Import and processing of cotton MON15985 x MON1445

COGEM advice CGM/081027-02

Summary

COGEM has been asked to advice on an application concerning the import and processing for use in food and feed of cotton line MON15985 x MON1445. Cultivation is not part of this application.

The hybrid cotton line was produced by crossbreeding of the two genetically modified parental lines MON15985 and MON1445. As a consequence, the hybrid line contains the genes cry2Ab2 and cry1Ac and therefore the line is resistant to certain insects of the Lepidopteran order. In addition, the hybrid cotton line expresses cp4 epsps which confers tolerance to herbicides containing glyphosate. Also, the marker genes nptII and uidA are present and simplify the selection of transformed cotton cells.

In Europe, no wild relatives of cotton are present and modern cotton cultivars do not possess any of the attributes commonly associated with problematic weeds. There are no reasons to assume that the genes inserted will increase the potential of the cotton to establish feral populations. Moreover, cotton cannot survive the climatological conditions in Northwest Europe. Therefore, COGEM is of the opinion that incidental spillage of seeds of this cotton line will not pose a risk to the environment in Northwest Europe.

In view of these considerations, COGEM is of the opinion that the import and processing of cotton line MON15985 x MON1445 does not pose a significant risk to the environment in the Netherlands.

Introduction

The present application by Monsanto Europe S.A., file EFSA/GMO/UK/2008/58, concerns the import and processing of cotton line MON15985 x MON1445. Cotton is mainly cultivated for the use of cotton lint. Cottonseeds are harvested as rest products and used as feed, or for the production of cottonseed oil for human consumption.

Cotton line MON15985 x MON1445 expresses the *cry1Ac* and *cry2Ab2* genes, both of which confer resistance to Lepidopteran pests. Furthermore, this line expresses the *cp4 epsps* gene, which confers tolerance to glyphosate. In addition, MON15985 x MON1445 cotton contains the *nptII* and *uidA* marker genes, which allow easy selection of transformed cotton cells.

Parental cotton line MON15985 has been authorized for commercial import, processing and cultivation in Australia (2002), the United States of America (2002), South Africa (2003) and India (2006) (1). No adverse effects concerning handling and

consuming of products and derivatives of this line have been reported. Parental line MON1445 has been authorized for commercial import, processing and cultivation in, among other countries, the United States (1995), Japan (1997) and Argentina (1999) (1).
In Australia, hybrid line MON15985 x MON1445 has been authorized for commercial import, processing and cultivation since 2002. This line has also been authorized for food and/or feed purposes in the European Union (2005), Japan (2005) and Mexico (2006) (1).
No adverse effects concerning handling and consuming of products and derivatives of parental lines MON15985, MON1445 and hybrid line MON15985 x MON1445 have been reported.

Aspects of the crop

Cotton is a member of the genus *Gossypium* and belongs to the *Malvaceae* family (2). More than 95% of commercial cotton is upland cotton, *G. hirsutum*, while long staple cotton, *G. barbadense,* covers a small area of less than 5% (3).

Major producers of seed cotton and lint are China, the United States of America, India, Pakistan, Brazil and Turkey. Together, these countries are responsible for 80% of the total cotton production (4). Within the European Union, cotton is mainly grown in Greece and on a smaller scale in Spain and Bulgaria (5). It should be noted that only non-genetically modified (gm) cotton is grown in Europe.

Depending on cultivar and climate, the growth period can range from 160 to 220 days. The crop will flower about eight weeks after planting. In the following two months, a cottonboll will develop and will finally open. About eight weeks later, the cotton fibers have reached full length and cellulose content and the cotton can be harvested (2).

Cotton is highly sensitive to temperature. It does not start its vegetative activity until the temperature reaches 15ºC and the activity is delayed when the temperature rises above 38ºC. For normal development, cotton needs an average of 150 days with temperatures between these values (6). 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 will be extended. Reduced light intensity will retard flowering and fruiting. Because cotton is susceptible to frost, the whole growth period of six months has to be free of frost (3,6).

In areas where the rainfall is less than 500 mm a year, irrigation should be applied (7). In places where cotton is grown as a rain-fed crop, the average rainfall is 800-1200 mm (2).

