Import and processing of cotton GHB614xLLCotton25

COGEM advice CGM/110325-01

Summary

The present application by BayerCropScience (file EFSA/GMO/NL/2010/77) concerns import and processing for use in feed and food of cotton GHB614xLLCotton25. Cultivation is not part of this application.

The hybrid cotton line GHB614xLLCotton25 was produced by conventional crossbreeding of parental cotton lines GHB614 and LLCotton25. GHB614xLLCotton25 expresses the genes 2mepsps *and* bar. *As a result, the hybrid line is tolerant to glyphosate and glufosinate ammonium containing herbicides.*

GHB614xLLCotton25 has not been previously assessed by COGEM. COGEM advised positively on import and processing of both parental cotton lines.

The molecular analysis of the inserts present in the hybrid cotton line was updated in 2010. The presence of open reading frames at the junction sites of the DNA introduced in cotton GHB614 and LLCotton25, and present in the hybrid cotton line, was analyzed. The amino acid sequence of the open reading frames was deduced in silico *and analyzed for similarity to known toxins or allergens. No similarity was found. The molecular characterization of cotton line GHB614xLLCotton25 meets the criteria of COGEM.*

Although the general surveillance (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 cotton line GHB614xLLCotton25.

In Northwest Europe, no wild relatives of cotton are present. Modern cotton cultivars do not possess any of the attributes commonly associated with problematic weeds. There are no reasons to assume that the introduced traits will increase the potential of cotton to establish feral populations. Moreover, cotton cannot survive the climatologic conditions in Northwest Europe. Therefore, COGEM is of the opinion that incidental spillage of GHB614xLLCotton25 seeds will not pose a risk to the environment in Northwest 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 cotton line GHB614xLLCotton25 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/2010/77) by BayerCropScience concerns import and processing of cotton line GHB614xLLCotton25. According to the applicant this hybrid cotton line is also referred to as GTxLL or GlyTolxLL. GHB614xLLCotton25 was produced by conventional crossbreeding of parental cotton lines GHB614 and LLCotton25. GHB614xLLCotton25 expresses the genes *2mepsps* and *bar*. As a result, the hybrid line is tolerant to glyphosate and glufosinate ammonium containing herbicides. GHB614xLLCotton25 has recently been authorized for food, feed, processing, storage and transportation in Japan (2010)[1] and is authorized for use as food in Mexico.[2]

GHB614xLLCotton25 has not been previously assessed by COGEM. Both parental lines have previously been assessed by COGEM. COGEM advised positively on import and processing of GHB614 cotton and LLCotton25.[3,4] EFSA also advised on import and processing of the two parental cotton lines and concluded that both GHB614 and LLCotton25 are unlikely to have any adverse effect on human and health or on the environment in the context of their intended uses.[5,6] In 2008, the European Commission published a decision authorizing the placing on the market of products containing, consisting of, or produced from LLcotton25.[7] A decision on the authorization of cotton GHB614 is currently pending.

Aspects of the crop

Cotton is a member of the genus *Gossypium* and belongs to the *Malvaceae* family. The majority of cultivated cotton (90%) is *Gossypium hirsutum*, but *Gossympium barbadense*, *Gossympium arboreum* and *Gossympium herbaceum* are cultivated as well.[8,9,10] In Europe *G. hirsutum* cotton is grown in Greece, Spain and Bulgaria.[11]

Cotton plants reproduce sexually.[10] Cotton is predominantly a self-pollinating species, but crosspollination may occur. The pollen of cotton is large, heavy and somewhat sticky. [9,10] The viability of *G. hirsutum* pollen decreases rapidly after eight hours.[10] Outcrossing rates for cotton are strongly influenced by the prevalence of insects[10] and dissemination of pollen by wind is (almost) absent.[9] Amongst others bumble bees (*Bombus*), honey bees (*Apis*) and other bee species (*Anthophora*, *Melissodes* and *Halictus*) are pollinators of cotton flowers.[9] Wild relatives of cotton (*G. hirsutum*) do not occur in Northwest Europe. Therefore, hybridization with wild relatives cannot occur in Northwest Europe.[9]

