an analysis of the formation of putative new ORFs is not carried out by COGEM.

COGEM was surprised with the revised sequence data and underlines that these subsequent deviations are a cause of great concern. However, both sequence datasets (old and revised) of the parental maize lines Bt11 and MIR604 give no reasons to expect any potential environmental risks.

In view of the above mentioned, COGEM is of the opinion that the molecular analysis of hybrid maize line Bt11 x MIR604 does not indicate that import and processing of this line would pose a risk to the environment. Regarding the risks for food and feed, the outcome of the assessment done by other organizations (EFSA, RIKILT, RIVM) was not known at the moment this advice was completed.

Environmental risk assessment

During the long process of domestication, maize has lost the ability to survive in the wild. In addition, maize needs human intervention to disseminate its seed. Maize kernels exhibit no dormancy and can only survive within a narrow range of climatic conditions. Furthermore, maize is very sensitive to weed competition and cannot persist as a weed (4, 8). In the Netherlands, volunteers are rarely found and establishment of maize plants in the wild has never been observed. There are no reasons to assume that the introduced traits will increase the potential of Bt11 x MIR604 to establish feral populations in case of incidental spillage.

General surveillance plan

Several organizations representing trade organizations that import or use viable maize are mentioned in the general surveillance plan. According to the applicant these organizations are ‘well-placed’ to detect unanticipated effects on human health or the environment. However, information concerning their expertise in the environment is not given. In addition, it is unclear whether these organizations have agreed to cooperate in the general surveillance of Bt11 x MIR604. As stated in previous

advices, COGEM is of the opinion that the applicant should ascertain that information on potential adverse effects is obtained. In addition, COGEM would prefer independent organizations which have expertise on the environment to be involved in general surveillance. In a previous advice on post-market monitoring, COGEM has outlined the standards that have to be met by a post-market monitoring system and has identified organizations which could be involved in post-market monitoring in the Netherlands (9).

Furthermore, according to the applicant indirect or delayed effects will be reported at the stage of re-evaluation or at the end of a given consent. As stated before, in COGEM's opinion all observed effects, including indirect and delayed effects, should be reported annually.

Advice

COGEM has been asked to advice on import and processing for use in feed and food of hybrid maize line Bt11 x MIR604.

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 the expression of the *cry1Ab, mcry3A, pat* and *pmi* genes in Bt11 x MIR604 increases the potential of maize to establish feral populations. In addition, introgression of the introduced genes into closely related species cannot occur, as wild relatives of maize are not present in Europe.

Regarding the current molecular characterization, COGEM is of the opinion that incidental spillage of maize line Bt11 x MIR604 poses negligible risks to the environment. Based on the considerations put forward in this advice, COGEM considers the risks for the environment associated with import and processing of maize line Bt11 x MIR604 negligible.

Additional remarks

The original sequence data of the parental maize lines Bt11 and MIR604 were revised and appeared to contain base pair changes. COGEM underlines that a proper risk assessment can only be carried out if correct and clear-cut information is provided to the competent authorities.

COGEM emphasizes that the information provided by the applicant is the foundation on which the European legislation regarding authorization of GMOs is based. If the information provided by the applicant later turns out to be incorrect, this can seriously hamper the risk analysis. Furthermore, this harms the confidence of the competent authorities assessing this information. This also applies to the confidence of European citizens in (future) consumer products containing GMOs and indirectly in the confidence in the European and national governments as well as in the industry involved.

References

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