Cultivated cotton is predominantly a self-pollinating species. But the prevalence of insects strongly influences outcrossing rates for cotton. Many field-based assessments estimate out-crossing rates at 10% or less, although rates up to 80% have been found.

The pollen remains viable up to a period of twelve hours (7). Cotton has some wild-relatives, however, they are not found in Europe (3).

Cotton is a domesticated crop. 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 (7). Cotton volunteers occur in cotton growing areas and may occur when cottonseed is used as livestock feed. Seeds that do not germinate are likely to be removed by seed predators or rot, rather than become incorporated into a persistent soil seed bank (7). There is no indication that these volunteers establish feral populations in Europe.

Previous COGEM advices

COGEM issued a positive advice on commercialisation of cotton line MON1445 in 1998 (8). Recently, COGEM also advised positively on the import and processing of parental cotton line MON15985 and on hybrid line MON88913 x MON15985 (9,10). In 1998, COGEM advised positively on the commercialisation of cotton line MON531 (11). MON531 was used to produce cotton line MON15985.

Molecular characterisation

The hybrid cotton line MON15985 x MON1445 was produced by crossbreeding of two genetically modified cotton lines. An overview of the sequences introduced in the parental lines, the properties of the introduced genes and the molecular analysis is given.

Introduced elements

The elements inserted in the parental lines are described below.

Summary of the elements inserted in MON15985

MON15985 cotton was produced by particle bombardment of the genetically modified cotton line MON531, which was previously produced by *Agrobacterium* mediated transformation. As a result of the particle bombardment, MON15985 contains the *cry2Ab2* and *uidA* genes.

- Overview of the elements inserted in MON15985:
  - enhanced 35S promoter, derived from *Cauliflower mosaic virus* (CaMV) and containing the duplicated enhancer region
  - *uidA* gene, coding for the β-D-glucuronidase GUS protein from *Escherichia coli* and used as a selection marker

- nos transcription terminator, derived from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens*
- enhanced 35S promoter, derived from CaMV and containing the duplicated enhancer region
- *hsp70* leader, derived from the heat shock protein 70 from petunia
- *ctp2* chloroplast targeting sequence from *Arabidopsis thaliana*
- *cry2Ab2* gene, encoding a synthetic version of the Cry2Ab2 protein of *Bacillus thuringiensis* subsp. *kurstaki*
- nos transcription terminator, derived from the nopaline synthase (*nos*) gene of *A. tumefaciens*

- In addition, MON15985 contains the *cry1Ac, aad* (non-functional) and *nptII* genes, which were introduced in MON531. Overview of the elements introduced in MON531:
  - 3'end of the *cry1Ac* gene, (non functional) synthetic version of the *cry1Ac* gene of *B. thuringiensis* subsp. *kurstaki*
  - 7S transcription terminator, derived from the 7S seed storage protein gene of soybean
  - 2 copies of a portion of the right border region from *A. tumefaciens* used for the transfer of T-DNA
  - 7S transcription terminator, derived from the 7S seed storage protein gene of soybean
  - *cry1Ac* gene, encoding a synthetic version of the Cry1Ac protein of *B. thuringiensis* subsp. *kurstaki*
  - enhanced 35S promoter, derived from CaMV and containing the duplicated enhancer region
  - *aad* gene, (non functional) bacterial gene (comprising its own regulatory elements) coding for an aminoglycoside-modifying enzyme from transposon Tn7
  - nos transcription terminator, derived from the nopaline synthase (*nos*) gene of *A. tumefaciens*
  - *nptII* gene, isolated from the bacterial transposon Tn5 and encoding a neomycin phosphotransferase type II. The *nptII* cassette also contains a 153 bp portion of the *ble* gene encoding the bleomycin binding protein
  - 35S promoter, derived from CaMV
  - oriV, origin of replication derived from plasmid RK2

<u>Summary of the elements introduced in MON1445</u>

MON1445 was produced by *Agrobacterium*-mediated transformation. The elements introduced are:

- E9 transcription terminator, derived from *Pisum sativum*

- *cp4 epsps*, gene confers resistance to glyphosate containing herbicides
- *ctp2*, chloroplast targeting sequence from *A. thaliana*
- CMoVb, 35S promoter, derived from *Figwort mosaic virus*
- *aad* gene, isolated from the bacterial transposon Tn7 and encoding an aminoglycoside-modifying enzyme
- nos transcription terminator, derived from the nopaline synthase (*nos*) gene of *A. tumefaciens*
- *nptII* gene, isolated from the bacterial transposon Tn5 and encoding a neomycin phosphotransferase type II. The *nptII* cassette also contains a 153 bp portion of the *ble* gene encoding the bleomycin binding protein
- 35S promoter, derived from CaMV
- oriV, origin of replication derived from plasmid RK2

Properties of the introduced genes conferring insect resistance

Cotton line MON15985 x MON1445 expresses the proteins Cry1Ac en Cry2Ab2. These δ-endotoxins provide increased resistance to certain insects of the Lepidopteran order, such as the cotton bollworm (*Helicoverpa armigera*) and pink bollworm (*Pectinophora gossypiella*). Larvae of these insects inflict damage to the plants through feeding.

The δ-endotoxins confer protection by solubilizing in the midgut of susceptible insects, after which activation by midgut proteases takes place 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 (12).

Properties of the introduced genes conferring herbicide tolerance

The hybrid cotton line contains a functional *cp4 epsps* gene. This gene encodes for a CP4 EPSPS protein possessing a high tolerance to glyphosate. EPSPS is a naturally occurring enzyme involved in the biosynthesis of aromatic amino acids. In non-transgenic soybean lines, glyphosate acts by binding to and inhibiting the function of naturally occurring EPSPS. Consequently, aromatic amino acids are no longer formed, leading to plant death. In contrast, CP4 EPSPS has a reduced binding affinity to glyphosate and thus the protein is not affected by this substance. Because MON1445 expresses *cp4 epsps,* it has acquired a high tolerance to glyphosate (13).

EPSPS proteins are active in the chloroplasts of a plant cell. The sequence encoding the chloroplast transit peptide is fused to the *epsps* gene, resulting in the transport of the transgenic CP4 EPSPS protein to the chloroplast (14).

Properties of introduced marker genes

Hybrid line MON15985 x MON1445 expresses the *nptII* and *uidA* genes. These genes are used as a marker during transformation and enable the selection of genetically modified cotton cells.

Expression of the *nptII* gene enables the MON15985 x MON1445 cotton cells to survive in the presence of aminoglycosides (e.g. kanamycin). Aminoglycosides disrupt protein synthesis by binding to the 30S ribosomal subunit which causes misreading of mRNA and results in non-functional, misfolded proteins leading to cell death (15). The *nptII* gene encodes neomycin phosphotransferase type II which modifies the aminoglycoside molecule. The modified molecule cannot bind to the 30S ribosomal subunit, protein synthesis is no longer disrupted and the cotton cells can survive if aminoglycosides are present (16).

The *uidA* gene encodes β-D-glucuronidase (GUS) an exohydrolase which catalyzes cleavage of β-glucuronides. Expression of the *uidA* gene enables selection of transformed cotton cells because these cotton cells turn blue in the presence of substrate 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (X-gluc) (17).

Also, MON15985 x MON1445 contains the *aad* sequence encoding the enzyme AAD (3'(9)-O-aminoglycoside adenylyltransferase). The *aad* gene is not expressed in MON15985 x MON1445 because it is under control of a prokaryotic promoter which is not functional in cotton. Analysis of protein levels in MON15985 x MON1445 confirmed that the AAD protein is not present.

Molecular analysis

The molecular analysis of the parental line MON1445 and the hybrid line MON15985 x MON1445 are described below. The molecular analysis of MON15985 was discussed extensively in a recent COGEM advice (9). COGEM concluded that the analysis of this cotton line contains some weaknesses.

<u>Parental line MON1445</u>

Cotton line MON1445 was generated by *A. tumefaciens* mediated transformation. The insert is genetically stable in multiple consecutive generations, as was shown by Southern blotting with probes detecting *cp4 epsps* en *nptII*.