The climate in Northwest Europe is not suited for cotton growth. Cotton is highly sensitive to temperature and susceptible to frost. Temperature is the main factor to determine the geographic range in which cotton can be grown. *G. hirsutum* seeds do not germinate until the temperature reaches 15ºC[9] and plant development ceases when temperatures are below 12ºC.[10] Activity is delayed when the temperature rises above 38ºC.[9] For normal development, cotton needs an average of 150 days with temperatures between these values.[9] The optimum temperature for germination is 34ºC, for growth of seedlings 24-29ºC and for later continuous growth 34ºC. When the crop is grown at lower temperatures, the production of vegetative branches increases and the cropping period is extended. Because cotton is susceptible to frost, the whole growth period (which can range from 160 to 220 days) has to be free of frost.[8] In places where cotton is grown as a rain-fed crop the average rainfall is 800-1200 mm.[8] In areas where the rainfall is less than 500 mm a year, irrigation should be applied.[9] In the seedling stage cotton does not tolerate shady circumstances, and in later plant stages reduced light intensity affects flowering and fruiting. [8]

Cottonseed may be dispersed by wind or water but may also be spread during transport or when feeding cattle. [10] In addition, cottonseed may be transported by birds or rodents. Germination is less likely to occur in undisturbed sites and roadsides than in disturbed sites.[10] Seeds from cotton cultivars do not possess dormancy[9,10] and will germinate in autumn if conditions are favourable. In addition, seeds will usually not survive in humid soil.[10] In regions with mild and dry winters, cottonseeds may overwinter and germinate in spring. Seedlings are sensitive to competition from weeds.[8]

Modern cotton cultivars do not possess any of the attributes commonly associated with problematic weeds, such as dormancy, persistence in soil banks, germination under adverse environmental conditions, rapid vegetative growth, a short life cycle, very high seed output,

high seed dispersal and long-distance dispersal of seeds. Cotton volunteers occur in cotton growing areas and may occur when cottonseed is used as livestock feed. The occurrence of volunteer cotton is limited by the availability of adequate soil moisture or the occurrence of frost[10]. There are reports that *G. hirsutum* and *G. herbaceum* cotton are naturalised in some Southern European countries, e.g. Greece and Spain.[12,13,14,15]

Molecular characterisation

GHB614xLLCotton25 was produced by crossing the two parental cotton lines GHB614 and LLCotton25 using traditional breeding methods. The molecular characterization of maize GHB614 was previously evaluated by COGEM. COGEM concluded that the molecular characterization of this cotton line was adequate.[3]

Southern blot analysis showed that the inserts from GHB614 and LLCotton25 are present in GHB614xLLCotton25. 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 GHB614 and LLCotton25 inserts and cotton genomic DNA were reanalyzed in the current application. The results from these analyses are described below.

Insert from parental cotton line GHB614

The genetically modified cotton line GHB614 was produced by *Agrobacterium tumefaciens* mediated transformation using the pTEM2 vector, which is derived from pGSC1700.

The following elements were introduced in GHB614:

- Left border region, derived from *A. tumefaciens* and used for integration of the T-DNA;
- Ph4a784At, constitutive promoter derived from the histone H4 gene from *Arabidopsis thaliana* with preferential expression in meristems of young seedlings and adult plants;
- TPotpC, chloroplast transit peptide based on sequences from sunflower (*Helianthus annuus*) and maize *(Zea mays*);
- Intron1 h3At, first intron of gene II of the histone H3.III variant of *A. thaliana;*
- *2mepsps*, modified *epsps* gene originally derived from *Z. mays*, encoding 5 enolpyruvylshikimate-3-phosphate synthase (2mEPSPS). The 2mEPSPS protein has two amino acids substitutions when compared to the wild type maize EPSPS protein gene. A methionine amino acid is added at the N-terminal site of the protein to restore the cleavage site of the chloroplast transit peptide;
- 3'histon At, transcription terminator derived from the 3' untranslated region of the histone H4 gene of *A. thaliana*;
- Right border region, derived from *A. tumefaciens* and used for integration of the T-DNA.

