



VKM Report 2016:08

Final health and environmental risk assessment of genetically modified cotton 281-24-236 x 3006-210-23 (MXB-13)

Scientific opinion on insect-resistant and glufosinate-tolerant, genetically modified MXB-13 from Dow AgroSciences for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (Application EFSA/GMO/NL/2005/16)

Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2016:08
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Food and feed safety assessment of insect resistant and glufosinate tolerant genetically modified cotton MXB-13 (EFSA/GMO/NL/2005/16)

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Assessed and approved

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The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed the Panel on Genetically Modified Organisms (GMO) to answer the request from the Norwegian Food Safety Authority and the Norwegian Environment Agency. Project leaders from the VKM secretariat have been Arne Mikalsen, Ville Erling Sipinen and Anne Marie Bakke.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Abstract

Genetically modified cotton 281-24-236 x 3006-210-23 (MXB-13) from Dow AgroSciences was produced by conventional crossing of the single-event GM-cotton cultivars 281-24-236, which expresses the *cry1F* and *pat* genes, and 3006-210-23, which expresses the *cry1Ac* and *pat* genes. The resulting stacked event cotton MXB-13 expresses all three proteins: Cry1Ac, Cry1F and the phosphinothricin-acetyl-transferase (PAT) enzyme. The Cry-proteins confer resistance against specific lepidopteran pests and the PAT-enzyme renders cotton MXB-13 tolerant to application of the herbicide glufosinate-ammonium.

Updated bioinformatics analyses of the inserted DNA and flanking sequences in cotton MXB-13 have not indicated potential production of putative harmful toxins or allergens caused by the genetic modification. Genomic stability of the functional inserts and consistent expression of the *cry1Ac*, *cry1F* and *pat* genes have been shown over several generations of cotton MXB-13.

Data from several field trials performed in the USA indicate that with the exception of the introduced traits, cotton MXB-13 is compositionally, phenotypically and agronomically equivalent to its conventional counterparts and other cotton cultivars.

A 90-day sub-chronic oral toxicity study with rats and a 42-day nutritional assessment trial with broilers have not revealed adverse effects of cottonseed meal from cotton MXB-13 compared to meal from the conventional counterpart PSC355 and other cotton cultivars. Toxicity testing of the Cry1Ac, Cry1F and PAT proteins in repeated-dose dietary exposures with mice and rats did not indicate adverse effects. The Cry1Ac, Cry1F and PAT proteins produced in cotton MXB-13 do not show amino acid sequence resemblance to known toxins or IgE-dependent allergens, nor have they been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the Cry1Ac, Cry1F and PAT proteins will cause toxic or IgE-mediated allergic reactions to food or feed containing cotton MXB-13 compared to conventional cotton cultivars.

Cotton is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of cotton in Europe.

Based on current knowledge and with the exception of the introduced traits, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterparts and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

Summary

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Scientific Committee for Food Safety (VKM) has been requested by the Norwegian Environment Agency and the Norwegian Food Safety Authority (NFSA) to conduct final food, feed and environmental risk assessments of all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC. The request covers scope(s) relevant to the Gene Technology Act. The request does not cover GMOs that VKM already has conducted its final risk assessments on. However, the Agency and NFSA requests VKM to consider whether updates or other changes to earlier submitted assessments are necessary.

The glufosinate-tolerant and lepidopteran-resistant genetically modified cotton MXB-13 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5) from Dow AgroSciences is approved in EU under Regulation (EC) No 1829/2003 for food and feed uses, import and processing since 22nd of December 2011 (Application EFSA/GMO/NL/2005/16, Commission Implementing Decision 2011/891/EU).

Cotton MXB-13 has previously been assessed by the VKM GMO Panel commissioned by the NFSA related to the EFSA's public hearing of the application EFSA/GMO/NL/2005/16 in 2005 (VKM, 2005).

The current food, feed and environmental risk assessment of the cotton MXB-13 is based on information provided in the application EFSA/GMO/NL/2005/16, relevant peer-reviewed scientific literature including scientific opinions and comments from EFSA (EFSA, 2010a), VKM (VKM, 2005) and statements provided by other member states made available on the EFSA GMO Extranet. Except for a synopsis of more recent literature, this draft opinion is to a large extent a summary of the above-mentioned VKM and EFSA opinions, which are provided in Appendix I and II respectively, and readers are referred to these for details.

The VKM GMO Panel has evaluated cotton MXB-13 with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed. VKM also takes account of the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006; EFSA, 2011b), the environmental risk assessment of GM plants (EFSA, 2010c), selection of comparators for the risk assessment of GM plants (EFSA, 2011a) and for the post-market environmental monitoring of GM plants (EFSA, 2011c).

The scientific risk assessment of cotton MXB-13 includes molecular characterisation of the inserted DNA and expression of novel proteins, comparative assessment of agronomic and phenotypic characteristics, nutritional assessments, toxicity and allergenicity, unintended

effects on plant fitness, potential for gene transfer, interactions between the GM plant, target and non-target organisms, and effects on biogeochemical processes.

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility or ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms. Likewise, the VKM mandate does not include evaluations of herbicide residues in food and feed from genetically modified plants.

Initially, the two parent single events, cotton 281-24-236 and cotton 3006-210-23, were developed by *Agrobacterium tumefaciens*-mediated transformation to express the genes *cry1F* and *pat*, and *cry1Ac* and *pat*, respectively. MXB-13 was developed by conventional crossing of the two parent events and expresses all three genes. Expression of the *cry*-genes encoding the proteins Cry1Ac and Cry1F confer resistance to specific Lepidopteran insect pests, whereas the *pat* gene(s) derived from *Streptomyces viridochromogenes*, a common soil bacterium, encode the enzyme phosphinothricin-acetyl-transferase (PAT), which renders cotton MXB-13 tolerant to glufosinate ammonium-based herbicides.

Molecular characterisation

Analyses performed by the applicant show that the integrity of the transgenic inserts in the parent events 281-24-236 and 3006-210-23 are retained in the stacked event MXB-13. The expression levels of the Cry and PAT -proteins in the stacked event were comparable to the levels expressed in the single events. Novel open reading frames (ORFs) in MXB-13 created due to the transformation process in the single events were identified by the applicant and theoretically predicted translation products were further investigated. According to the applicant, no relevant homologies were found to known toxins or allergens. Bioinformatic comparisons of the amino acid sequences of the Cry1Ac, Cry1F and PAT proteins do not reveal similarities to known allergenic or toxic proteins. Southern hybridisation, ELISA and segregation analyses show that the introduced gene elements were stably inherited and expressed over multiple generations in parallel with the observed phenotypic characteristics of the stacked event.

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in cotton MXB-13 have been sufficiently characterised, and that no unintended changes have been identified that requires particular attention in the further assessment.

Comparative assessments

Field trials have been conducted to assess the composition of whole delinted cottonseeds, toasted cottonseed meal, refined oil and hulls of the GM cotton MXB-13 compared to conventional counterparts. In the first year (2001), the control was a null-segregant that was selected in the F1 generation after stacking and further bred by four rounds of self-pollination. In the second and third years (2003, 2007), the control was the conventional counterpart PSC355, which was used in the development of the single-event parent lines and therefore has a comparable genetic background to the test lines. Field trials in 2002 were performed for agronomic and GM phenotype assessments of cotton MXB-13 compared the conventional counterpart PSC355.

With the exception of the changes caused by the introduced transgenic traits, data provided by the applicant revealed no biologically relevant differences between cotton MXB-13 and its conventional counterparts. Most statistically significant differences observed were only present in material from some of the locations in some years and the values were within or close to the range of historical values observed in conventional cotton cultivars. The differences were therefore considered to reflect the natural variability of the analytes.

Based on current knowledge and excluding the new proteins Cry1Ac, Cry1F and PAT, the VKM GMO Panel concludes that cotton MXB-13 is compositionally, agronomically and phenotypically equivalent to its conventional counterparts and other cotton cultivars.

Food and feed risk assessment

A 90-day subchronic oral toxicity study with rats, as well as a 42-day nutritional assessment trial with broilers did not reveal adverse effects or differences in the performance of animals fed cottonseed meal from cotton MXB-13 compared to its conventional counterpart PSC355 or other cotton cultivars. Toxicity testing of the Cry1Ac, Cry1F and PAT proteins with mice and rats has not shown adverse effects. Bioinformatics analysis of the amino acid sequences of Cry1Ac, Cry1F and PAT proteins did not show sequence resemblance to known toxins or IgE-dependent allergens, nor have these proteins been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the Cry1Ac, Cry1F and PAT proteins will cause toxic or IgE-mediated allergic reactions to food or feed containing cotton MXB-13 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally equivalent to and as safe as its conventional counterpart PSC355 and other cotton cultivars.

Environmental assessment

Considering the intended uses of cotton MXB-13, which exclude cultivation, the environmental risk assessment is concerned with the accidental release into the environment of viable seeds during transport and/or processing, and with indirect exposure to microorganisms in the gastrointestinal tract and in soil or water, mainly via intestinal content and faeces from animals fed feeds containing cotton MXB-13.

With the exception of the introduced insecticidal properties and tolerance to the herbicide glufosinate-ammonium, cotton MXB-13 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton, and there are no indications of an increased likelihood of spread and establishment of feral cotton plants in the case of accidental release of seeds from cotton MXB-13 into the environment. Cotton is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of cotton in Europe. Plant to plant gene flow is therefore not considered to be an issue. There are no indications that transfer of recombinant genes from MXB-13 products to microorganisms in the gastrointestinal tract or in soil or water could occur at higher frequencies than from naturally occurring microbial sources.

Based on current knowledge and considering its intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

Overall conclusion

Based on current knowledge and with the exception of the introduced traits, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterparts and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, Norwegian Food Safety Authority/Norwegian Environment Agency, GMO, cotton (*Gossypium hirsutum* L.), EFSA/GMO/NL/2005/16, genetically modified cotton 281-24-236 x 3006-210-23 (MXB-13), unique identifier DAS-24236-5 x DAS-21Ø23-5, 281-24-236, 3006-210-23, insect-resistance Cry1Ac- and Cry1F-, glufosinate-tolerant PAT-protein, herbicide glufosinate-ammonium, food/feed risk assessment, environmental risk assessment, Regulation (EC) No 1829/2003, Directive 2001/18, food and feed safety, import and processing

Sammendrag

Som en del av forberedelsene til implementering av forordning 1829/2003 i norsk rett, er Vitenskapskomiteen for mattrygghet (VKM) bedt av Miljødirektoratet og Mattilsynet om å utarbeide endelige helse- og miljørisikovurderinger av alle genmodifiserte organismer (GMOer) og avledete produkter som inneholder eller består av GMOer som er godkjent under forordning 1829/2003 eller direktiv 2001/18, og som er godkjent for ett eller flere bruksområder som omfattes av genteknologiloven. Miljødirektoratet og Mattilsynet har bedt VKM om endelige risikovurderinger for de EU-godkjente søknader hvor VKM ikke har avgitt endelige risikovurderinger. I tillegg er VKM bedt om å vurdere hvorvidt det er nødvendig med oppdatering eller annen endring av de endelige helse- og miljørisikovurderingene som VKM tidligere har levert.

Den genmodifiserte, glufosinattolerante og insektresistente bomullen 281-24-236 x 3006-210-23 (MXB-13; unik kode DAS-24236-5 x DAS-21Ø23-5) fra Dow AgroSciences er fremkommet ved tradisjonell kryssing av de genmodifiserte bomullssortene 281-24-236 og 3006-210-23. Hensikten med bomull MXB-13 er resistens mot enkelte skadegjørere i sommerfuglordenen *Lepidoptera* og motstandsdyktighet mot ugressmidler som inneholder glufosinat-ammonium.

MXB-13 ble den 22. desember 2011 godkjent for import, videreforedling og til bruk som mat og fôr under forordning 1829/2003 (Kommisjonsbeslutning 2011/891/EU). Søknaden og godkjenningen omfatter ikke dyrking.

Bomullen MXB-13 ble første gang vurdert av VKMs faggruppe for GMO i 2005 (VKM, 2005). Helse- og miljørisikovurderingen ble utført på oppdrag av Mattilsynet i forbindelse med EFSAs offentlige høring av søknad EFSA/GMO/NL/2005/16 i 2005. EFSAs endelige vurdering ble publisert i 2010 (EFSA, 2010a).

Risikovurderingen av den genmodifiserte bomullssorten er basert på søkers dokumentasjon som er gjort tilgjengelig på EFSAs GMO Extranet, og uavhengige vitenskapelige publikasjoner, samt vitenskapelige vurderinger fra EFSA (EFSA, 2010a) og VKM (VKM, 2005). Bortsett fra gjennomgang av nylig offentliggjort publikasjoner er resten av teksten i denne vurderingen en oppsummering av de tidligere VKM (VKM, 2005) og EFSA (EFSA, 2010a) vurderingene, som er vedlagt i hhv. Appendix I og II. For utfyllende detaljer henvises leserne til disse.

Vurderingen er gjort i henhold til tiltenkt bruk i EU/EØS-området, og i overensstemmelse med matloven, miljøkravene i genteknologiloven med forskrifter, først og fremst, forskrift om konsekvensutredning etter genteknologiloven. Videre er kravene i forordning 1829/2003/EF, utsettingsdirektiv 2001/18/EF (vedlegg 2, 3 og 3B) og veiledende notat til Annex II (2002/623/EF), samt prinsippene i EFSAs retningslinjer for risikovurdering av genmodifiserte planter og avledete næringsmidler (EFSA, 2006; EFSA, 2010c; EFSA, 2011a; EFSA, 2011b; EFSA, 2011c) lagt til grunn for vurderingen.

Den vitenskapelige vurderingen omfatter transformeringsprosess og vektorkonstruksjon, karakterisering og nedarving av genkonstruksjonen, komparativ analyse av ernæringsmessig kvalitet, mineraler, kritiske toksiner, metabolitter, antinæringsstoffer, allergener og nye proteiner. Videre er agronomiske egenskaper, potensiale for utilsiktede effekter på fitness, genoverføring, og effekter på målorganismer, ikke-målorganismer og biogeokjemiske prosesser vurdert.

Det presiseres at VKMs mandat ikke omfatter vurderinger av etikk, bærekraft og samfunnsnytte, i henhold til kravene i den norske genteknologiloven og dens konsekvensutredningsforskrift. Disse aspektene blir derfor ikke vurdert av VKMs faggruppe for genmodifiserte organismer. Vurderinger av mulige plantevernmiddelrester i den genmodifiserte planten som følge av endret sprøytemiddelbruk faller per i dag utenfor VKMs ansvarsområde og er derfor heller ikke vurdert.

Bomullssorten MXB-13 er utviklet ved konvensjonell kryssing av de to genmodifiserte bomullssortene 281-24-236 og 3006-210-23. Sortene 281-24-236 og 3006-210-23 ble hver for seg utviklet ved hjelp av *Agrobacterium*-mediert transformasjon til å uttrykke henholdsvis genene *cry1F* og *pat*, og *cry1Ac* og *pat*. Uttrykk av *cry*-genene fra bakterien *Bacillus thuringiensis*, som koder for insektstoksinene Cry1Ac og Cry1F, gir plantene resistens mot enkelte skadegjørere i sommerfuglordenen *Lepidoptera*, mens uttrykk av *pat* fra bakterien *Streptomyces viridochromogenes*, som koder for enzymet PAT (phosphinothricin-acetyltransferase), gir økt toleranse overfor glufosinat-ammonium baserte ugressmidler.

Molekylær karakterisering

Analyser utført av søker viser at integriteten til de transgene innskuddene fra hver av de to enkeltstortene 281-24-236 og 3006-210-23 er bevart i den kryssede bomullssorten MXB-13. Uttrykk av de introduserte Cry og PAT-proteinene i MXB-13 samsvarer med nivåene funnet i de respektive enkeltstortene. Sekvensanalyser har identifisert nye potensielle åpne leserammer (ORFs) i MXB-13 som en følge av genmodifiseringene i enkeltstortene. Ved eventuelt uttrykk av disse antatte genproduktene viser databasesøk ingen relevante samsvar eller homologier til kjente toksiner eller allergener. Databasesøk med aminosyresekvensene til Cry1Ac, Cry1F og PAT -proteinene har ikke avdekket relevante homologier til kjente toksiner eller allergener. Southern analyser, ELISA, og nedarvingsmønstre over flere generasjoner bekrefter at de introduserte genetiske elementene er stabilt nedarvet og samsvarer med de observerte fenotypiske egenskapene til bomull MXB-13.

Ut i fra dagens kunnskap og informasjon fra søker, konkluderer VKMs faggruppe for GMO med at den molekylære karakteriseringen av de tilsiktede endringene i bomull MXB-13 er tilstrekkelig og at det ikke er identifisert utilsiktede endringer som krever spesifikk oppfølging i den videre vurderingen.

Komparative analyser

Søker har utført feltforsøk i USA i 2001, 2003 og 2007 med påfølgende analyse av næringsstoffer, antinæringsstoffer og andre relevante, biologisk aktive stoffer i bomullsfrø, bomullsfrømel, rensed bomullsfrøolje og bomullsfrøskall. Registrering av agronomiske og fenotypiske egenskaper ble utført under feltforsøk i 2002. For alle feltstudiene ble data fra MXB-13 sammenlignet med konvensjonell kontroll PSC355 eller annen relevant ikke-genmodifisert bomull.

Tilgjengelig data fra søker viser at med unntak av de ønskede endringene, var det ingen biologisk relevante forskjeller i enkeltparametere mellom den genmodifiserte bomullen MXB-13 og konvensjonelle kontroller. De registrerte statistisk signifikante forskjellene varierte mellom lokalitet og/eller år, og nivåene lå innenfor eller svært nær spredningen i verdier rapportert for andre bomullssorter. Forskjellene skyldes sannsynligvis den naturlige variasjonen for de enkelte parameterne.

Ut i fra dagens kunnskap, og med unntak av de nye proteinene Cry1Ac, Cry1F, og PAT, konkluderer VKMs faggruppe for GMO med at bomull MXB-13 er vesentlig lik konvensjonell kontroll og andre bomullssorter med hensyn til næringsstoffsammensetning og agronomiske og fenotypiske egenskaper.

Helserisiko

Et 90-dagers sub-kronisk toksisitetsstudie med rotter og et 42-dagers fôringsforsøk med broilere har blitt utført med bomull MXB-13. Disse studiene har ikke vist uønskede eller skadelige effekter, eller indikert andre relevante forskjeller hos dyr gitt fôr med frømel fra bomull MXB-13 sammenlignet med konvensjonell kontroll. Repetert dose studier med Cry1Ac, Cry1F og PAT proteinene har ikke vist negative helseeffekter av disse hos mus eller rotter. Databasesøk viser ingen relevante sekvenslikheter mellom Cry1Ac, Cry1F og PAT proteinene og kjente toksiner eller IgE-avhengige allergener, og ingen av proteinene er rapportert å ha forårsaket IgE-medierte allergiske reaksjoner. Det foreligger derfor ikke data som tilsier at Cry1Ac, Cry1F og PAT proteinene vil føre til toksiske eller IgE-medierte allergiske reaksjoner fra mat og fôr som inneholder bomull MXB-13 sammenlignet med konvensjonelle bomullssorter.

Ut i fra dagens kunnskap og tiltenkt bruk, konkluderer VKMs faggruppe for GMO med at bomull MXB-13 er ernæringsmessig lik og like trygg som konvensjonell kontroll PSC355 og andre bomullssorter.

Miljørisiko

Miljøriskovurderingen av bomull MXB-13 er avgrenset til mulige effekter av utilsiktet spredning av spiredyktige frø i forbindelse med transport og prosessering, samt indirekte eksponering gjennom gjødsel fra husdyr fôret med den genmodifisert bomullen. Faggruppen har ikke vurdert mulige miljøeffekter knyttet til dyrking av MXB-13 i Norge.

Genmodifiseringene i MXB-13 har ikke medført endringer i egenskaper knyttet til fitness, oppformering eller spredning sammenlignet med konvensjonell bomull, og det er ingen indikasjoner på økt sannsynlighet for spredning og etablering av viltvoksende bomullplanter fra utilsiktet frøspill av bomull MXB-13. Bomull dyrkes ikke i Norge, og det eksisterer ikke viltvoksende populasjoner av bomull i Europa. Det er derfor ikke risiko for utkryssing med dyrkede sorter eller ville planter i Norge. Det er ingen indikasjoner på at transgener fra bomull MXB-13 vil kunne overføres horisontalt til mikroorganismer i mage-tarm eller i jord eller vann ved høyere frekvenser enn fra de naturlig forekommende mikrobielle kildene til de innsatte genene.

Med bakgrunn i tiltenkt bruksområde, som ekskluderer dyrking, konkluderer VKMs faggruppe for GMO med at bomull MXB-13 ikke vil medføre miljørisiko i Norge.

Samlet vurdering

Ut i fra dagens kunnskap, og med unntak av de introduserte egenskapene, konkluderer VKMs faggruppe for GMO med at bomull MXB-13 har lik næringsstoffsammensetning, og er ernæringsmessig, fenotypisk og agronomisk lik og like trygg som konvensjonell kontroll og andre bomullssorter.

Med bakgrunn i tiltenkt bruksområde, som ekskluderer dyrking, konkluderer VKMs faggruppe for GMO med at bomull MXB-13 ikke vil medføre miljørisiko i Norge.

Abbreviations and/or glossary

4ocsΔMas2	'Mannopine synthase promoter from <i>Agrobacterium tumefaciens</i> plasmid <i>pTi15955</i>
Abiotic	Of or characterised by the absence of life or living organisms
Annuals	A plant that complete its life cycle within one year, then dies
ARMG	Antibiotic resistance marker gene
Bt	<i>Bacillus thuringiensis</i>
bw	Body weight
Crude fiber	Fibrous food residue that is left over after treatment with dilute acid and alkali
Cultivar	A race or variety of a plant that has been intentionally created or selected and maintained through cultivation
Delinted	Pertains to cottonseed from which any leftover lint (see below) has been removed
DNA	Deoxyribonucleic acid
Dw	Dry weight
Dwt	Dry weight tissue
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERA	Environmental risk assessment
EU	European Union
FAO	Food and Agriculture Organisation
Fitness	Describes an individual's ability to reproduce successfully relative to that of other members of its population
Glandless cotton	Genotypes of cotton that are devoid of the gossypol-containing glands distributed in various tissues of the cotton plant
GM	Genetically modified
GMO	Genetically modified organism
GMP	Genetically modified plant
Hemizygous	The transformation process produces hemizygous plants, i.e. the transgene is inserted without an allelic counterpart (i.e. Cry1A/-; CryF/-; PAT/-) that are inbred to generate selected homozygotes for the transgene in the final GMOs
IgE	Immunoglobulin E
ILSI	International Life Sciences Institute
<i>In planta</i>	Within the living plant
Lint	Leftover fibres attached to the cottonseed following deseeding of the cotton boll
Linted	Cottonseed with leftover fibres (lint) attached
mRNA	Messenger RNA
MT/NFSA	Norwegian Food Safety Authority (Mattilsynet)

NDF	Neutral detergent fibre, measure of fibre used for animal feed analysis. NDF measures most of the structural components in plant cells (i.e. lignin, hemicellulose and cellulose), but not pectin.
Northern blot	A technique used to study gene expression by detection of RNA or cDNA separated in a gel according to size.
Novel gene(s)	Newly introduced gene(s) as a result of genetic modification
NTO	Non-target organism
Null-segregant (-/-)	T-DNA sequences lost by self-pollination of hemizygous GM-plants, or crosses between hemizygous and non-GM plants (EFSA 2011)
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame; a molecular reading frame that can code for amino acids between two successive stop codons.
PAT	Phosphinothricin-acetyl-transferase
PCR	Polymerase chain reaction, a technique to amplify DNA by copying
Perennial	Plant that lives for more than two years
Selfing	Self-pollination. Pollen grains from the anther are transferred to the stigma of the same flower
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Technique to separate proteins according to their approximate size
Southern blot	Method used for transfer of electrophoresis-separated DNA fragments to a filter membrane and possible subsequent fragment detection by probe hybridisation
Transgene copy number	Defined as the number of exogenous DNA insert(s) in the genome. If the exogenous DNA fragment inserts only once at a single locus of the genome, it is a single copy transgenic event.
Western blot	Technique used to transfer proteins separated by gel electrophoresis by 3-D structure or denaturated proteins by the length of the polypeptide to a membrane, where they might be identified by antibody labelling.

Background

On 28 June 2005, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands an application (Reference EFSA/GMO/NL/2005/16) for authorisation of the genetically modified insect resistant and glufosinate tolerant cotton 281-24-236 x 3006-210-23 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5), submitted by Dow AgroSciences within the framework of Regulation (EC) No 1829/2003.

The scope of the application covers:

- Food
 - ✓ GM plants for food use
 - ✓ Food containing or consisting of GM plants
 - ✓ Food produced from GM plants or containing ingredients produced from GM
 - ✓ Plants
- Feed
 - ✓ GM plants for feed use
 - ✓ Feed containing or consisting of GM plants
 - ✓ Feed produced from GM plants
- GM plants for environmental release
 - ✓ Import and processing (Part C of Directive 2001/18/EC)

After receiving the application EFSA/GMO/NL/2005/16 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed the EU- and EFTA Member States (MS) and the European Commission and made the summary of the dossier publicly available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of regulation (EC) No 1829/2003. Following receipt of additional information from the applicant, EFSA declared on 3 August 2005 that the application was valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the EC and consulted nominated risk assessment bodies of the MS, including the Competent Authorities within the meaning of Directive 2001/18/EC (EC 2001), following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Within three

months following the date of validity, all MS could submit via the EFSA GMO Extranet to EFSA comments or questions on the valid application under assessment. The VKM GMO Panel assessed the application in connection with the EFSA official hearing, and submitted a preliminary opinion in November 2005 (VKM, 2005). EFSA published its scientific opinion 10 June 2010 (EFSA, 2010a), and cotton 281-24-236 x 3006-210-23 was approved for food and feed uses, import and processing 22 December 2011 (Commission Implementing Decision 2011/891/EU).

