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MODIFICATIE

Aan de minister van
Volkshuisvesting, Ruimtelijke
Ordening en Milieubeheer
Mevrouw dr. J.M. Cramer
Postbus 30945
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DATUM: 20 oktober 2008
KENMERK: CGM/081020-01
ONDERWERP: Advies import en verwerking van katoen MON15985 (EFSA/GMO/UK/2008/57)

Geachte mevrouw Cramer,

Naar aanleiding van de adviesvraag betreffende het dossier EFSA/GMO/UK/2008/57, getiteld "Application for authorization to place on the market MON15985 cotton in the European Union, according to Regulation (EC) No 1829/2003 on genetically modified food and feed" door Monsanto Europe S.A., adviseert de COGEM als volgt.

Samenvatting:
De COGEM is gevraagd te adviseren over de import en verwerking van de genetisch gemodificeerde katoenlijn MON15985. Deze katoenlijn is voorzien van de *cry1Ac* en *cry2Ab2* genen waardoor de planten resistent zijn tegen bepaalde insecten uit de orde van de Lepidoptera. Daarnaast bevat MON15985 de genen *nptII* en *uidA*, die de selectie van getransformeerde cellen vereenvoudigen.
In Europa komen geen wilde verwanten van katoen voor en katoen bezit niet de eigenschappen om in Europa te kunnen verwilderen. Bovendien kan katoen de klimaatomstandigheden in Noordwest-Europa niet overleven. De katoenplant is namelijk sterk koudegevoelig en heeft hoge temperaturen nodig voor kieming en ontwikkeling. Bovendien is voor de teelt van katoen gedurende het hele jaar irrigatie of hoge neerslag noodzakelijk. Er zijn geen redenen om aan te nemen dat de modificatie het verwilderingspotentieel vergroot. De COGEM acht derhalve de kans verwaarloosbaar klein dat incidenteel morsen van de katoenzaden leidt tot verspreiding van de genetisch gemodificeerde katoenlijn MON15985 binnen Noordwest Europa.
Concluderend acht de COGEM de milieurisico's van MON15985 verwaarloosbaar klein en heeft zij derhalve geen bezwaar tegen import en verwerking van deze katoenlijn.

De door de COGEM gehanteerde overwegingen en het hieruit voortvloeiende advies treft u hierbij aan als bijlage.

Hoogachtend,

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Voorzitter COGEM

c.c. Drs. H.P. de Wijs
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Import and processing of MON15985 cotton

COGEM advice CGM/081020-01

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. Cultivation is not part of this application.

MON15985 cotton was produced by inserting the cry2Ab2 and uidA genes into the genome of the genetically modified cotton line MON531, which already contained the cry1Ac and nptII genes. As a result, this line is resistant to certain insects of the lepidopteran order. The presence of the nptII and uidA marker genes simplifies 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. In Europe, establishment of feral populations has never been observed. 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 import and processing of cotton line MON15985 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/2008/57, concerns import and processing of cotton line MON15985. 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.

MON15985 expresses the *cry1Ac* and *cry2Ab2* genes, both of which confer resistance to Lepidopteran pests. In addition, MON15985 cotton contains the *nptII* and *uidA* marker genes, which allow easy selection of transformed cotton cells.

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.

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). 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). Cotton volunteers occur in cotton growing areas and may occur when cotton seed is used as livestock feed. There is no indication that these volunteers establish feral populations in Europe.

Previous COGEM advices

Recently, COGEM advised positively on the application for import and processing of hybrid cotton line MON88913 x MON15985 (8). In 1998, COGEM advised positively on the commercialisation of cotton line MON531 (9). MON531 was used to produce cotton line MON15985.

Molecular characterisation

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 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 bleomycin binding protein
- 35S promoter, derived from CaMV
- V ori, origin of replication derived from plasmid RK2 of *Agrobacterium*

Properties of the introduced genes conferring insect resistance

Cotton line MON15985 contains genes encoding 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 (10).

Properties of introduced marker genes

MON15985 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 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 (11). 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 (12).

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 cotton cells that express the *uidA* gene turn blue in

the presence of the substrate 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (X-gluc) (13).

Molecular analysis

MON15985 was generated by particle bombardment of the genetically modified cotton line MON531. The applicant showed by Southern blot hybridization that no backbone fragments are present in MON15985 or in MON531.