GHB614 contains a single intact copy of the T-DNA. Upon integration of the T-DNA into the genomic DNA a 17 basepair fragment of cotton genomic DNA at the target site was deleted. No backbone fragments from the vector are present in GHB614.

The two junction sites between the cotton genomic DNA and the insert were reanalyzed in 2010. Twelve open reading frames (ORFs) were identified (stop to stop codon) and were translated *in silico* into amino acid sequences. Two of the ORFs located in the 5' junction region contain a start codon. Based on bioinformatic analysis the applicant concluded that it is highly unlikely that these two ORFs are expressed. The deduced amino acid sequences of the

twelve ORFs were analyzed for homology to known toxins and known allergens. No significant similarities were found between the twelve putative amino acid sequences and known toxins or allergens.

Insert from parental line LLCotton25
LLCotton25 was generated by *A. tumefaciens* mediated transformation using the vector plasmid pGSV71, which is derived from pGSC1700.

The following elements were introduced into LLCotton25:
- Right border region, derived from *A. tumefaciens* and used for integration of the T-DNA;
- P-35S3 promoter, constitutive promoter derived from Cauliflower Mosaic Virus (CaMV);
- *bar*, *bar* gene derived from *Streptomyces hygroscopius*, encoding phosphinotricin acetyltransferase (PAT). The N-terminal two codons of the wild type *bar* coding region were substituted (into ATG and GAC, respectively);
- 3'nos, transcription terminator derived from the 3' untranslated region of the nopaline synthase gene from *A. tumefaciens*;
- Left border region, derived from *A. tumefaciens* and used for integration of the T-DNA.

LLCotton25 contains a single intact copy of the T-DNA. The right border and left border regions of the T-DNA were not completely integrated in LLCotton25. Twenty-three basepairs of the right border region and 4 basepairs of the left border region are missing. In addition, upon integration of the T-DNA into the genomic DNA a 37 basepair fragment of cotton genomic DNA was deleted at the target site. No backbone fragments from the vector are present in LLCotton25.

The two junction sites between the cotton genomic DNA and the insert were reanalyzed in 2010. The applicant identified twelve ORFs (stop to stop codon) and translated these *in silico* into amino acid sequences. Three of the ORFs contain a start codon. Based on bioinformatic analysis the applicant concluded that translation of these three ORFs is very unlikely. One of the identified ORFs encoded a putative protein of less than 8 amino acids. The ORFs that encoded putative proteins of 8 or more amino acids were analyzed for homology to known toxins and known allergens. None of the deduced amino acid sequences from the identified ORFs showed sequence similarity or identity to known toxic or allergenic sequences. The applicant also used FGENESH software to predict the presence of putative genes. One putative gene sequence was identified using this software. The applicant concluded that it is highly unlikely that this putative gene sequence leads to the production of protein. The sequence was translated *in silico* into an amino acid sequence and analyzed for homology to known toxins and known allergens. No sequence similarity or identity to known toxic or allergenic sequences was found.

In conclusion, the applicant stated that putative polypeptides at the junction sites of the DNA introduced in cotton lines GHB614 and LLCotton25, and present in GHB614xLLCotton25, did not possess similarity to known toxins or allergens.

Properties of the introduced genes
GHB614xLLCotton24 expresses the *2mepsps* gene, which encodes a modified EPSPS protein, i.e. 2mEPSPS. The 2mEPSPS protein differs from the wild type EPSPS enzyme by

two amino acid substitutions. A chloroplast transit peptide sequence (TPotpC) is fused to the *2mepsps* gene, resulting in the transport of the 2mEPSPS protein to the chloroplast.[16] EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) is an enzyme involved in the biosynthesis of aromatic amino acids. EPSPS proteins are active in the chloroplasts of a plant cell. Glyphosate inhibits EPSPS, resulting in a lack of amino acids essential for growth and development of plants.[17] GHB614xLLCotton25 expresses the 2mEPSPS protein, which is not inhibited by glyphosate[18] and therefore GHB614xLLCotton25 is tolerant to glyphosate containing herbicides.