Cotton MXB-13, Trade name WideStrike™ Cotton, is authorised in several countries. Australia (Trade name: MBX-13, 2005, food, 2009, cultivation), Brazil (2009, food, feed, cultivation), Costa Rica (2009, cultivation), EU (2010, food, feed), Japan (2006, food, feed), Mexico (2004, food, feed, cultivation), New Zealand (2005, food), South Korea (2005, food, 2008, feed).

Terms of reference

The Norwegian Environment Agency has the overall responsibility for processing applications for the deliberate release of genetically modified organisms (GMOs). This entails inter alia coordinating the approval process, and to make a holistic assessment and recommendation to the Ministry of the Environment regarding the final authorisation process in Norway. The Agency is responsible for assessing environmental risks upon the deliberate release of GMOs, and to assess the product's impact on sustainability, benefit to society and ethics under the Gene Technology Act.

The Norwegian Food Safety Authority (NFSA) is responsible for assessing risks to human and animal health upon the deliberate release of GMOs pursuant to the Gene Technology Act and the Food Safety Act. In addition, NFSA administers the legislation for processed products derived from GMO and the impact assessment on Norwegian agriculture according to sector legislation.

The Norwegian Environment Agency

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency, by letter dated 13 June 2012 (ref. 2008/4367/ART-BI-BRH), requests VKM, to conduct final environmental risk assessments for all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC. The request covers scope(s) relevant to the Gene Technology Act.

The request does not cover GMOs that VKM already has conducted its final risk assessments on. However, the Norwegian Environment Agency requests VKM to consider whether updates or other changes to earlier submitted assessments are necessary.

The basis for evaluating the applicants' environmental risk assessments is embodied in the Act Relating to the Production and Use of Genetically Modified Organisms etc. (the Norwegian Gene Technology Act), Regulations relating to impact assessment pursuant to the Gene Technology Act, the Directive 2001/18/EC on the deliberate release of genetically modified organisms into the environment, Guidance note in Annex II of the Directive 2001/18 (2002/623/EC) and the Regulation 1829/2003/EC. In addition, the EFSA guidance documents on risk assessment of genetically modified plants and derived food and feed from the GM plants (EFSA, 2006; EFSA, 2010c; EFSA, 2011b; EFSA, 2011c), and OECD guidelines will be useful tools in the preparation of the Norwegian risk assessments.

The risk assessments' primary geographical focus should be Norway, and the risk assessments should include the potential environmental risks of the product(s) related to any changes in agricultural practices. The assignment covers assessment of direct environmental impact of the intended use of pesticides with the GMO under Norwegian conditions, as well as changes to agronomy and possible long-term changes in the use of pesticides.

The Norwegian Food Safety Authority

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency has requested NFSA to give final opinions on all GMOs and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC within the Authority's sectoral responsibility. The request covers scope(s) relevant to the Gene Technology Act.

NFSA has therefore, by letter dated 13 February 2013 (ref. 2012/150202), requested VKM to carry out final scientific risk assessments of 39 GMOs and products containing or consisting of GMOs that are authorised in the European Union.

The assignment from NFSA includes food and feed safety assessments of GMOs and their derivatives, including processed non-germinating products, intended for use as or in food or feed.

In the case of submissions regarding genetically modified plants (GMPs) that are relevant for cultivation in Norway, VKM is also requested to evaluate the potential risks of GMPs to the Norwegian agriculture and/or environment. Depending on the intended use of the GMP(s), the environmental risk assessment should be related to import, transport, refinement, processing and cultivation. If the submission seeks to approve the GMP(s) for cultivation, VKM is requested to evaluate the potential environmental risks of implementing the plant(s) in Norwegian agriculture compared to existing varieties (e.g. consequences of new genetic traits, altered use of pesticides and tillage). The assignment covers both direct and secondary effects of altered cultivating practices.

VKM is further requested to assess risks concerning coexistence of cultivars. The assessment should cover potential gene flow from the GMP(s) to conventional and organic crops as well as to compatible wild relatives in semi-natural or natural habitats. The potential for establishment of volunteer populations within the agricultural production systems should also be considered. VKM is also requested to evaluate relevant segregation measures to secure coexistence during agricultural operations up to harvesting. Post-harvest operations, transport and storage are not included in the assignment.

Evaluations of suggested measures for post-market environmental monitoring provided by the applicant, case-specific monitoring and general surveillance, are not covered by the assignment from NFSA. In addition, the changes related to herbicide residues of GMPs as a result of the application of plant-protection products fall outside the remit of the Norwegian VKM panels.

Assessment

1 Introduction

The food, feed and environmental risk assessment of the genetically modified, stacked event cotton 281-24-236 x 3006-210-23 (hereafter referred to as MXB-13) is assessed with reference to the intended use, which includes food, feed, import and processing, but excludes cultivation. The risk assessment is based on information provided by the applicant in the application EFSA/GMO/NL/2005/16, relevant peer-reviewed scientific literature, and scientific opinion and comments from VKM (VKM, 2005), EFSA (EFSA, 2010a) and other member states made available on the EFSA website GMO Extranet. Except for a synopsis of more recent literature, this draft opinion is to a large extent a summary of the above-mentioned VKM and EFSA reports, which are provided in Appendix I and II, respectively, and readers are referred to these for details.

MXB-13, (WideStrike™ Insect Protection)

MXB-13 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5) was produced by conventional crossing between lines of the single cotton events 281-24-236 (containing the genes *cry1F* and *pat*, and in addition one partial *pat* gene) and 3006-210-23 (containing the genes *cry1Ac* and *pat*). The commercial American cotton variety GC510 was used in the transformation of cotton 281-24-236 and cotton 3006-210-23. The *pat* gene was derived from *Streptomyces viridochromogenes*, a common soil bacteria (Lawrence, 2000), which can naturally develop the ability to detoxify glufosinate ammonium (Bartsch and Tebbe, 1989). MXB-13, 281-24-236 and 3006-210-23 contain the herbicide tolerance selectable marker gene, *pat*, that confers tolerance to the herbicide glufosinate ammonium (the active ingredient of Liberty® and Basta® herbicides).

The purposes of the modifications are to allow for effective weed and insect control during the cultivation of MXB-13.

The general mode of action of Cry proteins is to bind selectively to specific receptors on the epithelial surface of the midgut of susceptible lepidopteran species, leading to death of larvae through pore formation, cell burst and subsequent septicemia (OECD, 2007; Raymond et al., 2009). The expressed Cry1Ac and Cry1F proteins in MXB-13 therefore protect the plants from feeding damage caused by the lepidopteran insect species.

Glufosinate ammonium (also referred to as phosphinothricin; PPT) is a non-selective, contact herbicide that is phytotoxic to many broadleaf and grassy weeds. Glufosinate-ammonium inhibits glutamine synthetase, leading to glutamine deficiency, ammonia accumulation and eventually to plant death. The PAT protein in MXB-13 catalyses the conversion of glufosinate-ammonium to N-acetyl glufosinate. N-acetyl glufosinate is an inactive form that

does not bind to glutamine synthetase allowing plants to grow in the presence of glufosinate-ammonium.

The genetic modification in cotton MXB-13 is intended to improve agronomic performance only and is not intended to influence the nutritional properties, the processing characteristics or the overall use of cotton as a crop.

Cotton MXB-13 has been evaluated with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed.

VKM has also taken into account the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006; EFSA, 2011b), the environmental risk assessment of GM plants (EFSA, 2010c), the selection of comparators for the risk assessment of GM plants (EFSA, 2011a), and for the post-market environmental monitoring of GM plants (EFSA, 2011c).

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility or ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms.

2 Molecular characterisation

2.1 Previous molecular assessment

The stacked cotton event MXB-13 was developed by conventional crossing of the two single events 281-24-236 and 3006-210-23, to express the genes *cry1F* and *cry1Ac* encoding the proteins Cry1Ac and Cry1F that confer resistance to specific Lepidopteran insect pests. The *pat* gene encodes the enzyme phosphinothricin-acetyl-transferase (PAT) that acetylates L-glufosinate, which renders the plants tolerant to glufosinate ammonium based herbicides. The *pat* gene was used for selection of transformants during development of the single events.

The VKM and EFSA GMO Panels (VKM 2005, Appendix I; EFSA 2010a, Appendix II) have previously assessed the molecular characterisation of the stack with regards to the following:

1. The transformation system and vector constructs of the single events 281-24-236 and 3006-210-23
2. Characterisation of the transgene insertions and constructs of the single events 281-24-236 and 3006-210-23, and the constructs of the stacked cotton MXB-13
3. Information on the expression of the insert of the single events 281-24-236 and 3006-210-23, and the stacked cotton event MXB-13
4. Analyses of new open reading frames (ORFs) in the single events 281-24-236 and 3006-210-23
5. Inheritance and the stability of the inserted DNA in the single events 281-24-236 and 3006-210-23, and the stacked cotton event MXB-13

Both Panels concluded that the applicant had provided sufficient analyses for the molecular characterisation(s).

Initially, the Acala cotton line GC510 was transformed by *Agrobacterium tumefaciens* with the binary vectors pAGM281 and pMYC3006 to produce the cotton events 281-24-236 and 3006-210-23, respectively. Both single events were self-pollinated for one generation and then separately backcrossed three times to the commercial cotton line PSC355. These two backcrossed cotton lines were then crossed and self-pollinated five times to produce the stacked event MXB-13.

The breeding scheme for cotton MXB-13 is shown in Appendix III (Figure 1). Event 281-24-236 contains one functional copy of the T-DNA sequence from vector pAGM281, with a synthetically produced version of the *cry1F* gene from *Bacillus thuringiensis* (*Bt*) var. *aizawai*, a full-length *pat* gene sequence as well as a partial/incomplete *pat*-gene sequence from *Streptomyces viridochromogenes*. The synthetic *cry1F* gene was constructed by parts of the *cry1Fa*, *cry1Ca3* and *cry1A1* genes. Expression of the *cry1F* and *pat* genes were controlled by the synthetic promoter 4ocsΔMas2' and promoter UbiZm1 from maize, respectively.

Expression of the partial *pat* was at least 16 times lower than the full-length *pat* gene at the level of RNA, and was undetectable at the protein level. No vector backbone sequences were detected in event 281-24-236. The insertion is localized in the 3'untranslated region of a GA 20-oxidase gene. This gene belongs to a multigene family with several redundant genes. In addition cotton variety GC510 is tetraploid, i.e. containing four copies of the gene family. Therefore a putative changed expression of one GA 20-oxidase gene is not expected to have any functional impact. This is further supported by the compositional and agronomic analyses showing that event 281-24-236 is equivalent to its conventional counterpart.

Event 3006-210-23 contains one functional copy of the T-DNA sequence from vector pMYC3006, with a synthetically produced *cry1Ac* gene from *Bacillus thuringiensis* var. *kurstaki*. The synthetic *cry1Ac* is a chimeric combination of *cry1Ac*, *cry1Ca* and *cry1Ab* codon optimised for expression in plants. Expression of *cry1Ac* is driven by the ZmUbi1 promoter. Like event 281-24-236 the inserted sequence in event 3006-210-23 also contains the *pat* gene, identical to the one from vector pAGM281, it is however controlled by the 4OCSΔMas2' promoter instead of UbiZm1. Novel open reading frames (ORFs) created by the genetic modifications in the single events have been identified by the applicant and potential putative translation products further investigated. According to the applicant none of the induced ORFs indicated any relevant potential for the production of allergenic or toxic proteins. Bioinformatic comparisons of the amino acid sequences of the Cry1Ac-, Cry1F- and PAT proteins do not reveal similarities to known allergenic or toxic proteins.

Southern, 5' and 3' PCR of the flanking regions, and sequencing by the applicant show that the integrity of the transgenic inserts, including two full length copies and one partial *pat* gene, were retained in the stacked cotton event MXB-13. Levels of proteins in the stacked cotton measured by ELISA were also comparable to the levels in the single events.

Segregation analyses show that the introduced gene elements were stably inherited and expressed over multiple generations in parallel with the observed phenotypic characteristics in the stacked event.

2.2 Conclusions

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in cotton MXB-13 have been sufficiently characterised and that no unintended changes have been identified that requires particular attention in the further assessment.

3 Comparative assessments

Compositional and agronomic data provided by the applicant from various field trials with cotton MXB-13 has previously been assessed by the VKM GMO Panel (VKM, 2005), as commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency related to the EFSA's public hearing of the application EFSA/GMO/NL/2005/16 in 2005, and in EFSA's final opinion (EFSA, 2010a).

3.1 Production of material for comparative assessment

Field studies were conducted at six different sites located in major cotton producing states of the USA during the 2001, 2003 and 2007 growing seasons. In each year before the fields were sown, all field replicates underwent conventional maintenance and agrochemical application practices including herbicide and insecticide treatments, but glufosinate-containing herbicides were not used. Glufosinate was used only after sowing on MXB-13. Samples were collected from these studies to investigate the compositional equivalence and to characterise expression levels of Cry1F, Cry1Ac and PAT proteins.

For compositional studies, analysis was performed on delinted cottonseed, toasted meal, refined oil and hulls obtained from cotton MXB-13, and the single-event parental lines 281-24-236 and 3006-210-23 from which MXB-13 was produced. The MXB-13 and the single-event parent lines 281-24-236 and 3006-210-23 were compared to the null segregant (selected in the F1 generation after stacking and further bred by four rounds of self-pollination) during the growing season in 2001. In 2003 and 2007, MXB-13 and the single-event lines were compared to the conventional cotton cultivar PSC355, which is the recurrent parent in the breeding program to generate the two single-event GM cotton parental lines 281-24-236 and 3006-210-23 (see Figure 1 in Appendix III), and therefore considered to constitute a conventional counterpart. In addition, compositional analysis of hulls from all locations was performed in 2001.

The applicant also provided information on agronomic performance and phenotypic characteristics derived from several field trials in the US performed in 2002. The three GM cotton lines MXB-13, and the single-event parent lines 281-24-236 and 3006-210-23 were grown at 32 locations alongside the non-GM recurrent parental variety PSC355. Measurements of agronomic characteristics included field emergence, progeny seed germination, growth habit, vegetative vigor, flowering period, reproductive potential, and fiber quality.

3.2 Compositional analysis

The delinted cottonseeds were analysed for key nutrients, anti-nutrients, and toxicants as defined by the OECD consensus document for cotton (OECD, 2004). For cottonseeds, these are proximates (fat, ash, moisture, protein, fiber, carbohydrates and energy content), amino acids, fatty acids, micronutrients, such as vitamins and minerals, and anti-nutrients, such as gossypol, cyclopropenoid fatty acids and phytic acid. Hulls were analysed for proximates and minerals. Toasted meal was analysed for proximates, minerals, amino acids, and gossypol (both free and total gossypol in all three years). Analysis of refined oil included proximates (fat, moisture, and protein), fatty acids, antioxidants (tocopherols [alpha, beta, gamma, and delta in all three years; total tocopherols in 2003]), cyclopropenoid fatty acids, and gossypol (total gossypol in all three years, and free gossypol in 2001 and 2003).

Compared with the null segregant or its conventional counterpart PSC355, small but statistically significant differences in the composition of MXB-13 cottonseeds were observed. In 2001, MXB-13 cottonseeds contained statistically lower levels of crude fiber and the cyclopropenoid fatty acids sterculic acid and malvalic acid, but higher levels of stearic acid. Also in 2003, MXB-13 contained statistically significantly lower levels of sulfur, behenic acid, and total gossypol, but higher levels of alanine and tryptophan. And in 2007, statistically significant lower levels of calcium, manganese, phosphorus, linoleic acid, vitamin B1 (thiamin), free and total gossypol, but higher levels of stearic acid, oleic acid, arachidic acid, behenic acid, and dihydrosterculic acid.

Most of the above-mentioned differences, as well as those reported for toasted cottonseed meal and refined oil, were not observed at every site or in every year. The observed levels represented small differences and were generally within or very close to the range of natural variation reported in the literature for conventional cotton cultivars, and did not indicate an overall pattern of change.

3.3 Agronomic traits and GM phenotype

Since Norway does not cultivate cotton, only a short summary of previously reported assessments are provided. For further details the readers are referred to the previous VKM (VKM, 2005) and EFSA (EFSA, 2010a) assessments.

Whereas a number of statistically significant differences were observed between the stacked event MXB-13 and its single event parents on the one hand and their conventional counterpart on the other, these differences were considered by the applicant and EFSA (EFSA, 2010a) to be of minor magnitude and typical of variability among conventional cotton cultivars.

3.4 Conclusion

The VKM GMO Panel has considered the data supplied by the applicant on compositional, agronomic and phenotypic characteristics and confirms that with the exception of the introduced proteins, no biologically relevant differences were observed between cotton MXB-13, the null segregant and the conventional counterpart PSC355. The statistically significant differences observed were only present in material from some of the locations in some years and the values were within or close to the range of historical values observed in conventional cotton cultivars. The differences were therefore considered to reflect the natural variability of the analytes.

Based on current knowledge and excluding the new proteins Cry1Ac, Cry1F and PAT, the VKM GMO Panel concludes that cotton MXB-13 is compositionally, agronomically and phenotypically equivalent to its conventional counterparts and other cotton cultivars.

4 Food and feed safety assessment

Spain and Greece are the only two EU member states that grow cotton, and Greece is the largest cotton growing country in Europe. In Greece's marketing year 2013/2014 cotton production was 200,000 MT (Metric Tons) (Gain Report, 2014a), and in Spain's marketing year 2013/2014 cotton production was 145,000 MT (Gain Report, 2014b). No GM cotton is planted in these two countries.

Bulgaria produces cotton on less than 1 000 ha. Cotton production ceased in Italy in 1991 and in Portugal in 1996.

4.1 Previous evaluations by the VKM and EFSA GMO panels

Cotton MXB-13 was previously assessed for use as food and feed by the VKM GMO Panel commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency in connection with EFSA's public hearing of the application EFSA/GMO/ NL/ 2005/16 (VKM, 2005). EFSA has also published a final opinion on cotton MXB-13 (EFSA, 2010a). The VKM GMO Panel and EFSA concluded that MXB-13 was nutritionally equivalent to conventional cotton cultivars and it was unlikely that the inserted proteins would cause toxic or allergic reactions to food or feed containing cotton MXB-13 compared to conventional cotton.

4.2 Product description and intended uses

According to the applicant, the genetic modification in MXB-13 will not impact the existing post-harvest production processes used for cotton. Cotton is mainly grown for its commodity product the cotton boll. The fibres on the cotton boll are separated from the seeds by a cotton gin machine. The fibres, which consist mainly of cellulose, are primarily used for textiles, but also have some application for food or feed (see Figure 4.2-1). Especially the fibres that are too short to be spun into textiles can be used as food additives. Cellulose and methylcellulose can be used as thickeners, stabilisers, emulsifiers, or fillers. The protein- and oil-rich whole cottonseeds (WCS) are used for oil extraction and the oil is used in food and feed. Following oil extraction, the cottonseed can be processed into various other side-products, such as cottonseed meal, various protein preparations, and cottonseed milk, all used in food and feed. Protein-rich cottonseed meal is mostly used as an animal feed ingredient. Another major processed product derived from cottonseed is the fibre-rich hulls, which may also be used in animal feeds (Figure 4.2-1). For more information see Appendix IV.

Cottonseed and its derived products have a history of safe use in foods and feeds as long as dietary intake of the naturally occurring toxicants gossypol and cyclopropenoid fatty acids is restricted to acceptable levels. This is accomplished either by processing to reduce or

eliminate these toxicants or by limiting the inclusion level of cottonseed products in foods and feeds. Current EU regulations (Annex I of Council Directive 2002/32/EC; as assessed in EFSA, 2008) specifies maximum levels of free gossypol in various feed commodities and animal feeds. For more information see Appendix IV.

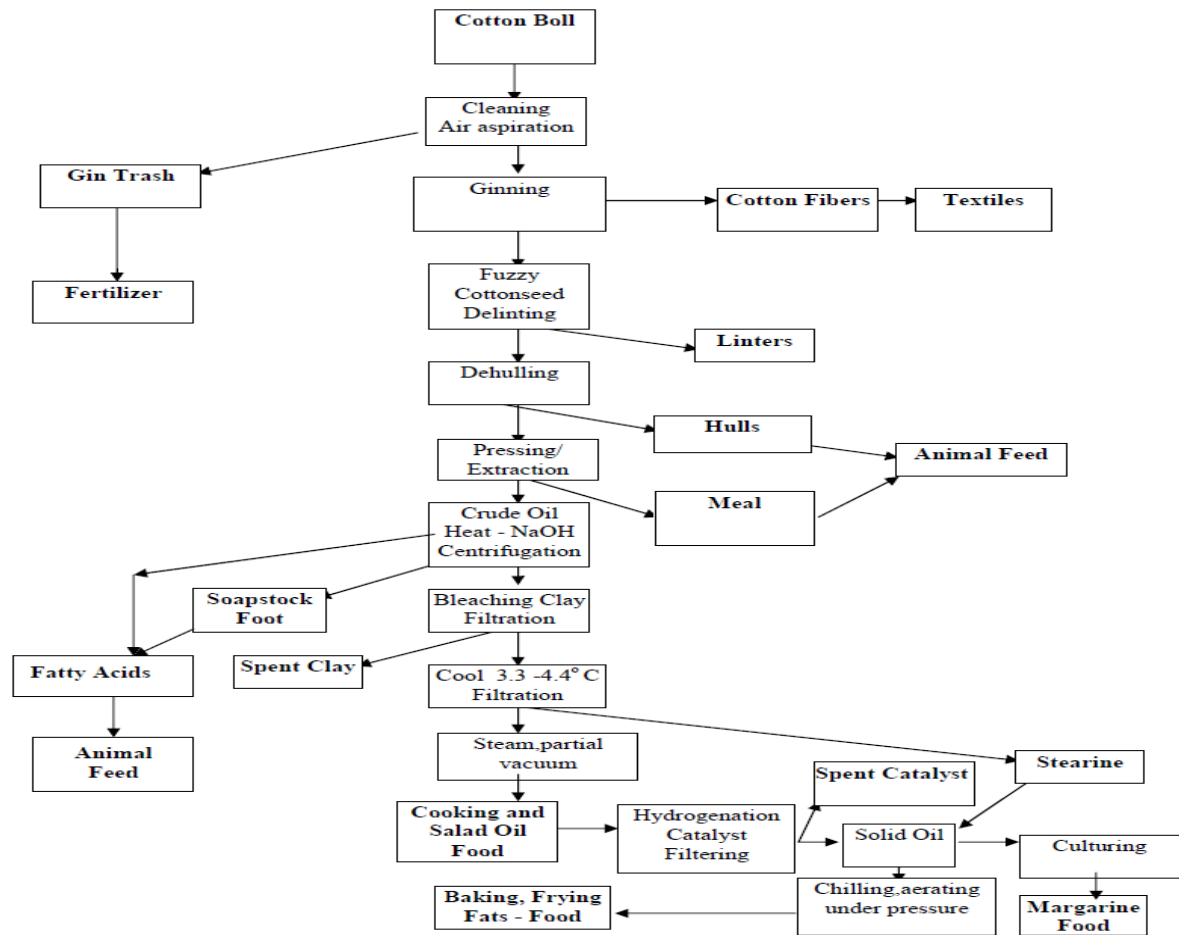


Figure 4.2-1 Processing of cotton boll, adapted from OECD (2004)

4.3 Effects of processing

According to the applicant, the commercial experiences have confirmed that the production and processing of MXB-13 cotton do not differ from the production and processing of the equivalent food and feed originating from conventional cotton cultivars.

4.3.1 Effects of processing on whole cotton products

The processing steps that are used to produce the various cotton products are shown in figure 4.2-1. The processing of whole cottonseed (WCS) may include delinting, dehulling, crushing, flaking, extruding, extracting, roasting, bleaching and deodorizing. WCS are first cracked and de-hulled, then heated to approximately 60°C, ground to flakes with rollers, and are then treated with solvent to remove the oil. The flakes are toasted, cooled and grounded. Roasting, extruding, and cracking whole cottonseed has improved digestibility in some trials but also has increased the availability of free gossypol in several circumstances. By-products of processing can be included in human diet, such as linters and oil, or in animal diet such as hulls and cottonseed meal. For more information see Appendix IV.

Cottonseed from cotton MXB-13 contains comparable levels of the naturally occurring toxicants gossypol and cyclopropanoid fatty acids relative to its conventional cotton counterpart and other cotton cultivars (see section 3.2). Therefore, processing to reduce or remove these toxicants, or practices used to limit their levels in foods and feeds are not expected to change.

4.3.2 Effect of processing on PAT, Cry1Ac and Cry1F proteins

The processing steps used to produce various cotton products are shown in Figure 4.2-1. According to information provided by the applicant, the processing conditions used for cottonseed and oil will reduce the PAT-, Cry1Ac- and Cry1F-protein to very low or non-detectable levels in hulls and cottonseed meal, and are not detectable in refined oil.

According to information provided by the applicant, Cry1F and Cry1Ac lost their insecticidal activity in a bioassay after being heated at 75 and 90°C at pH 7.5 for 30 minutes. On electrophoresis gels, protein bands were still observed in the heated solutions that corresponded to the intact forms of the Cry proteins. When lyophilized preparations of these Cry proteins were heated at 121°C for 30 minutes, bands corresponding to their higher molecular weight forms disappeared from the electrophoresis gels.

4.4 Toxicological assessment of cotton MXB-13

4.4.1 Toxicological assessment of the expressed novel proteins

The delta endotoxins Cry1Ac and Cry1F expressed in cotton MXB-13 are considered to be highly specific to certain Lepidopteran insect species. Humans and other mammals are considered unsusceptible due to the absence of receptors to these proteins in their intestines. These Cry proteins are expressed in numerous other genetically modified plants that have been assessed and considered safe by both VKM and EFSA. Their safety has also been reviewed by others (McClintock et al., 1995; Betz et al., 2000; Mendelsohn et al., 2003; US-EPA, 2005).