In addition, Southern blot analysis demonstrated that MON15985 contains one additional insert in comparison to MON531. This insert consists of one copy of the *uidA* and *cry2Ab2* expression cassettes.

Southern blot and sequence analysis indicated that 284 basepairs are missing at the 5' end of the insert. The promoter of the *uidA* gene was partially deleted (260 basepairs are missing). However, GUS protein was detected in leaf and seed samples, thus indicating that the deletion of these basepairs did not have an effect on the expression of the *uidA* gene. In addition, 66 basepairs of non-coding DNA were deleted at the 3' end of the insert. The *cry2Ab2* expression cassette was not affected by the deletion.

It is unclear whether deletions or rearrangements occurred at the site of insertion in the cotton genome because the site of insertion in MON15985 was not compared to the parental cotton line.

According to the applicant sequence analysis of 1598 basepairs flanking the 5' region of the insert showed that basepairs 75 to 463 were homologous to chloroplast DNA. Possibly, particle bombardment caused co-integration of chloroplast DNA. The applicant reported that sequence analysis of 636 basepairs flanking the 3' region of the insert indicated that these basepairs were homologous to cotton genomic DNA. 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.

In addition to the insert described above, MON15985 contains the elements present in MON531 cotton, which was used to produce MON15985.

Southern blot and sequence analysis show that the functional insert in MON15985 that originated from MON531 consists of one copy of the *nptII* and *cry1Ac* expression cassettes with the right border region coupled to a second copy of the right border region and a truncated *cry1Ac* expression cassette arranged as an inverted repeat. The truncated *cry1Ac* expression cassette consists of the transcription terminator plus the 3' end of the *cry1Ac* gene.

The 5' and 3' regions that flank this insert were obtained by PCR and the sequences were compared to conventional cotton genomic DNA. The analysis demonstrated that the flanking sequences are of cotton origin and showed that 85 basepairs of cotton DNA were deleted during the insertion. 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.

Southern blot analyses identified an additional insert that was present in event MON531 and also in event MON15985. This insert segregates independent from the functional insert and is, according to the applicant, not present in the commercial cultivars that originate from this event.

The additional insert consists of 242 basepairs and contains a portion of the 7S 3' transcriptional termination sequence. The sequences of the 5' and 3' regions that flank the 242 bp insert were obtained by PCR, compared to conventional cotton genomic DNA and shown to be of cotton origin. 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.

Southern blot analysis of MON15985 and MON531 using restriction enzymes *Bam*HI and *Bam*HI+*Sph*I showed that several fragments hybridized with the enhanced CaMV 35S promoter. Five fragments were present in both MON15985 and MON531. The applicant states that some hybridization is expected because the enhanced CaMV 35S promoter is present in the *cry1Ac* expression cassette of MON531. However, the presence of this promoter in MON531 does not explain all fragments revealed in the Southern blot. The Southern blot analyses of MON531 presented in the dossier are of such poor quality that they do not provide any answers to the origin of the additional fragments.

In COGEM's opinion, in MON15985 and MON531 the presence of additional fragments that contain the enhanced CaMV 35S promoter cannot be excluded based on the data presented. If additional fragments are present, new open reading frames may be formed at the junction of the fragment with the cotton genome.

The chance that such an open reading frame generates a stable and functional protein is extremely low. However, theoretically, new open reading frames could give rise to proteins with potential adverse effects like toxicity or allergenicity. MON531 has been authorized in the United States of America since 1995. No adverse effects concerning handling and consuming of products and derivatives of this line have been reported. In addition, there are no indications that MON531 has different biological characteristics apart from the introduced traits. Consequently, this cotton line has a history of safe use. If additional fragments are present, these fragments do not have an adverse effect on the characteristics of MON15985 and MON531 cotton.

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 concluded that the studies provided in the application for the renewal of the authorization for use as food or feed in general did not raise any concerns over the safety of MON15985. A comment was made on the absence of an update for some of the bioinformatics analysis.

General surveillance plan

General surveillance has been 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 either not, or hardly predictable.

In the present application, a detailed general surveillance plan is provided to observe and register adverse effects of the import of MON15985 timely. 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 which include the authorization holder and operators involved in the handling and use of viable MON15985 cotton. Operators involved in the import, handling and processing of MON15985 cotton 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 (14).