GHB614xLLCotton25 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] GHB614xLLCotton25 expresses the *bar* 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 GHB614xLLCotton25 is tolerant to L-phosphinothricin and thus to herbicides containing glufosinate ammonium.

Environmental risk assessment

Cotton is predominantly a self-pollinating species, but crosspollination may occur. Wild relatives of cotton (*G. hirsutum*) do not occur in Northwest Europe. Therefore, hybridization with wild relatives cannot occur in Northwest Europe.[9]

Cotton plants are very sensitive to temperature. A reasonably high temperature (an average of 150 days with a temperature between 15 and 38˚C) is required in all stages of development. The optimum temperature for germination is 34ºC, for growth of seedlings 24-29ºC and for later continuous growth 34ºC. In areas where the rainfall is less than 500 mm a year, irrigation should be applied for cotton growth. In the Netherlands, May, June, July, August and September have average monthly temperatures above 12ºC, but below 18ºC.[21] In addition, in May, June, July, August and September the average monthly precipitation does not exceed the 100 mm.[21] Based on the above, the Dutch climate is unsuited for cotton growth.

Climate conditions in other parts of the European Union e.g. Greece, Spain, Bulgaria and Portugal are more suitable for growing cotton.[11] Seeds from cotton cultivars do not possess dormancy[9,10] and will germinate in autumn if conditions are favourable. In addition, seeds will usually not survive in humid soil.[10] In regions with mild and dry winters, cottonseeds may overwinter and germinate in spring.[8] The occurrence of volunteer cotton is limited by the availability of adequate soil moisture or the occurrence of frost[10].

There is no indication that the introduced traits, which confer tolerance to glyphosate and glufosinate ammonium containing herbicides, will increase the ability of cotton to survive in the environment. The applicant carried out an agronomic assessment for GHB614xLLCotton25. This assessment does not give any indication to assume that GHB614xLLCotton25 has an increased survivability compared to conventional cotton lines.

In view of the above, there are no reasons to assume that GHB614xLLCotton25 has an increased potential for the establishment of feral populations in case of incidental spillage. In addition, the climate in Northwest Europe is not suited for cotton growth.

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 plan

General surveillance (GS) 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.

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 GHB614xLLCotton25. In 2010, COGEM formulated criteria which GS plans concerning Dutch applications for import and cultivation of GM crops have to comply with.[22] Although the general surveillance plan could be improved by a guarantee that operators will monitor for unanticipated effects, COGEM considers the general surveillance plan sufficient for import and processing of GHB614xLLCotton25.

Advice

The present application concerns import and processing for feed and food purposes of the genetically modified cotton line GHB614xLLCotton25. Cultivation is not part of the application. Therefore, the risk assessment focuses on the accidental spillage of cottonseeds.

Cotton plants are very sensitive to temperature. The Dutch climate is unsuited for cotton growth. There is no indication that the introduced traits, which confer tolerance to glyphosate and glufosinate ammonium containing herbicides, will increase the ability of cotton to survive in the environment. Therefore, there are no reasons to assume that GHB614xLLCotton25 has an increased potential for the establishment of feral populations in case of incidental spillage. The climate in Northwest Europe is not suited for cotton growth. Therefore, in COGEM's view there is no risk that incidental spillage of cottonseeds will lead to the spread of cotton within Northwest Europe.

The molecular analysis of the inserts present in GHB612xLLCotton25 has been updated. The molecular characterization of GHB614xLLCotton25 meets the criteria of COGEM.

In view of the above, COGEM is of the opinion that the risks for humans and the environment associated with import and processing of cotton line GHB614xLLCotton25 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.

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