The PAT protein expressed in cotton MXB-13 is also expressed in numerous other genetically modified plants that have been assessed and considered safe by both VKM and EFSA, including maize T25 (VKM, 2014; EFSA, 2013) and soybean A5547-127 (VKM, 2015; EFSA, 2011d), and has also been reviewed by others (OECD, 1999; Herouet et al., 2005). The toxicological evaluation of PAT protein produced by *E. coli* was originally conducted by Pfister et al. (1996), which has since then formed the basis for the safety assessment of other transgenic crops expressing the *pat* gene (see below).

The applicant's Technical Dossier provides the following data regarding the toxicological assessment of the expressed novel proteins in cotton MXB-13:

- Acute toxicity testing of a mixture of Cry1Ac and Cry1F proteins with mice
- Acute toxicity testing of PAT protein with mice
- Degradation in simulated digestive fluids
- Thermolability (see section 4.3.2)
- Amino acid sequence comparisons with known toxins and allergens (see also sections 2.1 and 4.4.3; EFSA, 2010a)

Otherwise the applicant refers to previously generated data from repeated dose toxicity trials conducted by others (see below).

Due to the low levels of Cry1Ac, Cry1F and PAT in cotton and the difficult task of isolating a sufficient quantity of purified proteins from the cottons, the acute toxicity testing studies described and referred to in the Applicant Dossier were conducted with Cry1Ac and Cry1F proteins produced in *Pseudomonas fluorescens* and PAT protein produced in *Escherichia coli*. The applicant has performed analysis of structural similarity, physicochemical and functional equivalence of the microbially-produced Cry1Ac, Cry1F, and PAT proteins and the proteins produced by the cotton. These indicate that plant-produced and bacterially-produced Cry1Ac, Cry1F, and PAT proteins are biologically, biochemically, and immunologically equivalent.

According to the applicant, Cry1Ac and Cry1F produced by *P. fluorescens* were digested *in vitro* within one minute of exposure to a simulated gastric fluid containing pepsin at a

pepsin-to-protein ratio of 6.3 to 1 (w/w). Both stable and labile reference proteins, bovine serum albumin and beta-lactoglobulin, respectively, were included in the study. Integrity of the proteins were analysed with the help of SDS-PAGE (electrophoresis) and Western blot.

PAT protein has also been shown to be rapidly degraded in simulated gastric fluid (Mendelsohn et al., 2003).

4.4.1.1 Acute toxicity testing of novel proteins

Acute oral toxicity study of PAT protein with mice. The acute oral toxicity study is performed according to OECD Guideline no. 401 (OECD, 1987), EPA Guidelines OPPTS 870.1100 1998, JMAFF Acute Oral Tox. Study 2000, and EEC Methods Nr. B.1 Acute Oral 1992. Groups of 5 male and 5 female CD-mice were administered a single oral gavage dose of PAT protein at 5000 mg/kg body weight. Body weights of the test animals were determined prior to dosing (day 0) and on days 7 and 14 after dosing, and the animals were observed daily for any clinical abnormalities or mortality. No mortality occurred during the study. Following scheduled euthanasia of test animals on day 14, no gross internal pathologies were observed. Based on this test, the acute oral LD₅₀ was estimated to be greater than 5000 mg of PAT/kg body weight.

The data indicates that the PAT protein up to 5000 mg/kg body weight did not cause acute oral toxicity in mice.

Acute oral toxicity of mixed Cry1Ac and Cry1F proteins. An acute oral toxicity study was performed according to OECD Guideline no. 401 (OECD, 1987), EPA Guidelines (OPPTS 870.1100; 1998), JMAFF (Acute Oral Tox. Study; 2000), and EEC Methods (Nr. B.1 Acute Oral; 1992). Five male and five female CD-1 mice received a 5000 mg/kg body weight dose of microbially-produced protein containing a mixture of Cry1Ac and Cry1F proteins. The amounts of Cry1Ac and Cry1F in 5000 mg microbial protein were 350 mg and 375 mg, respectively. Parameters evaluated during the two-week observation period included body weights, detailed clinical observations, and gross pathological changes. All mice survived to the end of the two-week observation period. No adverse clinical signs or pathological lesions were observed on any of the test animals. All mice gained weight over the duration of the study.

According to the applicant the acute oral LD₅₀ was greater than 375 mg/kg for Cry1F and 350 mg/kg for Cry1Ac.

The VKM GMO panel agrees with EFSA's guideline (EFSA, 2011b) that acute toxicity testing of newly expressed proteins is discouraged since this is of little additional or applicable value to the risk assessment for human and animal consumption of food and feed derived from GM plants. The VKM GMO panel recognises that the applicant submitted the application prior to the last guidance document from EFSA.

4.4.1.2 Repeated dose toxicity testing

The applicant has not provided data from repeated dose toxicity trials with the novel proteins Cry1Ac, Cry1F and PAT expressed in cotton MXB-13. However, such trials have been conducted with these proteins and used in the assessment of numerous other transgenic crops with the same inserted *cry1ac*, *cry1f* and/or *pat* genes. These are summarised below.

Fourteen day repeated dose feeding study with the PAT protein. A repeated dose feeding study of reduced duration (14-day) relative to prevailing guidelines (OECD, 1995) was conducted in rats with the PAT protein encoded by the *pat*-gene generated in *E. coli* (Pfister et al., 1999). Groups of five male and female Wistar rats (HanIbm: WIST) received diets containing the PAT protein (lyophilized powder) at levels of 0, 5 and 50 g/kg diet. The high level corresponded to a dose of 7.6 and 7.9 g/kg bw/day for males and females, respectively. A reference group received standard rat diet. No remarkable findings were observed apart from statistically significant increases in blood cholesterol levels in males of groups fed the 5 and 50 g PAT-supplemented diets and blood phospholipid levels in females fed the 50 g and males fed the 5 and 50 g PAT-supplemented diets. The applicant concluded that since these effects were also observed in the control group (fed diets without PAT protein), they were not regarded as toxicologically relevant.

Comments to the 14-day PAT- feeding study: According to the OECD guideline 407, the duration of exposure should normally be 28 days. Although a 14-day study may be appropriate in certain circumstances, justification for use of a 14-day exposure period should be provided. No justification for using 14-days was found in the report.

Repeated dose toxicity studies on Cry performed by independent investigators. A two-year chronic rat feeding study was undertaken with microbial Cry1Ac products from *Bacillus thuringiensis kurstaki* at doses of up to 8400 mg/kg bw/day. A decrease in weight gain was observed in female rats at the highest dose but, in the absence of any other adverse findings (e.g. survival, clinical observations or pathology), this was not considered to indicate Cry protein toxicity (reviewed by McClintock et al., 1995).

Two separate studies with *Bacillus thuringiensis kurstaki* spores orally administered to humans did not reveal any observable health effect at a dose of 1000 mg of microbial spores per day for 3 or 5 days (reviewed by McClintock et al., 1995; Betz et al., 2000).

4.4.2 Toxicological assessment of the whole GM food/feed

4.4.2.1 90-day subchronic toxicity study

A 90-day subchronic toxicity study with cottonseed meal was performed with Crl:CD(SD) rats (Dryzga et al., 2007).

This study was conducted according to the O.E.C.D. guideline, E.E.C. Directive 2001/59/EC, Method B.26 (August, 2001), U.S. E.P.A. OPPTS Series 870, Health Effects, Testing Guidelines, N°870.3100 (August, 1998) and M.A.F.F. in Japan notification 12 Nousan N°8147 guideline (November, 2000). This study was also conducted in compliance with the following Good Laboratory Practice Standards (GLP) of the European Parliament and Council Directive 2004/10/EC, The Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) – Good Laboratory Practice Standards, 11 NohSan, Notification No. 6283 - 1 October 1999 revised by 12 NohSan, Notification No. 8628 - 6 December, 2000, US Environmental Protection Agency - FIFRA GLPs Title 40 CFR, Part 160 – Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Good Laboratory Practice Standards, Final Rule, Organisation for Economic Co-Operation and Development (OECD) -- OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM(98)17.

Before preparation of the cottonseeds for inclusion in the experimental diets, molecular characterisation (PCR) and transgene expression (ELISA) analysis were conducted in MXB-13, conventional counterpart PSC355 and the three commercial non-transgenic cotton varieties PHY72, PHY78 and 98M-2983. The amounts of transgene protein expressed were measured by ELISA antibody assays and were only detected in MXB-13 cottonseeds. The reported levels in MXB-13 were 2.80 µg Cry1F/g, 0.96 µg Cry1Ac/g and 0.46 µg PAT/g.

These proteins were not detected in any of the cottonseed meal samples following processing, therefore no ELISA tests were performed on any of the prepared feed samples.

The cottonseed meals (isoline control, 3 commercial controls and MXB-13) were also analysed for nutrients, anti-nutrients, gossypol, mycotoxins and pesticide residues. All meals contained comparable levels of protein, fat, ash, carbohydrates, calories, dry matter, crude fiber, detergent fiber, amino acids, minerals, vitamins, fatty acids and heavy metals. Pesticides and mycotoxins were not detected in any cottonseed meal samples. Information regarding herbicide application was not provided by the applicant. Levels of the anti-nutrient gossypol in the cottonseed and the test diets are reported in Appendix IV.

Groups of 12 male and 12 female Sprague-Dawley rats per group were given diets containing 10% toasted cottonseed meal from either MXB-13, the conventional counterpart cotton (PSC355) or three commercial non-transgenic line controls (PHY72, PHY78 and 98M-2983) continuously for at least 90 days (Dryzga et al., 2007). The diets were provided ad libitum.

Animals were observed for clinical signs daily. Body weight was measured weekly and feed consumption was measured twice a week for the first 6 weeks and once a week thereafter. A detailed physical examination was performed on all animals once during the acclimatization phase and weekly throughout the study. All animals were subjected to a neurotoxicity assessment during week 12 of the study. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all animals of the control, high dose group and reference group during week 13. Urine samples were collected overnight during the week before necropsy from all animals. Before scheduled necropsy, a blood sample was collected from the retro-orbital venous plexus of each surviving animal for haematology and clinical chemistry determinations. All animals were necropsied, selected organs weighed and a range of tissues were sampled, fixed and examined microscopically.

There were no treatment-related effects on clinical signs, ophthalmological or neurotoxicological observations, body weights, feed consumption, haematology, prothrombin time, clinical chemistry, or urinalysis parameters. There were also no treatment related effects on organ weight, gross or histological observations.

According to the applicant the No-Observed-Adverse-effect level (NOAEL) in this study was 7.2 mg/kg bw/day for males and 7.9 mg/kg bw/day for females, for the Cry1F/Cry1Ac proteins.

4.4.3 Allergenicity

The strategies used when assessing the potential allergenic risk focus on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation or to elicit IgE-dependent allergic reactions in already sensitised persons and whether the transformation may have altered the allergenic properties of the modified food. A weight-of-evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (Codex Alimentarius, 2003; EFSA, 2006; EFSA, 2010b).

4.4.3.1 Assessment of allergenicity of the newly expressed proteins

In order to assess the potential for introduced IgE-dependent allergens in MXB-13, sequence evaluation scheme was used to assess the similarity of the Cry1Ac, Cry1F and PAT proteins to known protein allergen sequences contained in several widely accepted databases. An immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids. In studies conducted on the Cry1Ac, Cry1F and PAT proteins, no immunologically significant sequence identity was detected, indicating that no homology to known IgE-dependent allergens, based on amino acid sequences in Cry1Ac, Cry1F or PAT. *In vitro* simulated gastric fluid (SGF) digestibility studies were also conducted on the proteins. Within one minute of exposure to SGF Cry1Ac, Cry1F and PAT were rapidly digested and no longer detectable by SDS-PAGE or western blot analysis.

Thermolability results for these proteins also indicated that these proteins were not biologically active following exposure to elevated temperature (>75°C). These proteins are also rapidly degraded upon exposure to mixture of digestive tract enzymes, and they are not glycosylated.

The results of these studies indicate that the Cry1Ac, Cry1F and PAT proteins do not exhibit characteristics commonly attributed to an IgE-dependent allergenic protein.

In addition, the donor organism for the *pat* gene, *Streptomyces viridochromogenes*, is a common soil bacterium and does not have a history of causing allergy.

4.4.3.2 Assessment of allergenicity of the whole GM plant

Allergenicity of the whole crop could be increased as an unintended effect of the random insertion of the newly introduced genes in the genome of the recipient, for example through qualitative or quantitative modifications of the pattern of expression of endogenous proteins.

This issue does not appear relevant since cotton is not considered to be a common allergenic food, and only rare cases of occupational allergy have been reported.

4.4.3.3 Assessment of allergenicity of proteins derived from the GM plant

Food products from cottonseed are limited to highly processed products due to the presence of the natural toxicants gossypol and cyclopropenoid fatty acids in the seed. These substances are removed or reduced by processing (OECD, 2004).

The main cottonseed product in human food, cottonseed oil, is highly purified. Edible oils that are refined, bleached and deodorised do not appear to pose a risk to allergic individuals, as they contain virtually no proteins. Linters are also highly processed (alkaline pH, high temperature) to remove non-cellulose components. Linters are composed of greater than 99% cellulose, and are a major source of cellulose for chemical and food use.

Exposure to proteins through consumption of oil and linters derived from MXB-13 would be very low to negligible.

4.4.4 Assessment of adjuvanticity

According to the EFSA Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed from GM plants (EFSA, 2010b) and the VKM risk assessment of the adjuvant properties of Cry-protein (VKM, 2012), adjuvants are substances that, when co-administered with an antigen increases the immune response to that antigen and therefore might increase the allergic response. Adjuvanticity has not been routinely considered in the assessment of allergenicity of GMOs.

MXB-13 contains Cry1Ac and Cry1F proteins. Cry1Ac-protein has been administered intraperitoneally or intragastrically to mice at relatively high doses (Vazquez et al., 1999; Vazquez-Padron et al., 2000). In these studies IgG, IgM and mucosal IgA response were induced, but no IgE-mediated response was observed. This demonstrates that Cry1Ac has no or low allergenic potential. Moreover these Cry1Ac and Cry1F proteins are not glycosylated in cotton MXB-13.

Another Cry protein, Cry1Ab has been shown to act as an adjuvant, e.g. it enhances the mucosal and/or the systemic antibody response to a protein that is co-administered with the Cry protein (Vazquez-Padron et al., 1999; Moreno-Fierros et al., 2003).

A former VKM GMO Panel (VKM, 2012) was of the opinion that the adjuvant effect of Cry proteins, observed following intragastric or intranasal administration of higher doses than those present in Cry-containing GM plants, did not raise concerns regarding allergenicity. In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the possible role of these proteins as adjuvants should be considered. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.

Cotton MXB-13 also contains the PAT protein. Interaction between the newly expressed PAT protein impacting on allergenicity and/or adjuvanticity is not expected given the lack of indications of allergenicity and adjuvanticity of the protein. Also, there is no information available on the structure or function of the newly expressed PAT protein that would suggest an adjuvant effect resulting in or increasing an eventual IgE response to a bystander protein.

"Bystander sensitisation" can occur when an adjuvant in food, or an immune response against a food antigen, results in an increased permeability of the intestinal epithelium for other components in food. Previously it was assumed that the epithelial cells of the intestine were permanently "glued together" by the so-called "tight junctions". More recent knowledge shows that these complex protein structures are dynamic and can be opened up by different stimuli.

Both in vitro and in vivo experiments have demonstrated that when an IgG response that can result in a complement activation (among other) is not balanced by an IgA response, the epithelial barrier can become leaky and unwanted proteins are able to enter the body (bystander-penetration) and lead to allergic sensitisation (Brandtzaeg and Tolo, 1977; Lim and Rowley, 1982).

4.5 Nutritional assessment of GM food and feed

Cottonseed oil and processed cotton linters are the primary cotton products used for human food. Both products undergo extensive processing procedures before use for human consumption. The processed linter pulp product is composed of almost pure cellulose, and is used in food mainly in the production of casings for bologna, sausages, and frankfurters. The total amount of linters used is very small. Cotton fibre is used in ice cream and salad dressings to increase viscosity (OECD, 2004).

Cottonseed meal is an important ingredient in animal feed. Depending on the oil extraction process, cottonseed meal finds uses in feed for cattle, monogastrics, and laying hens. Cottonseed meal is not used for human consumption in the EU, however it has been approved for use in human food in the USA and other countries, when derived from gossypol-free varieties of cotton or after processing to remove the gossypol. Human consumption of cottonseed meal is reported mainly in Central American countries and India where it is used as a low cost, high quality protein ingredient.

Fat in cottonseed is mostly in the form of oil, and unsaturated fatty acids are the predominant fatty acids. The polyunsaturated fatty acid linoleic acid is the main fatty acid in cottonseed oil, and it represents up to 50% of the total fat. Smaller quantities of oleic and palmitic acids are found in cottonseed oil.

The oil of conventional cottonseeds, particularly those of *Gossypium hirsutum*, generally contain about 0.5-1% of cyclopropenoid fatty acids such as malvalic, sterculic and dihydrosterculic acids. These fatty acids have been found to have deleterious effects on animal performance and various harmful effects on health (reproductive disorders, growth retardation and altered fat metabolism) in rainbow trout, rodents and poultry (OGTR, 2008). Rainbow trout fed glandless cottonseeds showed reduced weight gain and an increased prevalence of liver carcinomas (Hendricks et al., 1980). Glandless cottonseeds do not contain gossypol so the resulting effects have been attributed to cyclopropenoid fatty acids (OGTR, 2008).

Analysis of cotton products derived from MXB-13 confirmed that there is no detectable level of protein in either cottonseed oil or processed cotton linters.

4.5.1 Intake information/exposure assessment

According to FAO statistics (www.faostat3.fao.org), the total human consumption of cottonseed oil in the European Union was 17 500 metric tonnes in 2011. Consumption data of cottonseed products are not available for Norway. In the last five years, no registered import of food or feed-grade cottonseed products into Norway was found in Statistics Norway's External Trade in Goods database (www.ssb.no). Thus, the intake of cottonseed products by humans and animals in Norway is considered to be negligible.

4.5.2 Nutritional assessment of feed derived from the GM-plant

The nutritional assessment study summarised below was not conducted according to the latest EFSA guidelines (EFSA, 2011b), but the VKM GMO panel recognises that the applicant submitted the application prior to the latest guidance document.

Data from a 42-day broiler nutritional assessment study with cotton MXB-13 was submitted by the applicant (McNaughton 2003). A total of 480 CobbxCobb chickens were distributed in 48 pens with 10 birds/pen, 5 males and 5 females per pen, 12 replicate pens per diet were given the same feed supplemented with cottonseed meal from four different cotton plant sources: conventional cotton counterpart (PSC355), two conventional controls (Pima and Acala), and the transgenic cotton MXB-13. These cottons were grown in six field sites in representative regions in USA. Appropriate insect, weed and disease control practices were applied to produce an agronomically acceptable crop.

The broiler starter- and grower feeds contained 10% cottonseed meals. The study was performed in compliance with US EPA Good laboratory practice regulations (21 CFR, Part 58). Routine water analyses for pesticides, PCBs and toxic metals were conducted using standard U.S. EPA procedures. These analyses were not conducted in accordance with GLP procedures (i.e. no distinct protocol, Study Director). The concentrations of these substances were analysed in the cottonseed meals. All mycotoxins analysed in cottonseed meals were below detection limits. Levels of the anti-nutrient gossypol in the cottonseed and the test diets are reported in Appendix IV.

Analysis of Variance (ANOVA) was used to compare parameter means from all test groups. Means were further separated using Least Significant Difference. Significant differences found at the $p < 0.05$ level were reported.

Collective data from day 0-42 indicated that feed was most efficiently converted to body weight gain in the broilers fed the diet containing cotton MXB-13, with statistically significant differences observed between the broilers fed the diets containing the conventional counterpart PSC355 and MXB-13. Otherwise, no statistically significant differences were observed in mortality, body weight gain or other performance parameters between broilers fed a diet containing cottonseed meal from MXB-13 or cottonseed meal from the PSC355 or the other non-transgenic cotton cultivars.

Feeding studies by independent investigators were not found by search in available databases.

4.6 Conclusion

A 90-day subchronic oral toxicity study with rats, as well as a 42-day nutritional assessment trial with broilers did not reveal adverse effects or differences in the performance of animals fed cottonseed meal from cotton MXB-13 compared to its conventional counterpart PSC355 or other cotton cultivars. Toxicity testing of the Cry1Ac, Cry1F and PAT proteins with mice and rats has not shown adverse effects. Bioinformatics analysis of the amino acid sequences of Cry1Ac, Cry1F and PAT proteins did not show sequence resemblance to known toxins or IgE-dependent allergens, nor have these proteins been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the Cry1Ac, Cry1F and PAT proteins will cause toxic or IgE-mediated allergic reactions to food or feed containing cotton MXB-13 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally equivalent to and as safe as its conventional counterpart PSC355 and other cotton cultivars.

5 Environmental risk assessment

5.1 Introduction

Considering the scope of the application for the cotton lines MXB-13, which excludes cultivation, the environmental risk assessment is concerned with the accidental release into the environment of viable cotton seeds during transport and/or processing, and with indirect exposure to microorganisms in the gastrointestinal tract and in soil or water. The line MXB-13 has resistance to certain lepidopteran pests and glufosinate tolerance. Use of glufosinate-ammonium is forbidden in Norway.

Genus *Gossypium* (Malvaceae) contains about 50 diploid or allotetraploid species, four of these (*G. arboretum*, *G. barbadense*, *G. herbaceum* and *G. hirsutum*) are domesticated and cultivated (Brubaker et al., 1999). *G. herbaceum* and *G. hirsutum* have been cultivated in Southern Europe since the 19th century (Davis, 1967). Globally *G. hirsutum* is the most cultivated species today, and China, India, USA and Pakistan are the main producers of cotton (FAOSTAT, 2015). In Europe cotton is mainly grown in Greece and Spain, but five other countries have minor production (FAOSTAT, 2015).

G. hirsutum is originally a perennial plant, but the cultivars used today are grown as annuals. Cotton is adapted to tropical and subtropical conditions. *G. hirsutum* is tetraploid and mainly self-pollinated. Pollen grains are heavy and sticky, but pollen can be carried by bumble bees and bees. The degree of out-crossing varies between the cultivars, but generally it is very low (0-25%) (Xanthopoulos and Kechagia, 2000; Turley and Kloth, 2002). There are no native plant species in Europe which could hybridize with *G. hirsutum*. However, single plants of *G. herbaceum* and *G. hirsutum* have been found outside cultivated areas (Davis, 1967).

Being a tropical-subtropical plant, cotton is sensitive to low temperature. The optimum temperature for seed germination is 25-30°C and germination is inhibited at temperatures below 12-18°C, root growth is strongly reduced at temperatures below 20°C. Temperatures below 18°C result in chilling injuries (Stewart et al., 2010). Most of the commercial cultivars of cotton do not have any seed dormancy. For production of ripe seed, cotton needs a growth period of 120-200 days.

According to the national statistics, no food or feed grade cottonseed products have been imported to Norway in 2011-2015 (www.ssb.no/statistikkbanken).

5.2 Unintended effects on plant fitness due to the genetic modifications

Cotton is not a weed in Europe. Generally in Europe, spreading of cotton outside the cultivated areas is limited by the lack of seed dormancy and lack of tolerance to low temperatures. The genetic modifications of the lines in this assessment do not have any effects on seed dormancy or on temperature requirement for germination and growth. The fitness properties of the transgenic line MXB-13 is similar to those of conventional, non-transformed cotton. Thus, under Norwegian conditions, it is highly unlikely that the seeds of the GM lines of cotton will germinate, the growing season is too cold and short for production of ripe seed, and the plants or seeds cannot survive the winter. Further, feral populations of the modified cotton line will have selective advantages only if exposed to specific herbicide glufosinate-ammonium, or if attacked by certain insect herbivores, which are not found in Norway. Consequently, the establishment of feral populations of these cotton lines in Norway is highly unlikely.

5.3 Potential for gene transfer

A prerequisite for gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA, or vertical gene flow via pollen or seed dispersal. Concerning the transgenic lines of cotton, gene transfer to microorganisms could take place in the digestive tract in humans and animals when cottonseed is used as food or feed, or in soil from faeces from animals fed with cottonseed. Under the Norwegian climatic conditions, gene flow via pollen or seed dispersal is not an issue. Use of extracted cottonseed oil as food or feed does not cause environmental concerns in Norway.

5.3.1 Plant to micro-organisms gene transfer

Experimental studies have shown that gene transfer from transgenic plants to bacteria rarely occurs under natural conditions and that such transfer depends on the presence of DNA sequence similarity between the DNA of the transgenic plant and the DNA of the bacterial recipient (Nielsen et al., 2000; De Vries and Wackernagel, 2002, reviewed in EFSA, 2004 and 2009; Bensasson et al., 2004; VKM, 2005).

DNA is effectively degraded during digestion. The stability and uptake of DNA from the intestinal tract has been studied in mice after M13 DNA was administered orally. The DNA introduced was detected in stool samples up to seven hours after feeding. Small amounts (<0.1%) could be traced in the blood vessels for a period of maximum 24 hours, and M13 DNA was found in the liver and spleen for up to 24 hours (Schubbert et al., 1994). Following oral intake, it has been shown that DNA from GM soybean is more stable in the intestine of persons with colostomy compared to a control group (Netherwood et al., 2004). No GM DNA was detected in the faeces from the control group. Rizzi et al. (2012) provides an extensive review of the fate of feed-derived DNA in the gastrointestinal system of mammals. Nordgård

et al. (2012) concluded that, even after extensive ingestion of DNA, natural transformation of microorganisms in the gastrointestinal tract of rats was not detectable.

Considering the low level of exposure to recombinant DNA in connection with feeding cottonseed meal, horizontal gene transfer in the gastrointestinal system is highly unlikely.

5.3.2 Plant to plant gene flow

Cotton is not grown in Norway, establishment of feral populations from spilled seeds is highly unlikely, and there are no close relatives of cotton in the flora of Norway. Thus, gene flow from plant-to-plant is not an issue in Norway.

5.4 Interaction between the GM plant and target organisms

Interaction between the transgenic lines of cotton and any target organisms is not an issue in Norway.

5.5 Interaction between the GM plant and non-target organisms

Interaction between the transgenic lines of cotton and any non-target organisms is not an issue in Norway.