The plasmid vector PV-GHGT07 (PV7) used for transformation consists of the coding sequences *cp4 epsps, nptII, aad* and *gox*, the sequences for CMoVb, and the sequences for the origins of replication ori V and pBR322. The applicant showed by Southern blotting analysis that *cp4 epsps*, *nptII*, *aad* and *CMoVb* are present in MON1445. Southern blotting also demonstrated that oriV is only partially transferred into MON1445. Approximately 200 bp of the 400 bp are inserted. The remaining sequences and coding regions of the plasmid are not transferred into MON1445.

The sequences of the insert and the regions flanking the insert in MON1445 were determined from PCR fragments and compared to the corresponding genomic sequence of the non-trangenic parental line. It was demonstrated that the flanking regions are of genomic cotton origin. The analysis showed that at the integration site 67 bp of genomic DNA were deleted and one base pair was added. The applicant states that the function of the DNA sequence at the integration site is unknown.

Sequences spanning the 5' and 3' junctions between the insert and the genomic DNA were translated from stop to stop codon in all frames. None of the putative polypeptides showed homology to known toxins or allergens.

COGEM remarks that the quality of several Southern blot hybridizations is very poor. In addition to the oriV fragment derived from the MON1445 insertion, the oriV hybridizations gave rise to several additional bands, which are also detected in the hybrid line MON15985 x MON1445. Their presence is explained by a partial digestion of the genomic DNA. Since no other hybridizations are shown to confirm this, additional small oriV inserts can not formally be excluded. Nevertheless, by combining results from different experiments, it can be assumed that there is only a single copy of the insert.

Furthermore, the applicant uses plasmid PV-GHGT06 (PV6) instead of PV7 (used for transformation of the cotton line) as a hybridization control in several Southern blot analyses. Moreover, PV6 was used as a probe in one analysis. The applicant states that PV6 is smaller than PV7 and the *gox* cassette is absent. It is unclear why the applicant uses PV6 in stead of PV7 in the analyses.

<u>Hybrid line MON15985 x MON1445</u>

The hybrid line was produced by traditional breeding of MON15985 and MON1445. In addition to the analyses of the various parental cotton lines which are described above and which apply also for the hybrid line, complementary tests were performed on MON15985 x MON1445.

Southern blot analyses demonstrated that the parental inserts are all present in the hybrid cotton line MON15985 x MON1445. COGEM is of the opinion that these analyses are also of poor quality. However, COGEM concludes that only a single copy of every insert is present.

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. 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 and RIVM.

RIKILT and RIVM requested additional information, including data on the absence of AAD protein in seed samples of MON15985 x MON1445 or data concerning the toxicity and allergenicity of AAD protein (18).

General surveillance plan

General surveillance was introduced to be able to observe unexpected effects of the cultivation of genetically modified crops on the environment. The setting or population in which these effects could occur is not or hardly predictable.

In the present application, a detailed general surveillance plan is provided to observe and register timely adverse effects of the import of MON15985 x MON1445. Following the initial placing on the market, the authorization holder will submit general surveillance reports on an annual basis for the duration of the authorization period.

Observations for unanticipated adverse effects will be monitored by existing systems including the authorization holder and operators involved in the handling and use of viable MON15985 x MON1445 cotton. Operators involved in the import, handling and processing of the hybrid line inform the European trade associations (COCERAL, UNISTOCK and FEDIOL) of observed adverse effects. The trade associations report these effects to the authorization holder via the European Association of Bioindustries (EuropaBio) or directly to the authorization holder. EuropaBio is an association of members of the plant biotechnology industry which hosts a website containing information on approved genetically modified plants subject to general surveillance. The website contains an e-mail address and a telephone number to exchange information on the plants. COGEM points out that to gather general surveillance data a questionnaire would be helpful. By placing such a list on the website, essential information on adverse effects can be collected in a more coherent and consistent manner.

As mentioned in previous advices, COGEM would prefer independent organizations, which have expertise on the environment and whose activities in general surveillance continue after the authorization period, 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 (19).