Advice

The present application concerns 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 (15). 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 the spillage 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 characterization of MON531 which was used to produce MON15985 contains weaknesses. Consequently, the molecular characterization of MON15985 contains weaknesses as well. Cotton line MON531 has a history of safe use. 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 does not pose a significant risk to the environment in the Netherlands.

Additional remarks

Marker gene encoding β-D-glucuronidase

MON15985 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 (16, 17). 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 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 does not pose a risk to the environment.

Marker genes conferring antibiotic resistance

Cotton line MON15985 expresses the marker gene *nptII* (which confers resistance to kanamycin and neomycin) (18). In addition, the *aad* marker gene (conferring resistance to streptomycin and spectinomycin) is present (18). The *aad* gene is not expressed in MON15985 because it is under control of a prokaryotic promoter which is not functional in cotton. Analysis of protein levels in MON15985 confirmed that the AAD protein is not present.

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, that 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 (19).

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 (19).

COGEM points out that the chance of gene transfer from plant to bacterium is not likely to occur and has only been observed during specific laboratory situations and not in practice (18, 20). Furthermore, antibiotic resistance genes are already present in the environment (18).

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 approved the commercialisation of MON531 (used for the production of MON15985 cotton) and of the cotton hybrid MON88913xMON15985, which both contain the *nptII* and *aad* genes (8, 9). 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.

References

1. Agbios (2008). Agbios database product description. Internet: www.agbios.com. (d.d. September 5th 2008)
2. Crop Protection Compendium (2004). *Gossypium* (cotton). 2004 edition CD-ROM edition. ©Cab International 2004, Nosworthy way, Wallingford, UK

3. ISAAA report. Global review of commercialized transgenic crops: 2001, chapter 9: Bt-cotton
4. NationMaster (2007). Cotton production most recent by country. Internet: www.nationmaster.com (d.d 18 march 2008)
5. European commission. http://ec.europa.eu/agriculture/capreform/cotton/index_en.htm (d.d. September 15th 2008)
6. OECD Consensus Document on the Biology of cotton (Gossypium spp.) Twelfth meeting on the working group on the harmonization of regulatory oversight in biotechnology, June 2002
7. Office of the gene technology regulator (2002) report. The biology and ecology of cotton (*Gossypium hirsutum*) in Australia
8. COGEM (2008). Import and processing of cotton MON88913 x MON15985 (CGM/080328-01)
9. COGEM (1998). Advies C/ES/96/02 (CGM/981203-03)
10. Broderick NA, Raffa KF and Handelsman J (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proceedings of the National Academy of Science 103, 15196-15199
11. Azucena E and Mobashery S (2001). Aminoglycoside-modifying enzymes: mechanisms of catalytic processes and inhibition. Drug Resistance Updates 4, 106-117
12. Jana S and Deb JK (2006). Molecular understanding of aminoglycoside action and resistance. Applied Microbiology and Biotechnology 70, 140-150
13. Karcher SJ (2002). Blue plants: Transgenic plants with the GUS reporter gene. in: Tested studies for laboratory teaching Volume 23
14. COGEM (2005). Post market monitoring van genetisch gemodificeerde gewassen in Nederland (CGM/050414-03)
15. Wereld Klimaat Informatie (WKI). Koninklijk Nederlands Meteorologisch Instituut (KNMI). www.knmi.nl/klimatologie (d.d 25 october 2005)
16. Alwen A, Benito Moreno RM, Vicente O and Heberle-Bors E (1992). Plant endogenous β-glucuronidase activity: how to avoid interference with the use of the *E. coli* β-glucuronidase as a reporter gene in transgenic plants. Transgenic Research 1, 63-70
17. Gilissen LJW, Metz PLJ, Stiekema WJ and Nap JP (1998). Biosafety of *E. coli* β-glucuronidase (GUS) in plants. Transgenic Research 7, 157-163
18. COGEM (2007). Gebruik van antibioticumresistentiegenen in genetische gemodificeerde gewassen voor veldproeven (CGM/070703-01)
19. European Food Safety Authority (2004). Opinion of the scientific panel on genetically modified organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants (Question N˚ EFSA-Q-2003-109). The EFSA Journal 48: 1-18

20. Faber F and Van Elsas JD (2005). Transfer of DNA from genetically modified plants to bacteria. COGEM Research Report 2005-02