5.6 Potential interactions with the abiotic environment and biogeochemical cycles

Considering the intended uses of the cotton line MXB-13, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with the abiotic environment and biogeochemical cycles are not considered an issue by the VKM GMO Panel.

5.7 Conclusion

Considering the intended uses of cotton MXB-13, which exclude cultivation, the environmental risk assessment is concerned with the accidental release into the environment of viable seeds during transport and/or processing, and with indirect exposure to microorganisms in the gastrointestinal tract and in soil or water, mainly via intestinal content and faeces from animals fed feeds containing cotton MXB-13.

With the exception of the introduced insecticidal properties and tolerance to the herbicide glufosinate-ammonium, cotton MXB-13 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton, and there are no indications of an increased likelihood of spread and establishment of feral cotton plants in the case of accidental release of seeds from cotton MXB-13 into the environment. Cotton is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of cotton in Europe. Plant to plant gene flow is therefore not considered to be an issue. There are no indications that transfer of recombinant genes from MXB-13 products to microorganisms in the gastrointestinal tract or in soil or water could occur at higher frequencies than from naturally occurring microbial sources.

Based on current knowledge and considering its intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

6 Post-market environmental monitoring

The objectives of a monitoring plan according to Annex VII of Directive 2001/18/EC are to confirm that any assumptions regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment are correct and to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment which were not anticipated in the environmental risk assessment.

The environmental risk assessment did not identify any potential adverse environmental effects of the transgene lines of cotton. Thus, the general surveillance plan is sufficient and there is no need for a specific surveillance plan.

7 Conclusions

Molecular characterisation

Analyses performed by the applicant show that the integrity of the transgenic inserts in the parent events 281-24-236 and 3006-210-23 are retained in the stacked event MXB-13. The expression levels of the introduced Cry and PAT -proteins in the stacked event were comparable to the levels expressed in the single events. Novel open reading frames (ORFs) in MXB-13 created due to the transformation process in the single events were identified by the applicant and theoretically predicted translation products were further investigated. According to the applicant, no relevant homologies were found to known toxins or allergens. Bioinformatic comparisons of the amino acid sequences of the Cry1Ac, Cry1F and PAT proteins do not reveal similarities to known allergenic or toxic proteins. Southern hybridisation, ELISA and segregation analyses show that the introduced gene elements were stably inherited and expressed over multiple generations in parallel with the observed phenotypic characteristics of the stacked event.

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in cotton MXB-13 have been sufficiently characterised, and that no unintended changes have been identified that requires particular attention in the further assessment.

Comparative assessments

Field trials have been conducted to assess the composition of whole delinted cottonseeds, toasted cottonseed meal, refined oil and hulls of the GM cotton MXB-13 compared to conventional counterparts. In the first year (2001), the control was a null-segregant that was selected in the F1 generation after stacking and further bred by four rounds of self-pollination. In the second and third years (2003, 2007), the control was the conventional counterpart PSC355, which was used in the development of the single-event parent lines and therefore has a comparable genetic background to the test lines. Field trials in 2002 were performed for agronomic and GM phenotype assessments of cotton MXB-13 compared to the conventional counterpart PSC355.

With the exception of the changes caused by the introduced transgenic traits, data provided by the applicant revealed no biologically relevant differences between cotton MXB-13 and its conventional counterparts. Most statistically significant differences observed were only present in material from some of the locations in some years and the values were within or close to the range of historical values observed in conventional cotton cultivars. The differences were therefore considered to reflect the natural variability of the analytes.

Based on current knowledge and excluding the new proteins Cry1Ac, Cry1F and PAT, the VKM GMO Panel concludes that cotton MXB-13 is compositionally, agronomically and phenotypically equivalent to its conventional counterparts and other cotton cultivars.

Food and feed risk assessment

A 90-day subchronic oral toxicity study with rats, as well as a 42-day nutritional assessment trial with broilers did not reveal adverse effects or differences in the performance of animals fed cottonseed meal from cotton MXB-13 compared to its conventional counterpart PSC355 or other cotton cultivars. Toxicity testing of the Cry1Ac, Cry1F and PAT proteins with mice and rats has not shown adverse effects. Bioinformatics analysis of the amino acid sequences of Cry1Ac, Cry1F and PAT proteins did not show sequence resemblance to known toxins or IgE-dependent allergens, nor have these proteins been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the Cry1Ac, Cry1F and PAT proteins will cause toxic or IgE-mediated allergic reactions to food or feed containing cotton MXB-13 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally equivalent to and as safe as its conventional counterpart PSC355 and other cotton cultivars.

Environmental assessment

Considering the intended uses of cotton MXB-13, which exclude cultivation, the environmental risk assessment is concerned with the accidental release into the environment of viable seeds during transport and/or processing, and with indirect exposure to microorganisms in the gastrointestinal tract and in soil or water, mainly via intestinal content and faeces from animals fed feeds containing cotton MXB-13.

With the exception of the introduced insecticidal properties and tolerance to the herbicide glufosinate-ammonium, cotton MXB-13 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton, and there are no indications of an increased likelihood of spread and establishment of feral cotton plants in the case of accidental release of seeds from cotton MXB-13 into the environment. Cotton is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of cotton in Europe. Plant to plant gene flow is therefore not considered to be an issue. There are no indications that transfer of recombinant genes from MXB-13 products to microorganisms in the gastrointestinal tract or in soil or water could occur at higher frequencies than from naturally occurring microbial sources.

Based on current knowledge and considering its intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

Overall conclusion

Based on current knowledge and with the exception of the introduced traits, the VKM GMO Panel concludes that cotton MXB-13 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterparts and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that cotton MXB-13 does not represent an environmental risk in Norway.

8 Data gaps

Filling data gaps would confirm and strengthen the conclusions drawn based on current knowledge. With added knowledge, VKM and its commissioning agencies could thereby provide greater certainty when communicating conclusions regarding the safety of the GM products.

Herbicide tolerant (HT) crops permit the use of broad-spectrum herbicides such as glufosinate-ammonium as an in-crop selective herbicide to control a wide range of broadleaf and grass weeds without sustaining crop injury. This weed management strategy enables post-emergence spraying of established weeds and gives growers more flexibility to choose spraying times in comparison with the pre-emergence treatments of conventional crops. As the broad-spectrum herbicides are sprayed on the plant canopy and spraying often takes place later in the growing season than is the case with selective herbicides associated with conventional crops, the residue and metabolite levels of herbicides in plants with tolerance to glufosinate-ammonium could be higher compared to plants produced by conventional farming practices. Limited data is available on pesticide residues in HT crops.

More research is also needed to elucidate whether the genetic modifications used to make a plant tolerant against certain herbicide(s) may influence the metabolism of this or other plant protection products, and whether possible changes in the spectrum of metabolites may result in altered toxicological properties.

At present, the potential changes related to herbicide residues in genetically modified plants as a result of the application of plant protection products fall outside the remit of the VKM GMO Panel.

Development of food allergies and intolerances involves the interplay of various factors such as genetic predisposition, the composition of the mucosa as well as infection status of the gastrointestinal tract, age, and the nutritional state of an individual (van Wijk and Knippels, 2007).

Although there is limited knowledge with regards to Cry proteins as immune-modulating substances, a study with mice revealed that *Bacillus thuringiensis* Cry1Ac protoxin activated macrophages by up-regulation of cell surface molecules and induction of proinflammatory cytokines (Moreno-Fierros et al., 2013). Previous studies have shown that Cry1Ac protein has immunogenic potential to elicit strong IgG-response (Vazquez et al., 1999) and the induction of IgG antibodies to food antigen and even cross-priming against a bystander antigen may be of biological significance (Brandtzæg, 2010). Experimental studies both *in vitro* and *in vivo* have demonstrated that IgG antibodies that are not balanced by a mucosal IgA response can enhance the epithelial penetration of bystander proteins (Brandtzæg, 2010).

Most of the immunologic adjuvant experiments have been performed using Cry1Ac. Whether the other Cry proteins have similar adjuvant properties is unknown. The quantities of Cry proteins in genetically modified maize and soya are marginal compared with the amounts of other adjuvants that are natural components of food. However, the extent to which these naturally occurring adjuvants and Cry proteins contribute to the development of allergies is largely unknown. Determination of their importance is hampered by the lack of validated methods for measuring adjuvant effects. The possibility that Cry proteins might increase the permeability of the intestinal epithelium and thereby lead to "bystander" sensitization to strong allergens in the diet of genetically susceptible individuals cannot be completely excluded. This possibility could be explored in a relevant animal model.

One element of uncertainty in exposure assessment is the lack of knowledge concerning exposure via the respiratory tract and the skin, and also the lack of quantitative understanding of the relationship between the extent of exposure to an adjuvant and its effects in terms of development of allergies.

VKM GMO Panel has assessed adjuvant properties of Cry proteins (VKM, 2012) but still perceives the need for further clarification on the possible role of Cry proteins as immune-modulating substances and their role in contributing to the development of food allergies and intolerances. This has practical implications since these proteins are expected to be present at higher concentrations in certain processed food and feed ingredients, especially in protein concentrates and isolates, from Cry-containing crops. The levels of Cry proteins in these ingredients have not been reported, but they are extensively used in feeds for monogastric animals, including farmed fish. A need to develop sensitive methods and models for investigating immune-modulating effects, and the health implications these may have, has been identified and requires basic research efforts.

These studies will provide a better basis for risk assessment of food and feed products containing Cry proteins, and they will provide greater certainty when communicating conclusions regarding the safety of the GM products.

9 References

- Arieli A (1998) Whole cottonseed in dairy cattle feeding: a review. *Anim Feed Sci Technol* 72:97-110.
- Bartsh K, Tebbe CC (1989) Initial steps in the degradation of phosphotricin (glufosinate) by soil bacteria. *Appl. Environm. Microbiol.* 55: 711-716.
- Bensasson D, Boore JL, Nielsen KM (2004) Genes without frontiers. *Heredity* 92:483-489.
- Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *Regul Toxicol Pharmacol* 32:156–173.
- Brandtzaeg P (2010) Food allergy: separating the science from the mythology. *Nat Rev Gastroenterol Hepatol* 7:380-400.
- Brandtzaeg P, Tolo K (1977) Mucosal penetrability enhanced by serum-derived antibodies. *Nature* 266: 262-263.
- Brubaker CL, Bourland FM, Wendel JE (1999) The origin and domestication of cotton. In: *Cotton: Origin, History, Technology and Production*. Ed. by CW Smith, JT Cothren, John Wiley and Sons, Inc., New York, pp. 3-31.
- Calhoun MC, Kuhlmann SW, Baldwin BC Jr. (1995) Assessing the gossypol status of cattle fed cotton feed products. *Proceedings Pacific Northwest Nutrition Conference*. October 11 1995.
- Codex Alimentarius (2003) *Codex principles and guidelines on foods derived from biotechnology*. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organisation: Rome.
- Davis PH (1967) *Flora of Turkey and East Aegaen Islands*. Vol 2, Edinburgh University Press, 581 pp.
- De Vries J, Wackernagel W (2002) Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proc Natl Acad Sci USA* 99:2094-2099
- Dryzga M.D., Yano B.L., Andrus A.K., Mattsson J.L. (2007) Evaluation of the safety and nutritional equivalence of a genetically modified cottonseed meal in a 90-day dietary toxicity study in rats. *Food Chem Tox* 45:1994-2004.
- EFSA (2004) Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *EFSA Journal*, 48, 1-18.
http://www.efsa.europa.eu/en/science/gmo/gmo_opinions/384.html
- EFSA (2006) Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. *EFSA Journal* 99:1-100.
http://www.efsa.europa.eu/en/science/gmo/gmo_guidance/660.html.

- EFSA (2008) Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on gossypol as undesirable substance in animal feed. EFSA Journal 908:1-56.
- EFSA (2009) Scientific Opinion of the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ). Use of antibiotic resistance genes as marker genes in genetically modified plants. EFSA Journal 1034: 1-82
- EFSA (2010a) EFSA Panel on Genetically Modified Organisms (GMO); Scientific Opinion on application (EFSA-GMO-NL-2005-16) for the placing on the market of insect resistant genetically modified cotton (*Gossypium hirsutum* L.) 281-24-236/3006-210-23 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Dow AgroSciences. EFSA Journal 8(6):1644.
- EFSA (2010b) EFSA Panel on Genetically Modified Organisms (GMO); Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 8(7):1700.
- EFSA. (2010c) Scientific Opinion of the EFSA Panel on Genetically Modified Organisms (GMO); Guidance on the environmental risk assessment of genetically modified plants. EFSA Journal 8(11):1879.
- EFSA (2011a) Scientific Opinion of the EFSA Panel on Genetically Modified Organisms (GMO); Guidance on selection of comparators for the risk assessment of genetically modified plants and derived food and feed. EFSA Journal 9(5):2149.
- EFSA (2011b) Scientific Opinion of the EFSA Panel on Genetically Modified Organisms (GMO); Guidance for risk assessment of food and feed from genetically modified plants. EFSA Journal 9(5):2150.
- EFSA (2011c) Scientific Opinion of the EFSA Panel on Genetically Modified Organisms (GMO); Guidance on the post-market environmental monitoring (PMEM) of genetically modified plants. EFSA Journal 9(8):2316.
- EFSA (2011d) EFSA Panel on Genetically Modified Organisms (GMO); Scientific Opinion on application (EFSA-GMO-NL-2008-52) for the placing on the market of herbicide tolerant genetically modified soybean A5547-127 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience. EFSA Journal 9(5):2147.
- EFSA (2013) EFSA Panel on Genetically Modified Organisms (GMO); Scientific opinion on applications EFSA-GMO-RX-T25 and EFSA-GMO-NL-2007-46 for the renewal of authorisation of maize T25, and for the placing on the market of herbicide-tolerant genetically modified maize T25, both for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience AG. EFSA Journal 11(10):3356.
- FAOSTAT (2015) <http://faostat3.fao.org/browse/Q/QC/E>

- Forster LA Jr., Calhoun MC Jr. (1995) Nutrient values for cottonseed products deserve new look. *Feedstuffs* 67:1-5.
- Gamboa DA, Calhoun MC, Kuhlmann SW, Haq AU, Bailey CA (2001) Tissue distribution of gossypol enantiomers in broilers fed various cottonseed meals. *Poult Sci* 80:920-925.
- Gain report (2014a) Greece. Cotton and products update 2014.
<http://www.thefarmsite.com/reports/contents/GreeceCotton&Products22Oct2013.pdf>
- Gain report (2014b) Spain. Cotton and products update 2014.
<http://www.thefarmsite.com/reports/contents/SpainCotton3April2014.pdf>
- Hendricks JD, Sinnhuber RO, Loveland PM, Pawlowski NE, Nixon JE (1980) Hepatocarcinogenicity of glandless cottonseeds and cottonseed oil to rainbow trout (*Salmo gairdneri*). *Science* 208:309-311.
- Herouet C, Esdaile DJ, Mallyon BA, Debruyne E, Schulz A, Currier T, Hendrickx D, van der Klis RJ, Rouan D (2005) Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. *Reg Toxicol Pharmacol* 41:134-149.
- Heuzé V, Tran G (2015) Cotton (general). Feedipedia.org. A programme by INRA, CIRAD, AFZ and FAO. <http://www.feedipedia.org/node/15960>. Last updated on January 27, 2015.
- Heywood R (1988) The toxicology of gossypol acetic acid and (-)-gossypol. *Contraception* 37:185-190.
- Lawrence E (2000) Henderson's Dictionary of Biological terms. Lawrence E. (eds). Pearson Education Limited Essex, England, 719 pp.
- Lim PL, Rowley D (1982) The effect of antibody on the intestinal absorption of macromolecules and on intestinal permeability in adult mice. *Int Arch Allergy Appl Immunol.* 68:41-46.
- McClintock JT, Schaffer CR, Sjoblad RD (1995) A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. *Pestic Sci* 45:95-105.
- McNaughton JL (2003) Nutritional equivalency study of Cry1F/Cry1Ac cottonseed meal: poultry feeding study. Confidential applicant report 021309, 2003-Dow-01-B (Annex III).
- Mendelsohn M, Kough J, Vaituzis Z, Matthews K (2003) Are Bt crops safe? *Nature Biotech* 21:1003-1009.
- Moreno-Fierros L, Ruiz-Medina EJ, Esquivel RL, Lopez-Revilla R, Pina-Cruz S (2003) Intranasal Cry1Ac protoxin is an effective mucosal and systemic carrier and adjuvant of *Streptococcus pneumoniae* polysaccharides in mice. *Scand J Immunol* 57:45-55.
- Moreno-Fierros L, Garcia-Hernandez AL, Ilhuicatz-Alvaradoa D, Rivera-Santiago L, Torres-Martínez M, Rubio-Infantea N, Legorreta-Herrera M (2013) Cry1Ac protoxin from *Bacillus thuringiensis* promotes macrophage activation by upregulating CD80

- and CD86 and by inducing IL-6, MCP-1 and TNF- α cytokines. *Internat Immunopharmacol* 17:1051-1066.
- Netherwood T, Martin-Orue SM, O'Donnell AG, Gockling S, Graham J, Mathers JC, Gilbert HJ (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nat Biotechnol* 22:204-209.
- Nielsen KM, van Elsas JD, Smalla K (2000) Transformation of *Acinetobacter* sp. 13 (pFG4deltanptII) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. *Appl Env Microbiol* 66:1237-1242
- Nordgård L, Bruseth L, Raddaki N, Traavik T, Avershoff B, Nilsen KM (2012) An investigation of horizontal transfer of feed introduced DNA to the aerobic microbota of the gastrointestinal tracts of rats. *BMC Res Notes* 5:170.
- OECD (1987) Test No. 401: Acute oral toxicity. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, Subsection 401, February 24, 1987; <http://www.oecd.org/env/ehs/testing/45122762.pdf>
Note: Following the OECD Council decision, the test 401 'Acute Oral Toxicity' was deleted on 17th December 2002.
- OECD (1995) Test No. 407: Repeated dose 28-day oral toxicity in rodent. OECD Guideline for the Testing of Chemicals, Section 4: Health Effects, Subsection 407, July 27, 1995; <http://www.oecd.org/chemicalsafety/testing/37477972.pdf>
- OECD (1999) Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. Paris Environment Directorate, Organisation for Economic Co-operation and Development.
- OECD (2004) Consensus Document on Compositional Considerations for New Varieties of Cotton (*Gossypium hirsutum* and *Gossypium barbadense*): key food and feed nutrients and anti-nutrients. Series on the Safety of Novel Foods and Feeds, No. 11. Paris Environment Directorate, Organisation for Economic Co-operation and Development.
- OECD (2007) Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis* - derived insect control proteins. OECD Environment, Health and Safety Publications Series on Harmonization of Regulatory Oversight in Biotechnology, 2007 No. 42. Paris Environment Directorate, Organisation for Economic Co-operation and Development.
- OGTR (2008) Office of the Gene Technology Regulator, Department of Health, Australian Government. The Biology and Ecology of Cotton (*Gossypium hirsutum*) in Australia. 30 pp.
- Pfister T, Schmid H, Luetkemeier H, Biedermann K, Weber K (1999) PAT-protein – Repeated dose oral toxicity (14-day feeding) study in rats. Internal report. 267 pp, M-140484-07-1.

- Raymond B, Johnston PR, Wright DJ, Ellis RJ, Crickmore N, Bonsall MB (2009) A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae. *Env Microbiol* 11:2556-2563.
- Rizzi A, Raddadi N, Sorlini C, Nordgård L, Nielsen KM, Daffonchio D. (2012) The stability and degradation of dietary DNA in the gastrointestinal tract of mammals: implications for horizontal gene transfer and the biosafety of GMOs. *Crit Rev Food Sci Nutr* 52:142-161.
- Saxena DK, Sharma SK, Sambhi SS (2011) Kinetics and thermodynamics of cottonseed oil extraction. *Grasas Y Aceites* 62(2):198-205.
- Schubbert R, Lettmann C, Doerfler W (1994) Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Mol Gen Genet* 242:495-504.
- Stewart JM, Oosterhuis DM, Heitholt JJ, Mauney J (2010) *Physiology of Cotton*. 573 pp. Springer Verlag, Dordrecht. E-ISBN-978-90-481-3195-2.
- Turley RB, Kloth RH (2002) Identification of a Third Fuzzless Seed Locus in Upland Cotton (*Gossypium hirsutum* L.). *J Heredity* 93:359-364.
- US-EPA (2005) Biopesticide registration action document *Bacillus thuringiensis* Cry1F and Cry1Ac WideStrike Cotton Registration Action Document.
http://www.epa.gov/opppbpd1/biopesticides/ingredients_keep/tech_docs/brad_0065_12.pdf
- van Wijk F, Knippels L (2007) Initiating mechanisms of food allergy: Oral tolerance versus allergic sensitization. *Biomed Pharmacother* 61:8-20.
- Vazquez RI, Moreno-Fierros L, Neri-Bazan L, de la Riva GA, Lopez-Revilla R (1999) *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. *Scand J Immunol* 49:578-584.
- Vazquez-Padron RI, Moreno-Fierros L, Neri-Bazan L, de la Riva GA, Lopez-Revilla R (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induce systemic and mucosal immune response in mice. *Life Sciences* 64:1897-1912.
- Vazquez-Padron RI, Moreno-Fierros L, Neri-Bazan L, Martinez Gil AF, de la Riva GA, Lopez-Revilla R (2000) Characterization of the mucosal and systemic immune response induced by Cry1Ac protein from *Bacillus thuringiensis* HD 73 in mice. *Brazil J Med Biol Res* 33:147-155.
- VKM (2005) Risikovurdering av genmodifisert bomull 281-24-236/3006-210-23 (EFSA/GMO/NL/2005/16) fra Dow AgroScience. Uttalelse fra Faggruppe for genmodifiserte organismer i Vitenskapskomiteen for mattrygghet,
http://vkm.no/eway/default.aspx?pid=277&trg=Content_6504&Main_6177=6504:0:31,2365&Content_6504=6187:2032224::0:6271:10:::0:0

- VKM (2012) Helserisikovurdering av Cry-proteiners adjuvanseffekter. Uttalelse fra Faggruppe 3 i Vitenskapskomiteen for mattrygghet, ISBN: 978-82-8259-055-6, Oslo, Norway. <http://www.vkm.no/dav/d6991c1718.pdf>
- VKM (2014) Helse- og miljørisikovurdering av genmodifisert maislinje T25 fra Bayer CropScience (C/F/95/12/07). Uttalelse fra Faggruppe for genmodifisert organismer i Vitenskapskomiteen for mattrygghet, ISBN: 978-82-8259-146-1, Oslo, Norway. <http://vkm.no/dav/369107174d.pdf>
- VKM (2015) Food/feed and environmental risk assessment of herbicide tolerant genetically modified soybean A5547-127 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (EFSA/GMO/NL/2008/52). Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety, ISBN: 978-82-8259-163-8, Oslo, Norway. <http://vkm.no/dav/85209a3c09.pdf>
- Xanthopoulos FP, Kechagia UE (2000) Natural crossing in cotton (*Gossypium hirsutum* L.). Austral J Agricult Res 51:970-983.
- Yu F, McNabb WC, Barry TN, Waghorn GC (1995) Effect of condensed tannin in cottonseed hulls upon the in vitro degradation of cottonseed kernel proteins by rumen micro-organisms. J Sci Food Agricult 69:223-234.
- Yu F, Moughan PJ, Barry TN (1996) The effect of cottonseed condensed tannins on the ileal digestibility of amino acids in casein and cottonseed kernel. Brit J Nutr 75:683-698.

Appendix I

UTTALELSE OM DOW AGROSCIENCES GENMODIFISERTE BOMULL 281-24-236/3006-210-23 (EFSA/GMO/NL/2005/16)

Vurdert og godkjent av Faggruppe for genmodifiserte organismer

Dato: 17.11.05

Sammendrag

Vurderingen av den genmodifiserte herbicidresistente og insektstolerante bomullslinjen 281-24-236/3006-210-23 fra Dow AgroSciences er utført av Faggruppe for genmodifiserte organismer under Vitenskapskomiteen for mattrygghet. Mattilsynet ber Vitenskapskomiteen for mattrygghet om å vurdere den genmodifiserte bomullslinjen 281-24-236/3006-210-23 til bruk i næringsmidler og fôrvarer.

Hybriden 281-24-236/3006-210-23 er fremkommet ved krysning mellom 281-24-236 og 3006-210-23. Hensikten med 281-24-236/3006-210-23 er motstandsdyktighet mot enkelte insektsarter og sprøytemiddelet Roundup.

Vurdering av den genmodifiserte bomullen er basert på den dokumentasjonen som er gjort tilgjengelig på EFSA's nettside GMO EFSA-net. 281-24-236/3006-210-23 er vurdert i henhold til tiltenkt bruk og de prinsipper som er lagt til grunn i EFSA's retningslinjer for risikovurdering av genmodifiserte planter (EFSA 99, 2004) og Organisation for Economic Co-operation and Development (OECD) konsensusdokument for bomull (OECD 2004). Den vitenskapelige vurderingen omfatter transformeringsprosessen, bruk av vektor og det transgene konstruktet, komparativ analyse av ernæringsmessig kvalitet, mineraler, kritiske toksiner, antinæringsstoffer, allergener og nye proteiner.

Det er hovedsakelig oljen fra bomullsfrø som brukes som menneskeføde, mens avfallet fra oljeproduksjonen brukes som fôr. Analysene av ernæringsmessige viktige komponenter i frø ble vurdert. Det ble bemerket at noen av de komponenter som OECDs konsensusdokument (OECD 2004) anbefaler analysert for bomull ikke er utført. Det er funnet statistiske forskjeller for enkelte komponenter. De statistiske forskjellene for disse komponentene er ikke konsistente da forskjellene som er påvist i enkelte forsøksfelt, ikke er påvist i de andre forsøksfeltene. Faggruppen anser analysene for å være tilstrekkelige for en vurdering av hybrid 281-24-236/3006-210-23 til bruk som mat og fôr.

Informasjon vedrørende allergenisitet viser at for de parametere som er målt, har ikke de uttrykte proteinene likheter med kjente allergener eller egenskaper som tilsier at de er allergener.