Advice

The present application concerns the import and processing for feed and food purposes of a genetically modified cotton line. Cultivation is not part of the application. Therefore, the risk assessment focuses on the accidental spillage of cottonseeds. As mentioned before, 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 Dutch climate is unsuitable for

cotton growth. During the warmest months (April to October), the average temperature is around 14ºC. The average rainfall for spring and summer is 375 mm and is below the required 500 mm (20). Moreover, the frost periods during the winter make it impossible for cotton to survive and establish itself in the Netherlands.

Climate conditions in other parts of the European Union are more suitable for growing cotton. At the moment cotton is grown in Greece, Spain and Bulgaria (5). However, it is not to be expected that sillage of cottonseeds in these countries leads to the establishment of feral populations because no feral populations have been observed in Europe. All European cotton is irrigated due to shortage of rainfall in the growing season. Furthermore, modern cotton cultivars do not possess any of the attributes commonly associated with problematic weeds and there are no reasons to assume that the inserted genes will increase the potential of the cotton to run wild.

In view of the above, COGEM is of the opinion that there is no risk that incidental spillage of cottonseeds will lead to the spread of cotton within Northwest Europe. The molecular characterizations of MON15985 x MON1445 and of both parental lines contain weaknesses. However, parental lines MON1445 and MON531 (used for the production of parental line MON15985) have a history of safe use. Also, the hybrid cotton line has been authorized for commercial import, processing and cultivation since 2002. No adverse effects have been reported. In addition, cotton cannot grow in the Netherlands and the current application concerns import and processing. Therefore, COGEM is of the opinion that the proposed import and processing of the genetically modified cotton line MON15985 x MON1445 does not pose a significant risk to the environment in the Netherlands.

Additional remarks

Marker gene encoding β-D-glucuronidase

MON15985 x MON1445 contains the *uidA* gene from *E. coli* which expresses β-glucuronidase (GUS). β-Glucuronidases are found in many other bacterial species, a wide range of animals and in various higher plants (21,22). Adverse effects of the GUS protein on the environment have not been reported. The current application concerns import and processing and therefore the interaction of MON15895 x MON1445 with the environment is limited. In view of the above mentioned considerations, COGEM is of the opinion that the presence of the GUS protein in MON15985 x MON1445 does not pose a risk to the environment.

Marker genes conferring antibiotic resistance

Cotton line MON15985 x MON1445 expresses the marker gene *nptII* (which confers resistance to kanamycin and neomycin) (23). In addition, the *aad* marker gene (conferring resistance to streptomycin and spectinomycin) is present (23).

In 2004, the European Food Safety Authority (EFSA) published an opinion on the use of antibiotic resistance genes as marker genes in genetically modified plants. They conclude that the frequency of horizontal gene transfer of antibiotic resistance genes from genetically modified plants to other organisms is very low. Furthermore, they state that it has been shown, or is extremely likely, there is a considerable pool of resistance genes already present in the microbiota in the environment. In spite of these considerations, EFSA is of the opinion that the *aad* gene should be restricted to field trial purposes and should not be present in gm-plants which will be placed on the market (24).

On the other hand, EFSA states that there is no rationale for restricting or prohibiting the use of *nptII* in plants to be placed on the market; in particular, because *nptII*, among others, has a history of safe use in food crops (24).

COGEM is also of the opinion that the chance of gene transfer from plant to bacterium is not likely to occur. It has only been observed during specific laboratory situations and not in practice (23,25). Furthermore antibiotic resistance genes are already present in the environment (23).

In cultivation, plant material is in close contact with soil bacteria; however such contact does not take place in case of import. The present application concerns the import and processing for feed and food purposes. Moreover, consumption of cotton products is limited.

In view of the above, COGEM has already advised positively on the commercialisation of MON531 (used for the production of MON15985 cotton) and of the cotton hybrid MON88913xMON15985. Both cotton lines contain the *nptII* and *aad* genes (10,11). Furthermore, MON15985 and MON531 have been approved for food and feed purposes in the European Union (notified as existing products). In COGEM's opinion, the presence of both genes poses no risk to the environment or to human health.

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