Faggruppen finner det vanskelig å vurdere om fôrvarer fra bomullen 281-24-236/3006-210-23 er mer allergifremkallende enn fôrvarer fra umodifisert bomullsfrø. På bakgrunn av foreliggende eksperimentelle studier i mus, finner Faggruppen imidlertid at muligheten for økt allergifremkallende aktivitet hos fôrvarer fra 281-24-236/3006-210-23 i forhold til umodifisert bomull med den informasjon vi har tilgang til, ikke med rimelig sikkerhet kan utelukkes. Da mengde Cry1Ac og Cry1F i bomullsfrø kan henholdsvis være 0,89 og 6,6 µg/g fersk vekt, mener Faggruppen at det må kreves av Dow AgroSciences å kommentere forsøkene som viser adjuvanseffekt av Cry1Ac.

Faggruppen konkluderer med at bomullsolje fra 281-24-236/3006-210-23 er vesentlig lik olje fra umodifiserte bomullsfrø, og finner ikke at bruk av olje fra 281-24-236/3006-210-23 utgjør noen større helserisiko enn kommersiell olje fra umodifiserte bomullsplanter.

Nøkkelord

Genmodifisert bomull, 3006-210-23, 281-24-236, 281-24-236/3006-210-23, insektsresistens, herbicidtoleranse, PAT, Cry1Ac, Cry1F, helsemessig trygghet, helse.

Bakgrunn

Faggruppe for genmodifiserte organismer under Vitenskapskomiteen for mattrygghet er blitt bedt av Mattilsynet om en vitenskapelig risikovurdering av EFSA/GMO/NL/2005/16 genmodifisert bomull (281-24-236/3006-210-23) til bruk i næringsmidler og fôrvarer. Vurdering av den genmodifiserte bomullen er basert på den dokumentasjonen som er gjort tilgjengelig på EFSA's nettside GMO EFSA-net. 281-24-236/3006-210-23 er vurdert i henhold til tiltenkt bruk og de prinsipper som er lagt til grunn i EFSA's dokument "Guidance document for the risk assessment of genetically modified plants and derived food and feed" (EFSA 99, 2004). Ved vurdering av vesentlig likhet har Faggruppen lagt vekt på OECDs konsensusdokument for bomull (OECD 2004), som gir anbefalinger over hvilke parametre som bør undersøkes.

I henhold til Vitenskapskomiteen for mattrygghets uttalelse på møtet 23. april 2004 har Faggruppe for genmodifiserte organismer vedtatt at i de sakene hvor EFSA har kommet med sine uttalelser før Faggruppe for genmodifiserte organismer får sakene til behandling, skal søknadene behandles på samme måte som i EU-landene, dvs. ved en noe forenklet risikovurdering. Det vil imidlertid bli tatt hensyn til særnorske forhold der slike kan påvises.

Det er kun medlemmene i Faggruppen som har vurdert den genmodifiserte bomullen.

Oppdrag fra Mattilsynet

I sitt brev ber Mattilsynet Vitenskapskomiteen for mattrygghet om å vurdere den genmodifiserte bomullen. Bruksområdet som søknaden gjelder for er prosessering, næringsmidler og fôrvarer i henhold til EUs Forordning (EC) nr. 1829/2003, artiklene 3(1) og 15(1). Søknaden gjelder ikke for import og kultivering, og krever derfor ikke vurdering for miljørisiko i henhold til Direktiv 2001/18/EØF. Mattilsynet ber VKM om vurdering av helseaspekter ved disse produktene, og legge risikovurderingen inn på EFSA-net, og sende kopi av vurderingen til Mattilsynet.

Linjen er fremkommet ved tradisjonell krysning mellom de genmodifiserte bomullslinjene 3006-210-23 og 281-24-236.

Produktet som ønskes vurdert, er:

Genmodifisert bomull, EFSA/GMO/NL/2005/16 (281-24-236/3006-210-23). Unik kode er. DAS-24236-5 x DAS-21023-5

Status i EU: Søknad under 1829/2003/EF. EFSA's frist for innspill er 17.11.05.

Risikovurdering

Innledning

Den genmodifiserte bomullshybriden 281-24-236/3006-210-23 ble vurdert ut fra Mattilsynets oppdrag. I henhold til Dow AgroSciences er søknaden kun for import og bruk som næringsmidler, fôrvarer og industrielle produkter, ikke for utsetting. Primærbruken av produkter fra bomullsfrø i Norge i dag er til matolje, men avfall fra bomullsoljeproduksjonen brukes til dyrefôr.

Faggruppe for genmodifiserte organismer har på faggruppemøtet 02.02.05 vedtatt å bruke EFSA's retningslinjer som gruppens retningslinjer for vurdering av genmodifiserte planter. Prinsippene som er lagt til grunn for vurderingen, er derfor hentet fra EFSA's dokument "Guidance document for the risk assessment of genetically modified plants and derived food and feed" (EFSA 99, 2004).

Faggruppe for genmodifiserte organismer vurderer søknaden om markedsføring av genmodifisert bomull (EFSA/GMO/NL/2005/16) til bruk i næringsmidler og fôrvarer under rådsforordning 1829/2003.

Bakgrunnsinformasjon

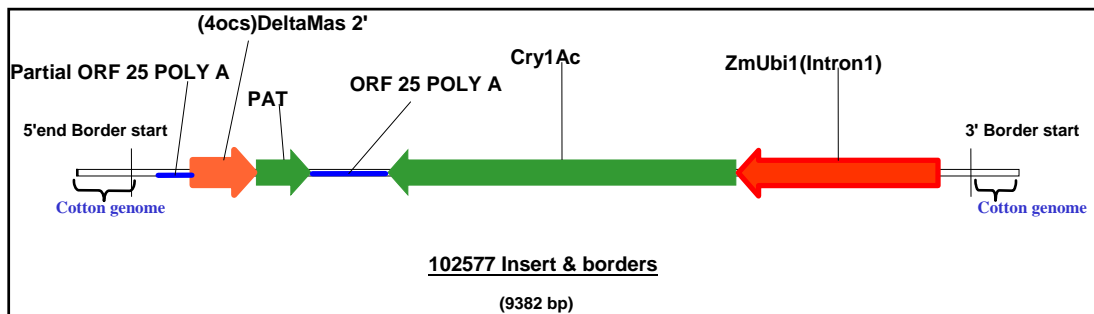
Beskrivelse av de innsatte genene

3006-210-23 (foreldrelinje):

Den molekylærbiologiske karakteriseringen viser at det er satt inn ett rekombinant DNA-fragment i 3006-210-23 bomull. Fragmentet inneholder en ekspresjonskasset.

Cry1Ac-ekspresjonskassetten inneholder:

- a) *UbiZm1*-promoter fra mais, inneholder exon 1 og intron 1
- b) et modifisert *cry1Ac*-gen som finnes i én kopi i genomet. *cry1Ac*-genet stammer fra jordbakterien *Bacillus thuringiensis*. Inneholder også deler av protoksinene *cry1Ca3* og *cry1Ab1*.
- c) *Orf25* – terminator sekvens fra *A. tumefaciens* plasmidet pTi15955
- d) *pat* – syntetisk versjon av glufosinatresistensgenet *pat* fra den gram-positive jordbakterien *Streptomyces viridochromogenes*.
- e) *(40C)deltaMas2'* – mannopin syntase promoter fra *A. tumefaciens* plasmidet pTi15955, inkludert 4 kopier av octopin syntase enhancer fra plasmidet pTiAch5



Figur 1: Rekombinant DNA-fragment i bomull 3006-210-23.

Det rekombinante DNA-fragmentet som er satt inn i planten inneholder som vist på figuren ett fullengde *pat*- og *cry1Ac* gen, samt en partiell åpen ORF25 poly A leseramme.

Genenes funksjon:

pat-genet:

Sprøytemidler basert på glufosinat-ammonium gir en irreversibel hemming av planters eget enzym glutaminsyntetase. Glutaminsyntetase lager aminosyren glutamin fra glutamat og ammoniakk. Enzymet hindrer dermed opphopning av den giftige ammoniakken som dannes ved fotorespirasjon. Sprøyting fører til at planten dør på grunn av akkumulering av ammoniakk. *pat*-genet koder for enzymet PAT (Phosphinothricin-Acetyl-Transferase) som acetylerer og inaktiverer glufosinat, den aktive komponenten i sprøytemidler som Liberty, Basta og Finale. Den genmodifiserte bomullslinjen overlever sprøyting med glufosinat-ammonium siden virkestoffet acetyleres og plantens eget glutamat syntetase-enzym ikke inhiberes, men fortsatt kan utføre sin syntese av glutamat og detoksifisering av ammoniakk.

cry1Ac-genet.

Bacillus thuringiensis (*B.t.*) er en vanlig jordbakterie som danner et intracellulært proteinkrystall [for eksempel Cry1Ac] som har entomopatogen effekt. Cry-proteinkrystallet som er et protoksin, løses opp i larvens tarm for så å spaltes av proteaser til det aktive toksinet. Cry-toksinet binder seg med høy affinitet til spesifikke reseptorer på plasmamembranen i tarmepitelceller. Cry-toksinet fører til osmotisk ubalanse i cellen og massiv vevsødeleggelse opptrer. Cry1Ac har størst effekt mot enkelte sommerfuglarter.

Påvisning av åpne leserammer (ORF)

Det gjort studie for å påvise åpne leserammer. Det er påvist 3 åpne leserammer, ORF3, ORF4 og ORF10 i områdene hvor DNA fragmentet er koblet til genomisk DNA. Homologi til de hypotetiske uttrykte aminosyresekvensene som kan stamme fra disse 3 åpne leserammene ble sammenlignet med aminosyresekvenser i en "in house" database for allergener (2414 aminosyresekvenser fra allergener) og GenPept fra GenBank (1846720 aminosyresekvenser) for homologi til toksiner. ORF-4 har en 6-mer likhet til pollenallergenene Car b I (*Carpinus betulus*) og Cor a I (*Corylus avellana*). Søk etter 7-mer likhet ga ingen likhet til noen allergener. BlastP søk for sekvenshomologi viste ingen domener som har likhet med toksiner. BlastP søk indikerer at ORF3 og ORF10 har henholdsvis noe homologi til en transposase fra *Streptococcus mutans* og en hypotetisk transkripsjonsfaktor fra *Prunus persica*. Fordi et eventuelt uttrykk fra disse tre ORFene har færre enn 80 aminosyrer, ble det ikke foretatt søk for 35 % eller større homologi i et vindu med 80 aminosyrer, som er anbefalt i FAO/WHO prosedyre for vurdering av potensiell allergenisitet (FAO/WHO 2001).

Molekylærbiologiske analyser viser at det rekombinante fragmentet i planten inneholder de samme gener og genelementer som er på det tilsvarende fragmentet i bakterien. Genene på det rekombinante DNA-fragmentet i 3006-210-23 uttrykker Cry1Ac- og PAT-protein som er identisk med proteinene som uttrykkes i bakterien. Analysene viser også at det er fjernet 16 bp ved innsettsstedet. Det var ingen åpne leserammer i de 16 bp.

Mengde Cry1Ac protein i frø fra vekstsesongene 2001 og 2003 er henholdsvis $0,57 \pm 0,09$ $\mu\text{g/g}$ ferskvekt (Range= 0,33-0,78) og $0,43 \pm 0,12$ $\mu\text{g/g}$ ferskvekt (Range=0,16-0,89). Prøvene som er analysert stammer fra seks feltforsøk utført i USA. I hver bomullsåker ble det plantet fire blokker, en blokk for hver av kontroll- og de tre genmodifiserte bomullsplantene. Verdiene er et gjennomsnitt av de seks forsøksfeltene og gjennomsnitt av tre paralleller fra hver de fire blokkene. Forsøksfeltene var lokaliserte på områder som representerer forskjellige vekstmiljøer for bomull.

Mengde PAT protein i frø fra vekstsesongene 2001 og 2003 er henholdsvis lavere enn 0,09 $\mu\text{g/g}$ ferskvekt og lavere enn 0,04 $\mu\text{g/g}$ ferskvekt. Prøvene som er analysert stammer fra de seks feltforsøk utført i USA. Statistisk analyse er som for Cry1Ac proteinet.

Det ble ikke påvist Cry- eller PAT-protein i olje.

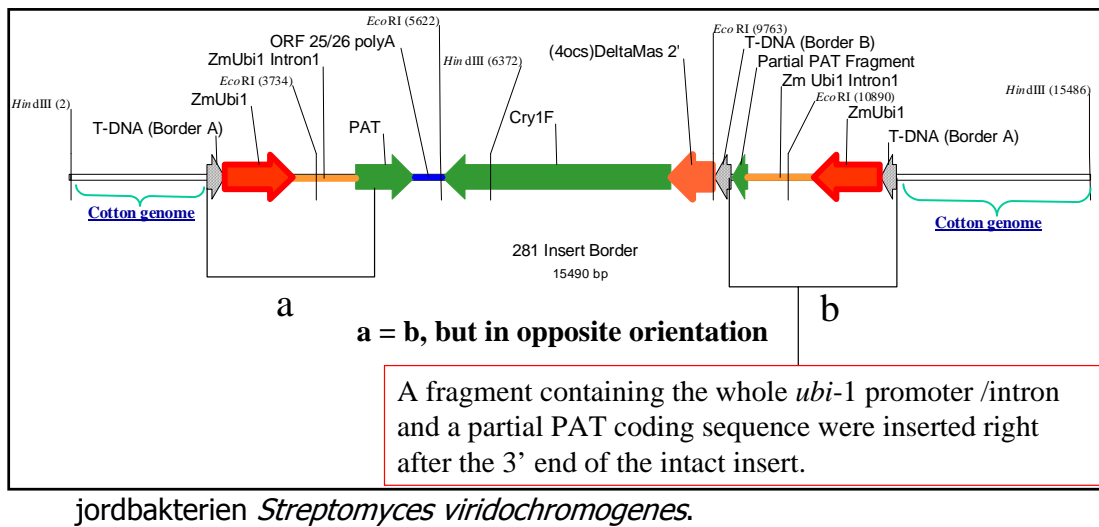
Faggruppen mener at karakteriseringen av det rekombinante innskuddet i 3006-210-23 er tilfredsstillende.

281-24-236 (foreldrelinje):

Den molekylærbiologiske karakteriseringen viser at det er satt inn ett rekombinant DNA-fragment i 281-24-236 bomull. Fragmentet inneholder en ekspresjonskasset.

Cry1F-ekspresjonskassetten inneholder:

- f) *(4OC)deltaMas2'* – mannopinsyntase promoter fra *A. tumefaciens* plasmidet pTi15955, inkludert 4 kopier av octopin syntase enhancer fra plasmidet pTiAch5
- g) *cry1F* - et modifisert *cry1F*-gen som finnes i én kopi i genomet. *cry1F*-genet stammer fra jordbakterien *Bacillus thuringiensis*. Inneholder også deler av protoksinene *cry1Ca3* og *cry1Ab1*.
- h) *Orf25* – terminator sekvens fra *A. tumefaciens* plasmidet pTi15955
- i) *pat* – syntetisk versjon av glufosinatresistensgenet *pat* fra den gram-positive



- j) *UbiZm1*-promoter fra mais, inneholder exon 1 og intron 1

Figur 2: Rekombinante DNA fragment i bomullsplanten 281-24-236.

Det rekombinante DNA fragmentet som er satt inn i planten inneholder som vist på figuren inneholder ett fullengde *pat* og *cry1F* gen, og ett partielt *pat*-gen (231 bp) drevet av *UbiZm1* promoteren.

Partielt *pat* mRNA ble påvist med revers transkriptase-PCR i en mengde som tilsvarer 1/16 av full-lengde *pat* mRNA. Potensielt partielt PAT protein kunne imidlertid ikke påvises med Western blot i noe bomullsvev. Det er blitt påvist en ny åpen leseramme (ORF-7). ORF-7 har en 6-mer likhet med aminosyrer til ett soyagen som har 40 % homologi til et rug pollenallergen (Lol P I, *Lolium perenne*). Ved analyse av flankesekvenser rundt innsetningsstedene for det rekombinante fragmentet er det funnet at *Cry1F*-genet er innsatt på en plass i bomullsgenomet som inneholder genet GA 20-oksidasase. GA 20-oksidasase er involvert i produksjonen av giberelliner, en gruppe av plantehormoner som har mange ulike funksjoner. Det er derfor vanskelig å forutsi effekten av en mulig endring i uttrykket av

GA20-oksidasen. Søker mener det likevel er lite trolig at plasseringen av genkonstruksjonen med Cry1F-genet har innvirkning på de ernæringsmessige egenskapene til bomullslinjen.

Mengde Cry1F protein i frø fra vekstsesongene 2001 og 2003 er henholdsvis $5,13 \pm 1,2$ $\mu\text{g/g}$ ferskvekt (Range= 3,2-8,2) og $2,27 \pm 0,6$ $\mu\text{g/g}$ ferskvekt (Range=0,99-3,86). Prøvene som er analysert stammer fra seks feltforsøk utført i USA. I hver bomullsåker ble det plantet fire blokker, en blokk for hver av kontroll- og de tre genmodifiserte bomullsplantene. Verdien er et gjennomsnitt av de seks forsøksfeltene og gjennomsnitt av tre paralleller fra hver de fire bomullsplantene. Forsøksfeltene var lokaliserte på områder som representerer forskjellige vekstmiljøer for bomull.

Mengde PAT protein i frø fra vekstsesongene 2001 og 2003 er henholdsvis $0,47 \pm 0,17$ $\mu\text{g/g}$ ferskvekt (Range= 0,23-1,07) og $0,43 \pm 0,20$ $\mu\text{g/g}$ ferskvekt (Range=0,16-0,69). Prøvene som er analysert stammer fra de seks feltforsøk utført i USA. Statistisk analyse er som for Cry1F proteinet.

Det ble ikke påvist Cry- eller PAT-protein i bomullsolje.

Molekylærbiologiske analyser viser at ett rekombinant fragment er satt inn i planten. Dette fragmentet inneholder de samme gener og genelementer som er på det tilsvarende fragmentet i bakterien. Genene på det rekombinante DNA-fragmentet i 281-24-236 uttrykker PAT- og Cry-protein som er identisk med proteinene som uttrykkes i bakterien.

Faggruppen mener at karakteriseringen av det rekombinante innskuddet i 281-24-236 er tilfredsstillende.

281-24-236/3006-210-23:

281-24-236/3006-210-23 er dannet ved konvensjonell kryssing mellom 281-24-236 og 3006-210-23. Analyse etter 6 generasjoner viser at de rekombinante fragmentene er stabilt integrert.

Det er foretatt Southern blot-analyse, PCR og sekvensering for å undersøke tilstedeværelsen og antall kopier av 281-24-236- og 3006-210-23-ekspresjonskassetten i 281-24-236/3006-210-23, samt å undersøke for fravær eller tilstedeværelse av andre potensielle mRNA transkript som følge av gensekvensene i de innsatte rekombinante DNA fragmentene. Det er påvist tilsvarende rekombinante ekspresjonskassetter som dem i 281-24-236- og 3006-210-23, pluss at det med *pat*-proben er påvist tre ekstra bånd på henholdsvis 1300 bp, ~ 8500 bp og ~9800 bp. Søker tilskriver 1300 og 9800 båndene til det partielle *pat*-genet, mens det ukarakteriserte 8500 båndet som stammer fra kutting med *HpaI*, tilskrives *HpaI* enzymets måte å kutte på.

Søker mener det er lite trolig at plasseringen av genkonstruksjonen med Cry1F-genet i GA 20-oksidasen genen har innvirkning på de ernæringsmessige egenskapene til bomullslinje 281-24-236/3006-210-23. Dette fordi 1) plasseringen av Cry1F er i den ikke-kodende 3'-delen av GA 20-oksidasen og dermed ikke forstyrrer den kodende sekvensen, 2) bomullslinje 281-24-

236/3006-210-23 er tetraploid (inneholder fire kromosomsett) og planten har derfor ytterligere tre kopier av GA 20-oksidasase, 3) GA 20-oksidaser tilhører en større genfamilie, noe som innebærer at det finnes enda flere varianter av denne gentyperen i bomull, 4) utover motstandsdyktighet overfor enkelte insekter og sprøytemidler av type glufosinat er det ikke observert endrede agronomiske egenskaper hos linje 281-24-236/3006-210-23 sammenliknet med ikke-genmodifisert kontroll og 5) næringsinnholdet i frø fra 281-24-236/3006-210-23 avviker ikke vesentlig fra kontrollfrøene.

Det konkluderes med at begge ekspresjonskassetene fra henholdsvis 3006-210-23 og 281-24-236 er satt inn i 281-24-236/3006-210-23. Sammenlignende Southern blot-analyser mellom hybridene 281-24-236/3006-210-23 og de to foreldrelinjene viser at bruttostørrelsen på de innsatte DNA-fragmentene er intakte, og at det er påvist med *pat*-proben et ekstra bånd på ~8500 bp som ikke er karakterisert.

Mengde Cry1Ac protein i frø fra vekstsesongene 2001 og 2003 er målt til henholdsvis $0,57 \pm 0,09$ µg/g ferskvekt (Range=0,33-0,78) og $0,46 \pm 0,12$ µg/g ferskvekt (Range=0,16-0,89). Mengde Cry1F protein er målt til henholdsvis $4,13 \pm 1,1$ µg/g ferskvekt (Range=1,4-6,6) og $2,34$ µg/g $\pm 0,52$ ferskvekt (Range=1,5-4,15). Mengde PAT protein er målt til henholdsvis $0,53 \pm 0,21$ µg/g ferskvekt (Range=0,20-1,31) og $0,53 \pm 0,17$ µg/g ferskvekt (Range=0,22-1,02). Prøvene som er analysert stammer fra de samme feltforsøk som for 281-24-236 og 3006-210-23.

Det ble ikke påvist DNA, Cry- og PAT protein i bomullsolje.

Dokumentasjon av "vesentlig likhet"

Søkerens hoveddokument er presentert som anbefalt i EFSA's retningslinjer for risikovurdering av genmodifiserte planter og rådsforordning 1829/2003. Analyser av sammensetning i bomullsfrø er fra bomullslinjene 281-24-236/3006-210-23, 3006-210-23, 281-24-236 og en umodifisert kontrollhybrid. Prøvene som er analysert, stammer fra seks feltforsøk utført på seks forskjellige dyrkningsområder i USA. Dyrkningsområdene representerer forskjellige vekstmiljøer for bomull, og undersøkelsene ble utført i 2001 og 2003. I hvert av de seks forsøksfeltene ble forskjellige bomullssortene plantet i fire blokker. Det er tatt ut tre prøver fra de fire blokkene fra hvert felt, og disse analysene omfatter bomullssortene 281-24-236/3006-210-23, 3006-210-23, 281-24-236 og en umodifisert kontrollhybrid. Alle bomullssortene ble dyrket på de samme forsøksfeltene. I søknadsdokumentet er det imidlertid kun dokumentert sammenligninger mellom 281-24-236/3006-210-23 og umodifisert kontrollhybrid, som er i henhold til EFSA's retningslinjer (EFSA 99, 2004).

Hovedkomponenter i bomullsfrø:

Søker har ikke for 281-24-236/3006-210-23, 3006-210-23, 281-24-236 og umodifisert kontrollhybrid gitt uttrykk for at valget av analyseparametere er gjort i henhold til aksepterte internasjonale standarder og OECDs konsensusdokument for bomull (OECD 2004). Imidlertid henviser søker til verdier i tabeller over analyserte parametre i OECD dokumentet. Det er foretatt forskjellige analyser av hovedkomponenter i en rekke vev. Faggruppen har konsentrert sin vurdering av frø, og avlede produkter fra frø som benyttes til mat (olje) og fôr (frøskall, frøkake og mel). Det ble analysert for aske, fett, protein, vann, karbohydrater, total fiber, kalorier, syrestabil fiber (ADF), nøytralstabil fiber NDF), aminosyrer, fettsyrer, vitaminene (kun for 2003) A, B₁, B₂, B₆, C, folat, niacin, tokoferoler (alfa, beta, gamma, delta), fosfor, jern, kalium, kalsium, kobber, magnesium, mangan, molybden, natrium, sink, svovel, anti-næringsmidlene gossypol (totalt og fritt) og cyklopropenoid fettsyrer (sterculic, malvalic og dihydrosterculic), samt aflatoksinene B₁, B₂, G₁ og G₂. Analysene ble utført under god laboratoriepraksis (GLP).

For hovedkomponentene vann (2001) og fiber (2001) funnet statistiske forskjeller som er mindre enn 10 %.

Fettsyresammensetning i bomullsfrø:

Fettsyresammensetningen i frø for 281-24-236/3006-210-23, 3006-210-23, 281-24-236 og umodifisert kontrollhybrid er målt er i henhold til OECDs konsensusdokument for bomull. Det ble analysert for 22 fettsyrer. Fettsyrenivået er i noen tilfelle lavere enn litteraturverdier, imidlertid er nivåene svært like for 281-24-236/3006-210-23 og foreldrelinjene.

Aminosyrer i bomullsfrø:

Både essensielle og ikke-essensielle aminosyrer ble analysert. De aminosyrer som er målt er i henhold til OECD dokumentet. Det er ikke funnet store statistiske forskjeller for alle forsøksfeltene. Verdien ligger innenfor 20 %, og for alle aminosyrene ligger verdiene innenfor de typiske verdiene som er rapportert i litteraturen.

Vitaminer:

Vitaminer som det i henhold til OECDs konsensusdokument for bomull bør undersøkes for, er vitamin E. For feltforsøkene i 2003 er det målt for vitaminene A, B₁, B₂, B₆, C, folat, niacin, tokoferoler (alfa, beta, gamma, delta). Det er ikke funnet store statistiske forskjeller. Vitamin A, alfa - og delta tokoferol var ikke påvisbare med de måle metodene som ble brukt.

Mineraler:

Med unntak for selen er mineralene som er målt i henhold til OECDs konsensusdokument for bomull. Det er funnet statistiske forskjeller for flere mineraler, men forskjellen ligger innenfor 5 %. For noen mineraler ligger verdiene utenfor typiske verdier for andre bomullssorter som er rapportert i litteraturen. Imidlertid er forskjellene mellom verdiene for umodifisert kontrollhybrid og genmodifisert hybrid mindre enn 5 %.

Antiernæringsstoffer:

Det er ikke funnet statistiske forskjeller for gossypol. Det er små forskjeller for cyklopropenoidsyrene for vekstsesongen 2001, men ikke for 2003.

Toksiner:

Aflatoksinene B₁, B₂, G₁ og G₂ er ikke påvist over påvisningsgrensen på 1 ppb.

Analyse av protein og DNA i raffinert bomullsolje.

Dow AgroSciences har analysert raffinert bomullsolje for protein og DNA. Det er ikke påvist protein eller DNA over påvisningsgrensene.

Konklusjon

Det er funnet statistiske forskjeller i enkeltparametere. Enkelte av verdiene for noen av komponentene ligger utenfor typiske verdier for andre bomullssorter som er rapportert i litteraturen. Imidlertid er forskjellene mellom genmodifisert bomull og umodifisert kontrollhybrid mindre enn 10 %. Faggruppen anser derfor at de forskjellene som er påvist ikke har noen helsemessig betydning.

Dokumentasjon av toksisitet og allegenisitet

Toksisitet:

Siden det kun er olje som benyttes som mat, krever søker at fôringsforsøk med de nye proteinene utføres i sammenheng med dyrehelse. Søknaden inneholder dokumentasjon på fôringsforsøk av mus med renfremstilt Cry1Ac, Cry1F og PAT- proteiner, og en 42 dagers studie med broilere.

Fôringsforsøkene med renfremstilte proteiner er gjort i henhold til OECDs retningslinjer nr. 401 for akutt oral toksisitet (1987), EPAs retningslinjer for helseeffekter OPPTS 870.1100 (1998), JMAFF retningslinjer for akutt oral toksisitet (2000) og EUs retningslinjer for akutt oral toksisitet (EEC Methods Number B.1, 1992). Det er ikke funnet noen testrelaterte endringer hos musene ved fôring med 350 mg Cry1Ac/kg kroppsvekt(kv), 375 mg Cry1F/kg kv og 6000 mg PAT/kg kv.

Fôringsforsøk på broiler:

Søknaden inneholder dokumentasjon fra 42-dagers fôringsforsøk på broilere, 480 dyr, fordelt i fire grupper. Dyrene ble fôret med henholdsvis bomullsmel fra 281-24-236/3006-210-23, en umodifisert kontrollhybrid og to kommersielle umodifiserte referansehybride bomullssorter.

Det ble påvist testrelaterte endringer for de dyrene som ble fôret med 281-24-236/3006-210-23 genmodifisert bomullsmel. Fôring med genmodifisert bomullsmel ga lavest og best fôromdannelsen. For de andre målte parametrene var det ingen statistiske forskjeller mellom dyrene. Faggruppen konkluderer med at det er ingen grunn til å anta at den ernæringsmessige kvaliteten til fôr fra genmodifiserte bomull er dårligere enn fôr fra umodifisert bomull.

Allergisitet:

Bt-proteiner

Til tross for vel 50 års bruk av B.t.k. som sprøytemiddel er det ingen bekreftede rapporter over øyeblikkelige eller forsinkede allergiske reaksjoner til tross for betydelig human oral-, dermal- og inhalasjonseksposering. (EHC 1999) Laboratoriestudier med pattedyr indikerer heller ingen potensielle allergiske reaksjoner mot *Bacillus thuringiensis* eller dets komponenter innbefattet delta-endotoksinet i krystallproteinet. Allergiske reaksjoner mot *Bacillus thuringiensis* har vært rapportert, men disse har ikke vært tilskrevet krystallproteinet (EHC 1999).

Det har ikke vært utført immunologiske studier med de transgene produktene. Det er vist at Cry1Ac-proteinet binder seg til musetarmoverflaten og induserer immunologiske reaksjoner mot seg selv og mot proteiner gitt samtidig (Vazquez-Padron *et al.* 2000a, Vazquez *et al.* 1999, Moreno-Fierros *et al.* 2003, Rojas-Hernández *et al.* 2004). Immunologisk kartlegging av systemisk og mucosal immunreaksjon på Cry1Ac har videre påvist at mus lager både systemisk IgM, IgG og sekretorisk IgA etter intraperitoneal og intragastrisk immunisering (Vazquez-Padron *et al.* 2000b). Det er ukjent om Cry1Ac-proteinet som er benyttet i disse studiene, tilsvarer Cry1F-toksinet som den transgene maislinjen lager. Det er vist at domene II fra Cry1Ab og Cry1Ac genererer ulik immunologisk respons i kanin (Vazquez-Padron *et al.* 1998). I en annen studie er det vist at Cry1Ac hadde utpreget mucosal adjuvanseffekt ved å potensere IgM-, IgG- og IgA-responsen mot hepatittvirusantigen og bovint serumalbumin som ble gitt med sondefôring samtidig med Cry1Ac (Vazquez *et al.* 1999). Produksjonen av IgE-antistoff, som er knyttet til allergisk reaksjon, ble ikke målt. Også i tidligere studier (Prasad & Shetna 1975) er det påvist adjuvanseffekt av krystallprotein fra *Bacillus thuringiensis*. Adjuvanseffekten av Cry1Ac er bekreftet i to senere publikasjoner med henholdsvis pneumokokk-antigen (Moreno-Fierros *et al.* 2003) og amøbe-lysate (Rojas-Hernández *et al.* 2004). Adjuvanseffekten av Cry1Ac ble funnet å være like sterk som adjuvanseffekten av koleratoksin (Vazquez-Padron *et al.* 1999), som er et mye brukt slimhinneadjuvant i eksperimentelle studier av vaksinasjon og av allergi, og som regnes for å være det sterkeste slimhinneadjuvant vi kjenner.

De mengder Cry1Ac som ga mucosal adjuvanseffekt ved sondefôring av mus var fra 0,1 µg til 100 µg (Vazquez *et al.* 1999). De adjuvansdoser som brukes for immunisering av mus og mennesker i andre sammenhenger er ofte av samme størrelsesorden, det vil si om lag

samme dose brukes til mus og menneske. Det er mulig at også Cry1F som benyttes i 281-24-236/3006-210-23 kan ha tilsvarende effekter som vist for det beslektede Cry1Ac-proteinet, som induserer immunologiske reaksjoner mot seg selv og økt reaksjon mot proteiner gitt samtidig. Dersom Cry1F har tilsvarende adjuvanseffekt som det beslektede Cry1Ac-proteinet er rapportert å ha, vil dette teoretisk kunne føre til økt utvikling av allergi for dyrene mot fôrvarer spist sammen med for eksempel bomullsmel. Man ville vente at adjuvanseffekten kom til syne først og fremst som økt forekomst av allergi mot de matvarene der matallergi fra før er vanligst. IgE ble ikke målt i de refererte studiene av adjuvanseffekt av Cry1Ac-proteinet.

Konklusjon:

Faggruppen finner det ut fra tilgjengelige data vanskelig å vurdere om bomullsmel fra 281-24-236/3006-210-23 er mer allergifremkallende for dyr enn umodifisert bomullsmel. På bakgrunn av foreliggende eksperimentelle studier i mus, finner Faggruppen imidlertid at muligheten for økt allergifremkallende aktivitet til 281-24-236/3006-210-23 med den informasjon vi har tilgang til, ikke med rimelig sikkerhet kan utelukkes. Faggruppen mener at siden adjuvanseffekt av Cry1F ikke med rimelig sikkerhet kan utelukkes, må det kreves av Dow AgroSciences å kommentere forsøkene som viser adjuvanseffekt av Cry1Ac i forhold til muligheten for adjuvanseffekt av Cry1F

Konklusjon

Det er funnet statistiske forskjeller i enkeltparametere. Noen av verdiene for de enkelte analyserte komponentene ligger utenfor typiske verdier for andre bomullssorter som er rapportert i litteraturen. Faggruppen finner at disse forskjellene er små og anser at de ikke har noen helsemessig signifikans. Da det ikke er funnet store statistiske forskjeller mellom genmodifisert – og umodifisert kontrollhybrid i enkeltparametere for olje konkluderer faggruppen derfor med at det ikke er grunn til å anta at den ernæringsmessige kvaliteten til olje fra den genmodifiserte bomullsplanten er forskjellig fra olje fra umodifisert bomullsplante.

Flere studier viser at proteinene PAT, Cry1Ac og Cry1F ikke er akutt toksiske. Dow AgroSciences har utført akuttstudier på mus med disse proteinene. Disse studiene viser at disse proteinene, ikke fører til påvisbare helseeffekter på dyrene. Dow AgroSciences har utført fôringsforsøk med broilere, men ikke utført sub-kroniske studier på rotter med fôr fra 281-24-236/3006-210-23. Faggruppen konkluderer med at det er lite sannsynlig at eksponering for PAT-proteinet i seg selv og i de mengder som tilføres via fôr fra den genmodifisert bomullen, er helsemessig betenkelige for dyr. Faggruppen finner imidlertid at for Cry1Ac- og Cry1F proteinene er det ut fra tilgjengelige data vanskelig å vurdere om bomullsmel fra 281-24-236/3006-210-23 er mer allergifremkallende for dyr enn umodifisert bomullsmel.

Faggruppen konkluderer med at bomullsolje fra 281-24-236/3006-210-23 er vesentlig lik olje fra umodifiserte bomullsfrø, og finner ikke at bruk av olje fra 281-24-236/3006-210-23 utgjør noen større helserisiko enn kommersiell olje fra umodifiserte bomullsplanter.

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Appendix II

EFSA, 2010a

SCIENTIFIC OPINION

Scientific Opinion on application (EFSA-GMO-NL-2005-16) for the placing on the market of insect resistant genetically modified cotton (*Gossypium hirsutum* L.) 281-24-236 x 3006-210-23 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Dow AgroSciences¹

EFSA Panel on Genetically Modified Organisms (GMO)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

This scientific opinion reports an evaluation of a risk assessment for placing on the market of insect resistant genetically modified (GM) cotton (*Gossypium hirsutum* L.) 281-24-236 x 3006-210-23 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5) for food and feed uses, import and processing. The GM cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of the single cotton events 281-24-236 and 3006-210-23. The cotton 281-24-236 x 3006-210-23 expresses combined traits encoded by *cry1F* in event 281-24-236 and by *cry1Ac* in event 3006-210-23, both genes conferring resistance to certain lepidopteran pests. Both single events contain *pat* genes used as a selectable marker. The structure of the inserts in the single cotton events was retained in the stack 281-24-236 x 3006-210-23. Expression levels of Cry1Ac, Cry1F and PAT proteins were demonstrated to be comparable to those of the respective single events. The results of comparative analyses indicated that cotton 281-24-236 x 3006-210-23 and its respective single events are compositionally, agronomically and phenotypically equivalent to its conventional counterpart, except for the expression of Cry1Ac, Cry1F and PAT proteins. The safety assessment identified no concerns regarding toxicity and allergenicity of cotton 281-24-236 x 3006-210-23. A feeding study on broiler chickens confirmed the nutritional equivalence of this GM cotton to its conventional counterpart and two commercial non-GM cotton varieties. The intended uses of cotton 281-24-236 x 3006-210-23 exclude cultivation within the European Union. The environmental risk assessment is therefore restricted to the indirect exposure through manure and faeces mainly from animals fed with cotton products of 281-24-236 x 3006-210-23 and with the accidental release into the environment of cotton

1 On request from the Competent Authority of the Netherlands on an application (EFSA-GMO-NL-2005-16) submitted by Dow AgroSciences, Question No EFSA-Q-2005-124, adopted on 26 May 2010.

2 Panel members: Hans Christer Andersson, Salvatore Arpaia, Detlef Bartsch, Josep Casacuberta, Howard Davies, Patrick du Jardin, Gerhard Flachowsky, Lieve Herman, Huw Jones, Sirpa Kärenlampi, Jozsef Kiss, Gijs Kleter, Harry Kuiper, Antoine Messéan, Kaare Magne Nielsen, Joe Perry, Annette Pötting, Jeremy Sweet, Christoph Tebbe, Atte Johannes von Wright, and Jean-Michel Wal. Correspondence: gmo@efsa.europa.eu

3 Acknowledgement: The EFSA GMO Panel wishes to thank the members of the Working Groups on Molecular characterisation, Food and Feed and Environment for the preparation of this opinion, Marco Nuti and Willem Seinen as external experts and EFSA's staff members Yi Liu, Sylvie Mestdagh and Nancy Podevin for the support provided to this EFSA scientific output.

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281-24-236 x 3006-210-23 grains during transportation and processing. There are no indications of increased likelihood of establishment or survival of feral cotton plants. If accidental spillage and subsequent release into the environment of cotton 281-24-236 x 3006-210-23 seeds occur, cotton 281-24-236 x 3006-210-23 plants would have a selective advantage only under infestation of target pest species or in presence of glufosinate-ammonium herbicides, which are not commonly used on cultivated cotton or in most areas where the GM cotton might be spilled. In conclusion, the EFSA GMO Panel considers that information available for cotton 281-24-236 x 3006-210-23 addresses the scientific comments raised by Member States and that the cotton 281-24-236 x 3006-210-23 as described in this application is as safe as its conventional counterpart and other appropriate comparators with respect to potential effects on human and animal health and the environment in the context of its intended uses. The EFSA GMO Panel concludes that cotton 281-24-236 x 3006-210-23 is unlikely to have any adverse effect on human and animal health and the environment, in the context of its intended uses.

KEY WORDS

GMO, cotton (*Gossypium hirsutum* L.), 281-24-236, 3006-210-23, 281-24-236 x 3006-210-23, insect resistant, risk assessment, food and feed safety, environment, import and processing, Regulation (EC) No 1829/2003.

SUMMARY

Following the submission of an application (EFSA-GMO-NL-2005-16) under Regulation (EC) No 1829/2003 from Dow AgroSciences, The EFSA Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel) was asked to deliver a scientific opinion on the safety of the insect resistant genetically modified (GM) cotton 281-24-236 x 3006-210-23 (Unique identifier DAS-24236-5 x DAS-21023-5) for food and feed uses, import and processing.

In delivering its scientific opinion, the EFSA GMO Panel considered the application EFSA-GMO-NL-2005-16, additional information supplied by the applicant, scientific comments submitted by Member States, and relevant scientific publications. The scope of application EFSA-GMO-NL-2005-16, includes the cotton 281-24-236 x 3006-210-23 and its derived-products, to be used for animal feed (e.g. cake of cottonseed, hulls), for human food products (e.g. oil, linters) and for industrial uses (e.g. textile fibre), but excluding cultivation in the EU. The EFSA GMO Panel assessed cotton 281-24-236 x 3006-210-23 with reference to the intended uses and principles described in the Guidance Documents of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a) and for the risk assessment of genetically modified plants containing stacked transformation events (EFSA, 2007). The evaluation of the risk assessment included molecular characterisation of the inserted DNA and data for the newly expressed proteins. An evaluation of the comparative analysis of composition, agronomic and phenotypic traits was undertaken, and the safety of the newly expressed proteins and the whole food/feed were evaluated with respect to potential toxicity, allergenicity and nutritional quality. An evaluation of environmental impacts and of the post-market environmental monitoring plan was undertaken.

Cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of the single events 281-24-236 and 3006-210-23, and subsequently self-pollinated for five generations to obtain the GM cotton 281-24-236 x 3006-210-23 homozygous for both inserts to achieve insect resistance traits against certain lepidopteran pests, such as *Heliothis zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm) and *Pectinophora gossypiella* (pink bollworm), by the expression of Cry1F and Cry1Ac proteins. PAT protein was used as a selectable marker during transformation processes. The EFSA GMO Panel evaluated the risk assessment on the stacked cotton 281-24-236 x 3006-210-23, as well as its respective single events.

The molecular characterisation data established that event 281-24-236 contained a single insertion with the *cryIF* and *pat* genes and an additional *pat* fragment. The event 3006-210-23 contained a single insertion with the *cryIAC* and *pat* genes. Analyses of the integration sites including sequence determination of the inserted DNA and flanking regions and bioinformatic analyses have been performed. Bioinformatic analyses of the flanking regions indicated that the 281-23-236 insertion occurred into the 3' untranslated region of a gibberellin 20-oxidase gene. Updated bioinformatic analyses of event 3006-210-23 did not indicate the interruption of any known endogenous coding or regulatory sequences. Bioinformatic analyses of junction regions demonstrated the absence of any new open reading frames (ORFs) potentially coding for known toxins or allergens. The expression of the genes introduced by genetic modification has been sufficiently analysed and the stability of the genetic modification has been demonstrated over several generations for both 281-24-236 and 3006-210-23 events. Moreover, the insert structure of both single events was retained in the stack 281-24-236 x 3006-210-23. The EFSA GMO Panel is of the opinion that the molecular characterisation of the DNA inserts and flanking regions of cotton 281-24-236 x 3006-210-23 does not raise any safety concern, and that sufficient evidence for the stability of the genetic modification was provided.

The comparative compositional analysis of cottonseed and derived products, as well as the analysis of agronomic and phenotypic characteristics, of the GM cotton 281-24-236 x 3006-210-23 and its respective single events in field trials at locations representative of commercial cotton cultivation in the USA, showed that cotton 281-24-236 x 3006-210-23 and both single events are compositionally, agronomically and phenotypically equivalent to their conventional counterpart. This also indicates that

the above mentioned 281-24-236 insertion into the 3' untranslated region of a gibberellin 20-oxidase gene did not alter the compositional and agronomic characteristics. Based on the assessment of data available, including the additional information provided by the applicant in response to the EFSA GMO Panel's requests for the GM cotton 281-24-236 x 3006-210-23, for the single events and for its conventional counterpart, the EFSA GMO Panel has found no indication that crossing of cotton 281-24-236 and 3006-210-23 results in interactions between the single events which causes compositional, agronomic or phenotypic changes. No indications of possible adverse effects of the newly expressed Cry1Ac, and Cry1F and PAT proteins were found in studies on potential toxicity and allergenicity, including bioinformatic studies and investigations on stability, digestibility and animal toxicity. Based on the mode of action of the Cry and PAT proteins and given all information provided, the EFSA GMO Panel concludes that interactions between the single cotton events that might impact on food and feed safety are unlikely, and data from a feeding study with broiler chickens showed that the nutritional properties are similar to those of its conventional counterpart, further confirming the outcomes of the compositional analysis.

There is therefore no requirement for scientific information on possible environmental effects associated with the cultivation of cotton 281-24-236 x 3006-210-23 as the environmental risk assessment is restricted to the indirect exposure through manure and faeces mainly from animals fed with cotton products of 281-24-236 x 3006-210-23 and with the accidental release into the environment of cotton 281-24-236 x 3006-210-23 grains during transportation and processing. There are no indications of increased likelihood of establishment or survival of feral cotton plants. If accidental spillage and subsequent release into the environment of 281-24-236 x 3006-210-23 cottonseed occur, cotton 281-24-236 x 3006-210-23 plants would have a selective advantage only under infestation of target pest species or in the presence of glufosinate-ammonium herbicides, which are not commonly used on cultivated cotton or in most areas where the GM cotton might be spilled. The EFSA GMO Panel therefore concludes that unintended environmental effects due to the establishment and spread of cotton 281-24-236 x 3006-210-23 will not be different from that of conventional cotton.

Considering the intended uses of cotton 281-24-236 x 3006-210-23, the monitoring plan provided by the applicant is in line with both the EFSA GMO Panel Guidance Document for the risk assessment of genetically modified plants and derived food and feed and the opinion of the EFSA GMO Panel on post-market environmental monitoring. However, the EFSA GMO Panel is aware that, due to physical characteristics of cottonseed and methods of transportation, accidental spillage cannot be excluded. Therefore the EFSA GMO Panel recommends that, within general surveillance, appropriate management systems are introduced to actively monitor the occurrence of feral cotton plants in areas where cottonseed spillage and plant establishment are likely to occur. The EFSA GMO Panel also recommends that appropriate management systems should be in place to restrict seeds of cotton 281-24-236 x 3006-210-23 entering cultivation as the latter requires specific approval under Directive 2001/18/EC or Regulation (EC) No 1829/2003.

In conclusion, the EFSA GMO Panel considers that the information available for cotton 281-24-236 x 3006-210-23 addresses the scientific comments raised by Member States; and that the cotton 281-24-236 x 3006-210-23 as described in this application is as safe as its conventional counterpart and other appropriate comparators with respect to potential effects on human and animal health and the environment. The EFSA GMO Panel thus concludes that cotton 281-24-236 x 3006-210-23 is unlikely to have any adverse effect on human and animal health and the environment in the context of its intended uses.

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BACKGROUND

On 28 June 2005, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands an application (Reference EFSA-GMO-NL-2005-16), for authorisation of the insect resistant genetically modified (GM) cotton 281-24-236 x 3006-210-23 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5), submitted by the applicant within the framework of Regulation (EC) No 1829/2003 on genetically modified food and feed. After receiving the application EFSA-GMO-NL-2005-16 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed Member States and the European Commission, and made the summary of the application publicly available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of Regulation (EC) No 1829/2003. On 3 August 2005, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the European Commission, and consulted nominated risk assessment bodies of Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Member State bodies had three months after the date of acknowledgement of the valid application (until 4 November 2005) within which to make their opinion known.

In parallel to the submission of the application EFSA-GMO-NL-2005-16 under Regulation (EC) No 1829/2003 in June 2005, the applicant submitted also a notification C/NL/04/01 for the placing on the market of cotton 281-24-236 x 3006-210-23 under Directive 2001/18/EC, which includes import of this cotton as well as its uses for feed and processing and was subject to the transitory measures of Article 46 of the Regulation. Following the clarification of scopes by the applicant and suggestion of DG SANCO and DG Environment, on 8 March 2006 the applicant withdrew its notification C/NL/04/01, and resubmitted the application EFSA-GMO-NL-2005-16 to EFSA with an updated scope, *i.e.*, for food and feed uses, import and processing, but excluding cultivation. EFSA then launched a second consultation with Member States for a period of 6 weeks (from 24 March 2006 until 5 May 2006).

The EFSA GMO Panel carried out a scientific assessment of the GM cotton 281-24-236 x 3006-210-23 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. When carrying out the safety assessment, the EFSA GMO Panel took into account the principles described in the Guidance Documents of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a) and for the risk assessment of genetically modified plants containing stacked transformation events (EFSA, 2007), the scientific comments of Member States and the additional information provided by the applicant.

On 21 October 2005, 15 December 2005, 7 July 2006, 14 November 2006, 17 March 2009 and 21 October 2009, the EFSA GMO Panel requested from the applicant additional information. The applicant provided the requested information on 2 November 2005, 21 March 2006, 25 August 2006, 9 December 2008, 29 September 2009, and 12 March 2010. After receipt and assessment of the full data package the EFSA GMO Panel finalised its risk assessment on cotton 281-24-236 x 3006-210-23.

In giving its scientific opinion on GM cotton 281-24-236 x 3006-210-23 to the European Commission, the Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of six months from the acknowledgement of the valid application. As additional information was requested by the EFSA GMO Panel, the time limit of six months was extended accordingly, in line with Articles 6(1), 6(2), 18(1), and 18(2) of Regulation (EC) No 1829/2003.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation and thus will be part of the EFSA overall opinion in accordance with Articles 6(5) and 18(5).

TERMS OF REFERENCE

The EFSA GMO Panel was requested to carry out a scientific risk assessment of cotton 281-24-236 x 3006-210-23 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environment and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The EFSA GMO Panel was not requested to give a scientific opinion on information required under Annex II to the Cartagena Protocol. Furthermore, the EFSA GMO Panel did also not consider proposals for labelling and methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to risk management.

ASSESSMENT

1. Introduction

The genetically modified (GM) cotton (*Gossypium hirsutum* L.) 281-24-236 x 3006-210-23 (Unique Identifier DAS-24236-5 x DAS-21Ø23-5) was assessed with reference to its intended uses, taking account of the principles described in the Guidance Documents of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a) and for the risk assessment of genetically modified plants containing stacked transformation events (EFSA, 2007). The evaluation of the risk assessment is based on the information provided in the application relating to cotton 281-24-236 x 3006-210-23, additional information from the applicant and information on the single events, as well as comments raised by Member States and relevant scientific publications.

The GM cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of the GM cotton events 281-24-236 and 3006-210-23. The applicant restricted the scope of this application to the stack 281-24-236 x 3006-210-23 excluding the single events 281-24-236 and 3006-210-23. Despite of the statement of the applicant not to commercialise the single events anywhere in the world, information on the single events has been assessed by the EFSA GMO Panel to support the evaluation of the stacked cotton 281-24-236 x 3006-210-23. Cotton (*G. hirsutum* L.) is predominantly a self-pollinator and the stacked cotton 281-24-236 x 3006-210-23 is homozygous for all traits (IRMM, 2006). Therefore, the produced and imported cottonseed of this GM cotton will contain all traits, and segregants are expected only at very low frequency.

2. Issues raised by Member States

The scientific comments raised by Member States are addressed in details in Annex G of the EFSA overall opinion and have been considered in this EFSA GMO Panel scientific opinion⁴.

3. Molecular characterisation

3.1. Evaluation of relevant scientific data

3.1.1. Method of production of the stacked cotton 281-24-236 x 3006-210-23

The GM cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of the GM cotton events 281-24-236 and 3006-210-23 to combine resistance to certain lepidopteran insect pests. Cotton 281-24-236 x 3006-210-23 contains the *cryIF* from event 281-24-236, the *cryIAC* from event 3006-210-23 and the *pat* genes from both events.

Agrobacterium-mediated transformation using the disarmed *Agrobacterium tumefaciens* (renamed *Rhizobium radiobacter*) strain LBA4404 carrying the binary vector pAGM281 or pMYC3006 was used to transform cotton variety GC510 and produce cotton 281-24-236 and cotton 3006-210-23, respectively. Both cottons were self-pollinated for one generation and backcrossed three times to cotton variety PSC355. These backcrossed lines 281-24-236 and 3006-210-23 were crossed to combine the insect resistance traits, and subsequently self-pollinated for five generations to obtain the GM cotton 281-24-236 x 3006-210-23 homozygous for both inserts.

⁴ <http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2005-124>

3.1.2. Evaluation of the single cotton events

3.1.2.1. Cotton 281-24-236

(a) Cotton 281-24-236: transformation process and vector constructs

The vector pAGM281 used to generate cotton 281-24-236 contained two expression cassettes on the T-DNA, one for the *cryIF* and one for the *pat*. This *cryIF* is a synthetic chimeric gene with the N-terminal core toxin *cryIFa* and the C-terminal part from *cryICa* and *cryIAb* (Gao *et al.*, 2006). The coding sequence was modified in order to introduce two restriction sites and this resulted in two amino acid substitutions (F604L and Q640R) (Gao *et al.*, 2006). The *cryIF* coding sequence is driven by the constitutive mannopine synthase promoter from *A. tumefaciens* pTi15955 (Barker *et al.*, 1983) fused with four copies of the octopine synthase (OCS) enhancer from *A. tumefaciens* pTiAch5 (Ellis *et al.*, 1987) ((4OCS) Δ Mas2' promoter). The *pat* expression cassette contained the synthetic plant-optimised *pat* gene based on the sequence from *Streptomyces viridochromogenes* Tu494 which results in tolerance to glufosinate-ammonium herbicides. The *pat* coding sequence is driven by the constitutive *Zea mays* ubiquitin 1 (ZmUbi1) promoter (Christensen *et al.*, 1992) enhanced by its exon1 and intron1 (Gao *et al.*, 2006). Termination of transcription of both transcripts is mediated by the bidirectional open reading frame 25 (orf25) terminator from *A. tumefaciens* pTi15955 (Barker *et al.*, 1983).

(b) Cotton 281-24-236: transgene constructs in the genetically modified plant

Southern analyses using multiple restriction enzyme and probe combinations covering the full-length plasmid pMYC3006 demonstrated the presence of a single intact copy of the pAGM281 T-DNA and in addition one partial *pat* gene. RT-PCR analysis indicated that transcriptional expression of partial *pat* was at least 16 times lower than the full-length *pat* gene. Western analysis of cotton 281-24-236 showed no detectable partial PAT in any of the plant tissues analysed. The absence of vector backbone sequences in event 281-24-236 was confirmed using probes spanning the vector backbone of pAGM281.

The analysis of the insert and the 5' and 3' flanking regions of cotton event 281-24-236 confirmed the sequence of the intact T-DNA except for a 2 base pair (bp) difference within the ZmUbi1 promoter region compared to the plasmid sequence. In addition, the DNA sequences confirmed the presence of a 231 bp partial *pat* coding sequence and as well as the entire ZmUbi1 promoter. The partial *pat* cassette is located downstream of the T-DNA border at the 3' end of the intact T-DNA in the opposite orientation. Sequence analysis indicated that the pre-insertion locus was preserved except for the deletion of 53 bp from the original locus. Updated bioinformatic analyses (2010) of the DNA sequences from the flanking regions in 281-24-236 cotton against public databases showed that a majority of the 3' flanking sequences plus 37 bp in the 5' flanking sequences had more than 90% homology to a cotton cDNA encoding gibberellin 20-oxidase (GA 20-oxidase, GenBank Accession AY603789). The 281-23-236 insertion occurred into the 3' untranslated region of the GA 20-oxidase gene. The EFSA GMO Panel considers that this insertion may have altered the expression of this gene. However the polyploid nature of cotton together with the fact that this gene belongs to a multigene family, is likely to compensate for a possible modification of the expression of this GA 20-oxidase. This is supported by the compositional and agronomic analysis of 281-24-236 (see section 4.2), which did not suggest any unintended effects from the genetic modification. Therefore, the EFSA GMO Panel does not consider that the insertion in event 281-24-236 raises any safety concern.

(c) Cotton 281-24-236: open reading frame (ORF) analysis

Bioinformatic analyses (2010) were performed to assess the potential for allergenicity and toxicity of putative peptides encoded by the ORFs spanning the insert-plant genomic DNA junctions. Putative peptides from all reading frames were compared to allergen, toxin, and public domain database sequences using bioinformatic tools. No biologically relevant identity to allergens, toxins, or bioactive proteins was observed for any of the putative peptides.

3.1.2.2. Cotton 281-24-236: conclusion

Cotton 281-24-236 contained one intact copy of the pAGM281 T-DNA, with the *cryIF* and *pat* genes, and in addition one partial *pat* gene. The expression of the partial *pat* was at least 16 times lower than the full-length *pat* gene at the level of RNA and was undetectable at the protein level. The absence of vector backbone sequences in event 281-24-236 was confirmed. Bioinformatic analyses of the regions flanking the insert indicated that the 281-23-236 insertion occurred into the 3'untranslated region of the GA 20-oxidase gene. However, compositional and agronomic analyses showed that event 281-24-236 is equivalent to its conventional counterpart except for the newly introduced traits (see section 4.2). No biologically relevant similarity to allergens, toxins, or bioactive proteins was observed for any of the ORFs spanning the junctions.

3.1.2.3. Cotton 3006-210-23

(a) Cotton 3006-210-23: transformation process and vector constructs

The vector pMYC3006 used to generate cotton 3006-210-23 contained the *cryIAc* gene on the T-DNA driven by the ZmUbi1 promoter and the *pat* coding sequence driven by the (4OCS) Δ Mas2' promoter. Termination of transcription of both genes is mediated by the bidirectional orf25 terminator. This *cryIAc* is a synthetic chimeric gene with the N-terminal core toxin from *cryIAc* and the C-terminal part from *cryICa* and *cryIAb* and codon optimised for plants (Gao *et al.*, 2006; Shan *et al.*, 2007). The *pat* coding sequence is identical to the one present in the pAGM281 vector.

(b) Cotton 3006-210-23: transgene constructs in the genetically modified plant

Southern analyses using multiple restriction enzyme and probe combinations covering the full-length plasmid pMYC3006 demonstrated the presence of a single intact copy of the pMYC3006 T-DNA and the absence of vector backbone sequences.

The analysis of the insert of cotton event 3006-210-23 confirmed the expected sequence of the insert. The analysis of the locus in the untransformed cotton genome showed that 16 bp from the original locus were deleted at the insertion site. Updated bioinformatic analyses (2010) on sequences flanking the insertion site of event 3006-210-23 did not indicate that known endogenous coding sequences or regulatory regions have been disrupted by the insertion

(c) Cotton 3006-210-23: open reading frame (ORF) analysis

Updated bioinformatic analyses (2010) were performed to assess the potential for allergenicity and toxicity of putative peptides encoded by the ORFs spanning the insert-plant genomic DNA junctions. Putative peptides from all reading frames were compared to allergen, toxin, and public domain database sequences using bioinformatic tools. No biologically relevant identity to allergens, toxins, or bioactive proteins was observed for any of the putative peptides.

3.1.2.4. Cotton 3006-210-23: conclusion

The cotton 3006-210-23 contains one intact copy of the pMYC3006 T-DNA, with the *cryIAc* and *pat* genes. The absence of vector backbone sequences in event 3006-210-23 was confirmed. Updated bioinformatic analyses on sequences flanking the insertion site of event 3006-210-23 did not indicate that any endogenous coding or regulatory sequences had been interrupted by the insertion. No biologically relevant similarity to allergens, toxins, or bioactive proteins was observed for any of the putative peptides spanning the junctions.

3.1.3. Evaluation of the stacked cotton 281-24-236 x 3006-210-23

3.1.3.1. Transgene constructs in cotton 281-24-236 x 3006-210-23

GM cotton 281-24-236 x 3006-210-23 was produced by conventional crossing between lines of the single cotton events 281-24-236 and 3006-210-23. No additional genetic modification has been introduced in this stacked cotton. The integrity of the individual inserts present in this cotton was confirmed using Southern analyses provided as additional information. This involved the use of DNA probes specific for the single inserts and restriction enzyme digestions informative of the structure of both events, including the junctions with the host genomic DNA. The predicted DNA hybridization patterns from each single event were retained in cotton 281-24-236 x 3006-210-23, demonstrating that integrity of the transgenic inserts was maintained.

3.1.3.2. Information on the expression of the inserts

Analyses of Cry1F, Cry1Ac and PAT protein levels was carried out by enzyme-linked immunosorbent assays (ELISA) using plants grown at six different sites in the major cotton growing regions of the USA during the 2001, 2003 and 2007 growing seasons. The trial locations provide a range of environmental conditions that would be encountered in commercial production of cotton. Protein expression levels were determined in young and terminal leaves, squares, bolls, whole plant, root, pollen, nectar, cottonseed and cottonseed processed fractions in 2001 and in cottonseed and cottonseed processed fractions in 2003 and 2007. Since the *pat* gene was used only as a selectable marker during transformation process, the EFSA GMO Panel does not require protein expression data on cotton 281-24-236 x 3006-210-23 treated with glufosinate-ammonium herbicides.

The scope of the application covers food and feed uses, import and processing, therefore protein expression data related to the cottonseed is considered most relevant, which are summarised in Table 1. Levels of proteins in the stacked cotton are comparable to levels in the single events.

Table 1. Summary of protein expression levels in cottonseed of 281-24-236 x 3006-210-23, 281-24-236 and 3006-210-23 ($\mu\text{g/g}$ dry weight)

	season	281-24-236 /3006-210-23	281-24-236	3006-210-23
Cry1F mean [range]	2001	4.13 [1.39-6.63]	5.13 [3.19-8.22]	NA
	2003	2.34 [1.5-4.15]	2.27 [0.99-3.86]	NA
	2007	3.11 [1.94-4.33]	3.06 [LOQ-5.30]	NA
Cry1Ac mean [range]	2001	0.55 [0.44-0.70]	NA	0.57 [LOQ-0.78]
	2003	0.46 [LOQ-0.89]	NA	0.43 [LOQ-0.69]
	2007	1.12 [0.68-1.97]	NA	1.13 [0.63-1.81]
PAT mean [range]	2001	0.54 [LOQ-1.31]	0.47 [LOQ-1.02]	0.06 [LOD-0.23]
	2003	0.53 [LOQ-1.02]	0.43 [LOQ-1.00]	LOQ [LOD-LOQ]
	2007	0.14 [0.09-0.21]	0.11 [LOD-0.19]	LOD [LOD-0.047]

LOD, values below detection limit; LOQ, values below lower quantification limit; NA, not applicable.

3.1.3.3. Inheritance and stability of inserted DNA

Stability of the inserts in cotton events 281-24-236 and 3006-210-23 was established by segregation analysis. The segregation ratios of the 281-24-236 and 3006-210-23 plants sprayed with glufosinate-ammonium herbicide showed the expected pattern for the dominant herbicide tolerant marker. In addition, segregation analysis was performed by crossing the hemizygous lines 281-24-236 and 3006-210-23 to produce the segregating F_1 generation. Both the F_1 and the F_2 plants, resulting from self-pollination of the F_1 , showed the expected segregation pattern using ELISA for the Cry1Ac and Cry1F proteins. In both generations the Chi square values indicated no significant difference from the expected ratios. Southern analyses were conducted on two different generations for the single and stacked events, confirming the stability of the inserts. Moreover, Southern analyses showed that the integrity of the inserts present in the single events was retained in 281-24-236 x 3006-210-23.

3.2. Conclusion

The molecular characterisation data establishes that the structure of the inserts in events 281-24-236 and 3006-210-23 is retained in cotton 281-24-236 x 3006-210-23. The phenotypic stability of the traits was shown over several generations. The levels of the newly expressed proteins in the cotton 281-24-236 x 3006-210-23 are comparable to levels in the single events. The EFSA GMO Panel concludes that the data were sufficient for the molecular characterisation and did not indicate any safety concerns.

4. Comparative analysis

4.1. Evaluation of relevant scientific data

4.1.1. Choice of comparator and production of material for the compositional assessment

In field trials, a comparison was made between the single events 281-24-236 and 3006-210-23 the stacked cotton 281-24-236 x 3006-210-23, and controls. In the first year (2001), the control was a null-segregant that was selected in the F_1 generation after stacking and further bred by four rounds of self-pollination. In the second and third years (2003, 2007), the control was cotton variety PSC355, which has a comparable genetic background to the test lines, but lacks all newly introduced genes. It is realized by the EFSA GMO Panel that because *G. hirsutum* L. is a tetraploid species, conventional counterparts derived from the same progenitor do not have a completely identical genetic background. In the opinion of the EFSA GMO Panel, variety PSC355 constitutes an appropriate conventional

counterpart, since it has been used as the recurrent parent for breeding the cotton 281-24-236 x 3006-210-23 (see section 3.1.1).

These field trials were carried out to investigate the compositional equivalence between the GM stack 281-24-236 x 3006-210-23, its respective single events cotton, and the control cotton; and to investigate the expression levels of the newly expressed Cry1F, Cry1Ac, and PAT proteins in cotton plants, cottonseed (see section 3.1.3.2) and the derived products obtained through seed processing (see section 5.1.2). The field trials were conducted at 6 locations each year within the major cotton growing regions in the USA during three growing seasons (2001, 2003 and 2007). One location was the same throughout all three seasons, and three locations were the same in the first two seasons (2001 and 2003), so that the total number of locations is equal to 13. In 2001 each location contained one replicate for the null segregant and three replicates for each of the transgenic cottons. Each location was therefore taken as a replicate in the statistical analysis of the compositional study in 2001. In 2003 and 2007, each treatment was replicated three times in every location (*i.e.* three separate plots for each treatment in a given location). In each year, all field replicates underwent conventional maintenance and agrochemical usage practices (including herbicide and insecticide treatments), but glufosinate-ammonium herbicide were not used. Since the *pat* gene was used only as a selectable marker during the transformation process, the EFSA GMO Panel does not require additional composition data on cotton 281-24-236 x 3006-210-23 treated with glufosinate-ammonium herbicide.

For compositional analysis on cottonseed and the derived products obtained through seed processing, three samples of each comprising three seed-containing bolls per plant were harvested manually or mechanically from each field. In 2001 samples from all locations of plants that had undergone the same treatment in all locations were further combined for processing, whilst, in 2003 and 2007, the samples taken from each field replicate were processed separately.

Cottonseed samples were further processed on pilot-scale into fractions consisting of kernel, hulls, meal, and oil. In 2003, expansion/extrusion of flaked kernels from cottonseed, which includes also steam injection, was omitted from the processing steps prior to oil extraction because of the smaller quantities being processed.

4.1.2. Compositional analysis

Compositional analysis was performed on delinted cottonseed, toasted meal, and refined oil obtained from cotton 281-24-236 x 3006-210-23, the single lines 281-24-236 and 3006-210-23, and the null segregant (in 2001) or their conventional counterpart (in 2003 and 2007). In addition, hulls from all locations were analyzed for their composition in 2001. By means of statistical analysis, the composition of the stacked cotton 281-24-236 x 3006-210-23 and the single lines 281-24-236 and 3006-210-23 were compared to that of the null segregant (in 2001) or their conventional counterpart (in 2003 and 2007) across all locations (in 2001, 2003 and 2007), as well as within each location (in 2003 and 2007).

Cottonseed analysis included proximates (ash, fat, moisture, protein, fibre, calculated carbohydrates and energy content), minerals, amino acids, fatty acids, and anti-nutrients/toxins (cyclopropenoid fatty acids [sterculic, malvalic and dihydrosterculic] and gossypol [total gossypol in all three years, free gossypol in 2003 and 2007]). In 2007 cottonseed was also analyzed for vitamins, including beta-carotene (pro-vitamin A), thiamin (vitamin B1), riboflavin (B2), niacin (B3), pyridoxine (B6), ascorbic acid (C), and folate, as well as for anti-oxidant tocopherols (alpha-, beta-, gamma-, and delta-tocopherol in 2003 and 2007; total tocopherols in 2003). Hulls were analyzed for proximates and minerals. Toasted meal was analyzed for proximates, minerals, amino acids, and gossypol (both free and total gossypol in all three years). Analysis of refined oil included proximates (fat, moisture, and protein), fatty acids, antioxidant (tocopherols [alpha, beta, gamma, and delta in all three years; total tocopherols in 2003]), cyclopropenoid acids, and gossypol (total gossypol in all three years, and free gossypol in 2001 and 2003). The measured compositional parameters are in line with recommendations laid down in the consensus document on key compositional parameters of cotton

varieties as published by the OECD Task Force on the Safety of Novel Foods and Feed (OECD, 2004).

Besides the compositional data mentioned above, supplementary data on the levels of anti-nutrients (total polyphenols and total gossypol) in terminal leaves and squares that were obtained from two locations in 2001 have also been provided. Given the limited number of data and the fact that these tissues are not representative of the cotton products within the scope of the application, these data are not further described in detail here.

4.1.2.1. Cotton 281-24-236

In 2001 cottonseed of the Cry1F-expressing cotton 281-24-236 had statistically significantly decreased levels of calcium and malvalic acid, as well as increased levels of manganese, stearic acid, palmitic acid, palmitoleic acid, oleic acid, linolenic acid, and arachidic acid, as compared to the null segregant. In 2003 cottonseed derived from cotton 281-24-236 had statistically significantly lower levels of palmitoleic acid, free gossypol, and total gossypol, as well as higher levels of vitamin C, whilst, in 2007 cottonseed of 281-24-236 showed statistically significantly lower levels of palmitoleic acid, free and total gossypol, as well as higher levels of moisture, copper, sodium, stearic acid, oleic acid, arachidic acid, and dihydrosterculic acid.

In 2003 toasted meal derived from cotton 281-24-236 contained statistically significantly decreased levels of iron, as well as increased levels of copper, alanine, isoleucine, leucine, lysine, serine, threonine, and tryptophan. In 2007 toasted meal derived from cotton 281-24-236 contained a statistically significantly lower level of total gossypol than its conventional counterpart.

In refined oil derived from 281-24-236 cottonseed grown in 2003, statistically significant decreases were observed in the levels of palmitoleic acid, gamma-tocopherol, and total tocopherol, as compared to its conventional counterpart. In 2007 the refined oil derived from cottonseed of 281-24-236 also showed several statistically significant differences, including lower levels of palmitic acid, palmitoleic acid, and gamma-tocopherol, as well as higher levels of arachidic acid and dihydrosterculic acid.

None of the statistically significant compositional differences observed for cotton 281-24-236 were consistently observed at each location or in each year. The measured values fall within the background range values (OECD, 2004), except for the levels of five fatty acids, *i.e.* palmitic acid, palmitoleic acid, oleic acid, stearic acid, and arachidic acid, in cottonseed of 281-24-236 and the null segregant in 2001 falling below the background ranges. Values outside the background ranges were also observed for three amino acids in meal derived from cotton 281-24-236 and its conventional counterpart in 2003, *i.e.* threonine falling below the range, and leucine and lysine above it, as well as for serine in meal from cotton 281-24-236 exceeding the background range. Also palmitoleic acid in seeds of cotton 281-24-236 was slightly below the background range in 2003. In addition, for vitamin C in seeds and for total tocopherol and dihydrosterculic acid in refined oil, no data on background ranges were available.

4.1.2.2. Cotton 3006-210-23

In cottonseed of the Cry1Ac-expressing cotton 3006-210-23 grown in 2001, the levels of crude fibre, sterculic acid, and malvalic acid were decreased, whilst those of fat, palmitic acid, stearic acid, and linolenic acid were increased as compared to the null segregant. Statistically significantly decreased levels of calcium, sulfur, palmitoleic acid, behenic acid, and total gossypol, as well as increased levels of fat, calculated energy content, alanine, and isoleucine, were observed in cottonseed of 3006-210-23 as compared to its conventional counterpart, when both were harvested in 2003. In 2007 cottonseed of 3006-210-23 contained statistically significantly lower levels of acid detergent fibre, calcium, myristic acid, palmitoleic acid, free and total gossypol, and higher levels of ash, fat, stearic acid, arachidic acid, vitamin B6 (pyridoxine), and dihydrosterculic acid.

In toasted meal derived from 3006-210-23 cottonseed grown in 2003, neutral detergent fibre and various minerals, *i.e.* calcium, iron, manganese, sulfur, and zinc, were present at statistically significantly lower levels, whilst potassium was present at higher levels, than in toasted meal derived from its conventional counterpart. In 2007, toasted meal obtained from 3006-210-23 cottonseed contained statistically significantly lower levels of crude fibre, carbohydrates, acid detergent fibre, and neutral detergent fibre, as well as higher levels of protein, than its conventional counterpart. Various amino acids in meal from cotton 3006-210-23 also showed statistically significantly elevated levels as compared to its conventional counterpart, namely alanine, aspartic acid, cysteine, glutamic acid, glycine, histidine, leucine, methionine, phenylalanine, proline, serine, threonine, and tryptophan. In the view of the EFSA GMO Panel, the higher values of specific amino acids in 2007 probably relate to the higher protein content of meal derived from cotton 3006-210-23 this year.

In refined oil derived from 3006-210-23 cottonseed grown in 2003, lower levels of palmitoleic acid, gamma-tocopherol, and total tocopherol were observed as compared to refined oil from its conventional counterpart. In 2007 the composition of refined oil derived from 3006-210-23 cottonseed showed various statistically significant differences in the comparison to its conventional counterpart, namely lower levels of myristic acid, palmitoleic acid, and gamma-tocopherol, as well as higher levels of stearic acid, arachidic acid, and dihydrosterculic acid.

None of these statistically significant differences in the seeds, meal, and oil derived from cotton 3006-210-23 were observed in each location and each year. Whilst most of the values showing differences fell within the background ranges, the levels of palmitic acid, stearic acid, and sterculic acid in seeds of both the cotton 3006-210-23 and the null segregant in 2001 were below the range of background values, and, in 2003 the average level of palmitoleic acid in cottonseed of 3006-210-23 was also slightly below the background range. In 2007 the values for leucine in meal from both the transgenic cotton and its conventional counterpart were slightly above the background range of literature values, whilst for two of the other amino acids showing differences (phenylalanine, proline); the values for meal derived from cotton 3006-210-23 were slightly above this range. For one compound showing a difference in 2003, *i.e.* total tocopherol in oil, and two compounds showing differences in 2007, *i.e.* vitamin B6 (pyridoxine) in seeds and dihydrosterculic acid in oil, no background ranges of literature values were available. In addition, the statistically significantly lower level of palmitoleic acid and higher level of stearic acid in 3006-210-23 cottonseed as compared to its conventional counterpart were consistently observed in all locations in 2007, as was the statistically significantly lower level of palmitoleic acid in the refined oil derived from 3006-210-23 cottonseed.

4.1.2.3. Cotton 281-24-236 x 3006-210-23

A number of statistically significant differences in the composition of 281-24-236 x 3006-210-23 cottonseed as compared with the null segregant or its conventional counterpart were observed. In 2001, for example, 281-24-236 x 3006-210-23 cottonseed contained statistically significantly lower levels of crude fibre, sterculic acid, and malvalic acid, as well as higher levels of stearic acid. In 2003 cottonseed of 281-24-236 x 3006-210-23 contained statistically significantly lower levels of sulfur, behenic acid, and total gossypol, as well as higher levels of alanine and tryptophan. The composition of 281-24-236 x 3006-210-23 cottonseed in 2007 showed statistically significantly lower levels of calcium, manganese, phosphorus, linoleic acid, vitamin B1 (thiamin), free and total gossypol, as well as higher levels of stearic acid, oleic acid, arachidic acid, behenic acid, and dihydrosterculic acid.

Toasted meal obtained from cotton 281-24-236 x 3006-210-23 in the year 2003 contained statistically significantly higher levels of calculated energy content, aspartic acid, alanine, proline, and lysine, and lower levels of ash, fibre (ADF, NDF), carbohydrates, calcium, iron, manganese, sulfur, and zinc. In 2007 the proximate analysis of cottonseed meal derived from cotton 281-24-236 x 3006-210-23 showed statistically significantly lower levels of total gossypol, as well as higher levels of moisture and protein.

Refined oil derived from cotton 281-24-236 x 3006-210-23 contained statistically significantly higher levels of stearic acid, linolenic acid, arachidic acid, and behenic acid, and lower levels of palmitoleic acid, gamma-tocopherol, in 2003. In 2007 the refined oil derived from cottonseed of 281-24-236 x 3006-210-23 showed statistically significantly lower levels of palmitoleic acid, linoleic acid, and gamma-tocopherol, as well as higher levels of stearic acid, oleic acid, arachidic acid, behenic acid, and dihydrosterculic acid.

Most of these differences occurred neither at each location nor in each year, except for the higher levels of stearic acid and oleic acid in cottonseed, and for the higher levels of oleic acid and arachidic acid in refined oil of cotton 281-24-236 x 3006-210-23, which were consistently observed within each location in one year (2007). In addition, these differences fell within the range of background values for non-GM cottonseed reported in literature, except for stearic acid and sterculic acid in cottonseed of 281-24-236 x 3006-210-23 and its conventional counterpart, which fell below the background range; for aspartic acid, proline, and lysine in toasted meal of cotton 281-24-236 x 3006-210-23 and its conventional counterpart in 2003, which exceeded the background range; as well as for moisture in toasted meal of cotton 281-24-236 x 3006-210-23 and its conventional counterpart in 2007, both of which fell below the background range. For thiamin in cottonseed and for linolenic acid and dihydrosterculic acid in oil, no background ranges were available.

Having considered data available on cottonseed and derived products obtained through seed processing (kernel, toasted meal and refined oil) of the GM cottons (281-24-236 x 3006-210-23, 281-24-236, and 3006-210-23), the null segregant and the conventional counterpart, the EFSA GMO Panel concludes that these GM cottons are compositionally equivalent to those from their conventional counterpart, except for the presence of the Cry1Ac, Cry1F and PAT proteins.

4.1.3. Agronomic traits and GM phenotype

Agronomic and phenotypic characteristics of cotton 281-24-236 x 3006-210-23, its respective single events, and their conventional counterpart (PSC355), were studied during field trials in the USA in 2002. Measurements of agronomic characteristics included field emergence, progeny seed germination, growth habit, vegetative vigor, flowering period, reproductive potential, and fibre quality. Whereas a number of statistically significant differences were observed between the GM stacked and single events on one hand and its conventional counterpart on the other, the EFSA GMO Panel considers these differences to be of minor magnitude and typical of variability among cotton lines.

For testing breeding performance, 14 lines of cotton 281-24-236 x 3006-210-23 were compared with their conventional counterpart (PSC355), and analyzed for linter and cottonseed characteristics, including yield and physical parameters. Of these parameters, micronaire (a measure of fibre quality) was different between all GM cotton lines and their conventional counterpart. The EFSA GMO Panel considers that this difference does not raise safety concerns.

The EFSA GMO Panel concludes that the agronomic performance and phenotypic characteristics of cotton 281-24-236 x 3006-210-23 and its single events 281-24-236 and 3006-210-23 are equivalent to their conventional counterpart except for the introduced traits.

4.2. Conclusion

The comparative assessment was mainly based on the compositional analysis of delinted cottonseed, toasted meal, and refined oil derived from cotton 281-24-236 x 3006-210-23, its respective single events and non-GM controls (a conventional counterpart and a null segregant) during three growing seasons. A number of statistically significant differences were observed in derived products obtained through seed processing of cotton 281-24-236 x 3006-210-23 and its single events when compared to their conventional counterpart. The EFSA GMO Panel did not consider these differences being

biologically relevant, because they were inconsistent (*i.e.* not in each year and/or location), and mostly within the background ranges. As a result of these analyses, the EFSA GMO Panel concludes that the compositional, agronomic and phenotypic characteristics of the GM cotton 281-24-236 x 3006-210-23 and the single cotton events 281-24-236 and 3006-210-23 are equivalent to those of their conventional counterpart, except for the newly expressed proteins (Cry1Ac, Cry1F and PAT). Based on the assessment of data available, the EFSA GMO Panel has found no indication that crossing of single cotton events 281-24-236 and 3006-23-310 to produce cotton 281-24-236 x 3006-23-310 would result in interactions which causes compositional, agronomic or phenotypic changes.

5. Food/Feed safety assessment

5.1. Evaluation of relevant scientific data

5.1.1. Product description and intended use

The scope of application EFSA-GMO-NL-2005-16 includes food and feed uses, import and processing of cotton 281-24-236 x 3006-210-23 and its derived products. Thus, the possible uses of cotton 281-24-236 x 3006-210-23 include the production of refined oil from seeds, production of cellulose from linters as food or food ingredient, and use of cottonseed meal, and hulls in animal feed. The genetic modification of cotton 281-24-236 x 3006-210-23 is intended to improve agronomic performance only, and is not intended to influence the nutritional properties, processing characteristics and overall use of cotton as a crop.

5.1.2. Effect of processing

The newly expressed proteins (Cry1Ac and Cry1F) were detectable in cottonseed, kernels and hulls but non-detectable in refined oil of the GM cotton 281-24-236 x 3006-210-23 in all three growing seasons of field trials in the USA; they were present in toasted meal at very low levels. PAT was present in cottonseed and kernels; non-detectable in refined oil; and either present at low levels or non-detectable in hulls and toasted meal, in all three years. Considering the toxicological profile and allergenic properties (see sections 5.1.3 and 5.1.4) the presence of Cry1Ac, Cry1F and PAT protein in derived products obtained through seed processing would not raise safety concern.

Since cotton 281-24-236 x 3006-210-23 is compositionally equivalent to its conventional counterpart except for the newly expressed proteins (see section 4.1.2), the effect of processing cotton 281-24-236 x 3006-210-23 is not expected to be different from that of conventional cotton.

5.1.3. Toxicology

5.1.3.1. Cry1Ac, Cry1F and PAT proteins used for safety assessment

The Cry1Ac, Cry1F and PAT proteins that were used in safety studies were produced in bacteria. Given that expression levels of these proteins in plants are low and that it is difficult to isolate in sufficient quantity purified proteins from the GM cotton, the EFSA GMO Panel considers it acceptable that bacterially produced recombinant proteins are used as substitutes, provided that equivalence between the proteins produced by bacteria and plants are demonstrated.

The biochemical and functional properties of the Cry proteins purified from *Pseudomonas fluorescens* (Cry1F and Cry1Ac), from the cottonseed (Cry1F) and leaf (Cry1F and Cry1Ac) of 281-24-236 x 3006-210-23 and from the leaf of both single events (Cry1F and Cry1Ac) were compared by various means. For example, the full-length proteins from both sources were compared for their activity in insect bioassays. In addition, the trypsinized core proteins were analyzed by Western analysis, N-terminal sequencing, glycosylation assay, and MALDI-TOF profiling of peptide masses after trypsin

digestion. Furthermore, the identity of several selected peptides was further confirmed by tandem mass spectrometry. In the opinion of the EFSA GMO Panel, each of the plant produced Cry1Ac and Cry1F proteins showed similar results to their counterpart from bacteria. Therefore, the bacterial recombinant proteins Cry1F and Cry1Ac are considered to be equivalent to the native plant proteins.

The equivalence of the recombinant PAT protein produced by *Escherichia coli* was studied using Western analysis and MALDI TOF peptide mass fingerprinting. The results show that, except for slightly higher molecular weight of the microbial produced protein due to an N-terminal poly-histidine extension, the pattern of peptides from the microbial produced PAT protein was as expected for the native plant protein.

5.1.3.2. Toxicological assessment of expressed novel proteins in cotton 281-24-236 x 3006-210-23

Both Cry1F and Cry1Ac that are newly expressed in cotton 281-24-236 x 3006-210-23 are full-length delta endotoxins with known highly specific insecticidal properties, to which humans and other mammalian species are considered to be unsusceptible because of the absence of delta endotoxin receptors in mammalian species. The third protein that is newly expressed in cotton 281-24-236 x 3006-210-23, the PAT protein, has been assessed by the EFSA GMO Panel and was then considered to be safe for human and animal consumption (EFSA, 2006c). This conclusion was based, among others, on a 14-day repeated-dose oral toxicity study with *pat*-derived PAT in rats. Furthermore, the safety of the PAT proteins derived from the *pat* genes in human food or animal feed has been reviewed (Herouet *et al.*, 2005). In the following sections, the data on the safety of the newly expressed proteins provided in the application on cotton 281-24-236 x 3006-210-23 are considered.

(a) Acute toxicity testing

A mixture of Cry1F and Cry1Ac proteins given to mice by gavages in amounts of 375 and 350 mg/kg body weight respectively did not induce relevant signs of toxicity, neither did PAT administered at a single dose of 5000 mg/kg.

(b) Degradation in simulated digestive fluids

Both Cry1Ac and Cry1F produced by *P. fluorescens* were tested for *in vitro* digestibility in simulated gastric fluid containing pepsin at a pepsin:protein ratio of 6.3:1 (w/w). In addition, reference proteins were included, *i.e.* bovine serum albumin as an example of a degradable protein and beta-lactoglobulin as that of a stable protein. The integrity of proteins was analyzed by SDS-PAGE and Western analysis. It was thus observed that both Cry1Ac and Cry1F were rapidly digested, *i.e.* within one minute. As regards the digestibility of the PAT protein, reference (Mendelsohn *et al.*, 2003) is made to previous evaluations in which it was considered that this protein is also rapidly degraded, *i.e.* within seconds, in simulated gastric fluid.

(c) Susceptibility to processing conditions

Cry1F and Cry1Ac lost their insecticidal activity in a bioassay after being heated at 75 and 90°C at pH 7.5 for 30 minutes. On electrophoresis gels, protein bands were still observed in the heated solutions that corresponded to the intact forms of the Cry proteins. When lyophilized preparations of these Cry proteins were heated at 121°C for 30 minutes, bands corresponding to their higher molecular weight forms disappeared from the electrophoresis gels.

In addition, the newly expressed proteins Cry1Ac, Cry1F, and PAT were not detected in refined cottonseed oil or in toasted cottonseed meal.

(d) Bioinformatic studies

An updated bioinformatic analysis (2010) for similarity of the transgenic proteins with amino acid sequences in general protein databases was performed. For Cry1Ac and Cry1F no significant sequence similarity to any known proteins that are harmful to humans or animals was found. However, they resembled related insecticidal Cry proteins. Bioinformatic analyses of the PAT protein and the partial PAT did not identify any significant sequence similarity with known toxic proteins.

5.1.3.3. Toxicological assessment of new constituents other than proteins

No new constituent other than the newly expressed proteins (Cry1Ac, Cry1F and PAT) have been identified in cotton 281-24-236 x 3006-210-23, therefore, relevant changes in the composition of cotton 281-24-236 x 3006-210-23 are unlikely.

5.1.3.4. Toxicological assessment of the whole GM food/feed

On the basis of the comparative analysis, the EFSA GMO Panel concluded that cotton 281-24-236 x 3006-210-23 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart except for the introduced traits. The applicant also submitted an argumentation for the low likelihood of possible interactions between the newly expressed proteins (Cry1Ac, Cry1F and PAT) in cotton 281-24-236 x 3006-210-23, including different modes of action, the absence of reported adverse health effects, and their low expression levels.

The EFSA GMO Panel has considered the outcomes of a 90-day rat feeding study. The animals received diets containing 10% cottonseed meal of the GM cotton 281-24-236 x 3006-210-23, conventional counterpart (PSC355) and three commercial non-GM cotton varieties, no relevant effects were observed. However, according to the EFSA GMO Panel's Guidance Document (EFSA, 2006a), animal safety studies with the whole food/feed are not necessary for this application.

The EFSA GMO Panel considered all the data available for the GM cotton 281-24-236 x 3006-210-23 and the newly expressed proteins (Cry1Ac, Cry1F and PAT), and is of the opinion that interactions between single events that might impact the food and feed safety of cotton 281-24-236 x 3006-210-23 are unlikely.

5.1.4. Allergenicity

The strategies used when assessing the potential allergenic risk focus on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons and whether the transformation may have altered the allergenic properties of the modified food. A weight-of-evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (CAC, 2003; EFSA, 2006a).

5.1.4.1. Assessment of allergenicity of the newly expressed proteins

Cottonseed oil and processed cotton linters are the primary cotton products used for human food. Analysis of cotton products derived from cotton 281-24-236 x 3006-210-23 confirmed that there is no detectable level of Cry1Ac, Cry1F and PAT proteins in both refined cottonseed oil and processed cotton linters. Thus, no significant human consumption of Cry1Ac, Cry1F and PAT proteins from cottonseed-oil-containing foods and food ingredients is expected. As described in section 5.1.3.2b, these proteins are rapidly degraded under simulated gastric conditions. In addition, the Cry1Ac and Cry1F proteins as part of their native bacterial hosts, *i.e.* *P. fluorescens* species, have no history of allergenicity.

For the assessment of allergenicity of the newly expressed proteins, their amino acid sequence was compared with an allergen database containing known and putative allergens as well as celiac-disease

inducing proteins residing in the FARRP dataset (2010). Potential identities between the newly expressed proteins and proteins in the allergen database were evaluated with the FASTA program. A greater-than-35%-identity threshold over any 80-or-more-amino-acid sequences between a query sequence and an allergen was used to indicate the potential for cross-reactivity. The protein sequences were also screened for any matches of 8 contiguous amino acids to the allergens in the FARRP dataset. Sequences of the transgenic proteins Cry1Ac and Cry1F showed no similarities of at least 8 identical contiguous amino acids or 35% in an 80-amino-acid window to known allergenic proteins.

In studies reported in literature, IgG, IgM and mucosal IgA response were induced, but no IgE response was observed after intraperitoneal or intragastric administration of Cry1Ac to mice at relatively high dosage (Vazquez-Padron *et al.*, 1999a; 2000), which demonstrates that Cry1Ac has no or low allergenic potential. Moreover these Cry1Ac and Cry1F proteins are not glycosylated.

Another Cry protein, Cry1Ab has been shown to act as an adjuvant, *e.g.* it enhances the mucosal and/or the systemic antibody response to a protein which is co-administered with the Cry protein (Vazquez *et al.*, 1999b; Moreno-Fierros *et al.*, 2003). However the EFSA GMO Panel is of the opinion that the adjuvant effect of Cry proteins, observed after high dosage intragastric or intranasal administration will not raise any concerns regarding allergenicity caused by cottonseed.

The potential allergenicity of the PAT protein has been assessed in previous applications (EFSA, 2006c) and considered to be of low potential or absent, among others on the basis of its fast degradability. In addition it was shown that the amino acid sequence of the PAT and the partial PAT protein do not share any significant similarity with known protein allergens.

Based on the available information, the EFSA GMO Panel considers it unlikely that the newly expressed proteins (Cry1Ac, Cry1F and PAT) in cotton 281-24-236 x 3006-210-23 are allergenic.

5.1.4.2. Assessment of allergenicity of the whole GM plant

Allergenicity of the whole crop could be increased as an unintended effect of the random insertion of the newly introduced genes in the genome of the recipient, for example through qualitative or quantitative modifications of the pattern of expression of endogenous proteins. This issue does not appear relevant to the EFSA GMO Panel since cotton is not considered to be a common allergenic food, and only rare cases of occupational allergy have been reported *e.g.*, (Atkins *et al.*, 1988; Malanin and Kalimo, 1988).

Furthermore, the main cottonseed product in human food, cottonseed oil, is highly purified and contains negligible levels of proteins, if any. Edible oils that are refined, bleached and deodorised do not appear to pose a risk to allergic individuals, as they contain virtually no proteins.

Based on the available information, the EFSA GMO Panel concludes that it is unlikely that the overall allergenicity of the whole GM cotton 281-24-236 x 3006-210-23 has been changed.

5.1.5. Nutritional assessment of GM food/feed

Comparative analysis showed the composition of cottonseed derived from the GM cotton 281-24-236 x 3006-210-23 to be equivalent to that derived from the conventional counterpart, except for the newly expressed proteins Cry1Ac, Cry1F and PAT. Therefore, in case of consumption of GM cottonseed 281-24-236 x 3006-210-23, the EFSA GMO Panel considers that nutritional properties are likely to be the same as for other cottonseed of similar gossypol content (EFSA, 2008). Apart from these considerations, a feeding study with GM 281-24-236 x 3006-210-23 cottonseed meal in broilers has been provided. Given its rapid growth, the broiler is considered as a suitable model to detect nutritional imbalances.

A total of 480 Cob x Cob broiler chickens (50% of each gender) were used in a 42-day study to evaluate the nutritional quality of cottonseed meal derived from the cotton 281-24-236 x 3006-210-23, its conventional counterpart (PSC355) and two commercial non-GM cotton varieties. There were 4 treatments, each with 12 replicates and 10 birds/replicate. The cottonseed meal formed 10% of the diet.

The analysis of the 42-day study showed no statistically significant differences when comparing the final bird weight, mortality rate and average weight gain of broilers fed with the GM cotton 281-24-236 x 3006-210-23, with its conventional counterpart and with each of the two commercial non-GM varieties. Whilst statistically significantly lower overall feed efficiency recorded for one of the commercial non-GM varieties when compared with GM cotton most likely was due to a higher initial start weight, there were no significant differences in feed efficiency when comparing the GM cotton with its conventional counterpart and the other commercial non-GM variety.

The EFSA GMO panel concludes that the data provided supports the view that cottonseed meal derived from the GM cotton 281-24-236 x 3006-210-23 is nutritionally comparable with that derived from its conventional counterpart.

5.1.6. Post-market monitoring of GM food/feed

An evaluation of the risk assessment concluded that no data have emerged to indicate that cotton 281-24-236 x 3006-210-23 is any less safe than its conventional counterpart. In addition, cotton 281-24-236 x 3006-210-23 is, from a nutritional point of view, substantially equivalent to commercial non-GM cotton. Therefore, and in line with the Guidance Document (EFSA, 2006a), the EFSA GMO Panel is of the opinion that post-market monitoring of the GM food/feed derived from cotton 281-24-236 x 3006-210-23 is not necessary.

5.2. Conclusion

Evidence has been provided that there is no safety concern regarding the newly expressed proteins in cotton 281-24-236 x 3006-210-23. The PAT protein has been previously examined in other applications and has been considered not to represent any health implications. The safety of the Cry1Ac and Cry1F proteins is supported by bioinformatic analysis and investigations on stability, digestibility and toxicity. The potential allergenicity of the expressed Cry1Ac, Cry1F, and PAT proteins has been assessed, and it was found unlikely that they are allergenic. As neither the molecular characterisation nor the compositional analysis of the GM-cotton showed any unintended effects, an alteration in allergenic properties of the GM-cottonseed appears to be very unlikely. In addition, a broiler study confirmed the nutritional equivalence of cottonseed meal of GM cotton 281-24-236 x 3006-210-23 to meal of non-GM cottonseed of similar gossypol content.

Based on the mode of action of the Cry and PAT proteins and given all the information provided, the EFSA GMO Panel concludes that interactions between the newly expressed proteins that might impact on food and feed safety of cotton 281-24-236 x 3006-210-23 are unlikely, and that the nutritional properties of cotton 281-24-236 x 3006-210-23 would not be different from those of the conventional counterpart.

In conclusion, the EFSA GMO Panel considers that cotton 281-24-236 x 3006-210-23 assessed in this application is as safe and nutritious as its conventional counterpart, and that it is unlikely that the overall allergenicity of the whole plant is changed. The EFSA GMO Panel concludes that cotton 281-24-236 x 3006-210-23 and its derived products obtained through seed processing are unlikely to have any additional adverse effects compared to conventional cotton on human and animal health in the context of its intended uses.

6. Environmental risk assessment and monitoring plan

6.1. Evaluation of relevant scientific data

The scope of application EFSA-GMO-NL-2005-16 includes food and feed uses, import and processing of cotton (*G. hirsutum* L.) 281-24-236 x 3006-210-23 and does not include cultivation. Considering the proposed uses of cotton 281-24-236 x 3006-210-23, the environmental risk assessment is concerned with the indirect exposure through manure and faeces mainly from animals fed with cotton products of 281-24-236 x 3006-210-23 and with the accidental release into the environment of cotton 281-24-236 x 3006-210-23 grains during transportation and processing.

As the scope of the present application excludes cultivation, environmental concerns related the use of glufosinate-ammonium herbicides on cotton 281-24-236 x 3006-210-23 would apply only to the use of these herbicides in the countries of origin. It is unlikely that glufosinate-ammonium herbicides would be used on this GM cotton, as the expression level of pat is low and the use of these herbicides may cause damage to the crop itself (EPA, 2004).

6.1.1. Environmental risk assessment

6.1.1.1. Unintended effects on plant fitness due to the genetic modification

Gossypium hirsutum is highly domesticated crop which has been grown in Southern Europe since the 19th century, giving rise to feral plants which can occasionally be found in the same area (Davies, 1967; Todaro, 1917). The main cultivated cotton (*G. hirsutum*) is an annual self-pollinating crop. In the absence of insect pollinators (such as wild bees, honeybees, bumblebees), cotton flowers are self-pollinating, but when these pollinators are present low percentages of cross-pollination occur (McGregor, 1959; Moffett and Stith, 1972; Moffett *et al.*, 1975; Van Deynze *et al.*, 2005).

Pollen and cottonseed dispersal are potential sources of vertical gene flow to cross-compatible wild cotton relatives, other cotton varieties, and to occasional feral cotton plants. However, in Europe, there are no cross-compatible wild relatives with which cotton can hybridise. Because cotton pollen is very large (120-200 micrometers), heavy and sticky, wind-mediated dispersal of pollen to other cotton varieties is negligible (Vaissiere and Vinson, 1994). In addition, cross-pollination percentages rapidly decrease with increasing distance from the pollen source (Hofs *et al.*, 2007; Kareiva *et al.*, 1994; Llewellyn and Fitt, 1996; Llewellyn *et al.*, 2007; Umbeck *et al.*, 1991; Van Deynze *et al.*, 2005; Xanthopoulos and Kechagia, 2000; Zhang *et al.*, 2005).

Seeds are the only survival structures. However seed-mediated establishment of cotton and its survival outside of cultivation in Europe is mainly limited by a combination of absence of a dormancy phase, low competitiveness, and susceptibility to diseases and cold climate conditions (Eastick and Hearnden, 2006). In regions where cotton is widely grown, such as Australia, the risk of GM cotton becoming feral along transportation routes, or a weed on dairy farms where raw cottonseed is used as feed has been shown to be negligible (Addison *et al.*, 2007). Adequate soil moisture is an additional factor affecting the survival of feral cotton seedlings. Since general characteristics of cotton 281-24-236 x 3006-210-23 are unchanged relative to its conventional counterpart, the inserted herbicide tolerance trait is not likely to provide a selective advantage outside of cultivation in Europe. If accidental release and subsequent release into the environment of cotton 281-24-236 x 3006-210-23 seeds occur, cotton 281-24-236 x 3006-210-23 plants would have a selective advantage only under infestation of target pest species or in the presence of glufosinate-ammonium herbicides which are not commonly used on cultivated cotton or in most areas where the GM cotton might be spilled. The resistance to certain target pests may also confer some increase in survival and fitness of plants under conditions of high infestation, but plant survival is also limited by sensitivity to a range of other environmental factors. It is thus considered very unlikely that cotton 281-24-236 x 3006-210-23, or its progeny, will differ from

other cotton varieties in their ability to survive until subsequent seasons or to establish feral populations under European environmental conditions.

In addition to the data presented by the applicant, the EFSA GMO Panel is not aware of any scientific report of increased fecundity, persistence (volunteerism) or ferality of GM cotton in regions where it is cultivated (Bagavathiannan and Van Acker, 2008; Eastick and Hearnden, 2006). There is no information to indicate change in survival capacity (including over-wintering). Experimental data provided by the applicant showed that seed germination of cotton 281-24-236 x 3006-210-23 was in some cases significantly lower than its conventional counterpart. However, average values from the test material were always within the ranges observed from conventional cotton produced at each respective location. Furthermore, there is no evidence that this herbicide tolerance trait introduced by the genetic modification results in increased persistence and invasiveness of any crop species, except in the presence of glufosinate-ammonium herbicides. Thus escaped plants and genes dispersed to other cotton plants would result in plant populations no different from existing populations and would not create additional agronomic or environmental impacts.

The EFSA GMO Panel is thus of the opinion that, even in case of accidental release into the environment, cotton 281-24-236 x 3006-210-23 is very unlikely to show any enhanced fitness and would behave as conventional cotton.

6.1.1.2. Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA, or vertical gene flow via cottonseed dispersal and cross-pollination.

(a) Plant to bacteria gene transfer

Genomic DNA is a component of many plant products and it is documented that DNA present in food and feed becomes substantially degraded in the process of digestion in the human or animal gastrointestinal tract. However, a low level of exposure of fragments of ingested DNA, including the recombinant fraction of such DNA, to microorganism in the digestive tract of humans, domesticated animals, and other animals feeding on the plant is expected (see section 5.1.3).

Current scientific knowledge indicates that horizontal gene transfer of non-mobile DNA fragments between unrelated organisms (such as plants to microorganisms) is extremely unlikely to occur under natural conditions (see EFSA, 2009 for further details). The concentration of DNA in the gastrointestinal tract is relatively low and most bacteria lack competence to take up and recombine foreign DNA.

Cotton 281-24-236 x 3006-210-23 contains the *cryIAc*, *cryIF* and *pat* genes originating from bacteria. Thus, in theory, the *cryIAc*, *cryIF* and *pat* genes of the recombinant DNA insert could provide sufficient DNA similarity for homologous recombination to take place in environmental bacteria. However, as discussed further below, such hypothesized horizontal gene transfer event is not likely to be maintained in bacterial population due to a predicted lack of efficient expression and no identified selective advantage of gene transfer recipients.

In case of illegitimate recombination into genomes of environmental bacteria, it is unlikely that the *cryIAc* gene regulated by eukaryotic plant promoter in cotton 281-24-236 x 3006-210-23 would be expressed. The *cryIF* and *pat* genes are regulated by *Agrobacterium* promoters. The activity of these promoters in bacteria may be possible. However, no selective advantage of a hypothesized bacterial uptake all of the above mentioned genes is anticipated, because *cry* and *pat* genes are distributed in various bacterial species in the natural environment. Thus, the hypothesized low level exposure of bacterial communities to the cotton 281-24-236 x 3006-210-23 *cryIAc*, *cryIF* and *pat* genes must be

seen in the context of the natural occurrence and level of exposure to alternative sources of genetically diverse *cry* and *pat* genes to which bacterial communities are exposed.

The wide environmental presence of genetically diverse natural variants of the recombinant DNA coding sequences, the use of regulatory sequences optimised for expression in eukaryotes, and the absence of an identified plausible selective advantage, suggest it is highly unlikely that the recombinant DNA will transfer and establish in the genome of bacteria in the environment or human and animal digestive tract.

(b) Plant to plant gene transfer

Considering the intended uses of cotton 281-24-236 x 3006-210-23 and the physical characteristics of cottonseed, a possible pathway of dispersal is from cottonseed spillage and pollen of occasional feral GM cotton plants originating from accidental cottonseed spillage during transportation and/or processing.

The genus *Gossypium* consists of at least four crop species: *Gossypium arboreum*, *Gossypium barbadense*, *Gossypium herbaceum* and *Gossypium hirsutum*. *G. herbaceum* is reported (Zohary and Hopf, 2000) to be a traditional fibre crop in the Eastern Mediterranean area already in the pre-Columbus period (before 1500 AD). In Southern Europe *G. herbaceum* and *G. hirsutum* have been grown since the 19th century giving rise to occasional feral plants in the same area (Davies, 1967; Todaro, 1917; Tutin *et al.*, 1992; Zangheri, 1976) but no sexually compatible wild relatives of *G. hirsutum* have been reported in Europe. Therefore, the plant to plant gene transfer from this GM cotton is restricted to cultivated and occasional feral populations. The EFSA GMO Panel also takes into account the fact that this application does not include cultivation of the GM cotton within the EU so that the likelihood of cross-pollination between the imported GM cotton and cotton crops and occasional feral cotton plants is considered to be extremely low. Even in case feral populations of cotton 281-24-236 x 3006-210-23 were established or transgene flow occurred to cultivated and feral cotton, a selective advantage would occur only under infestation of target pest species or in the presence of glufosinate-ammonium herbicides, which are not commonly used on cultivated cotton or in most areas where the GM cotton might be spilled.

6.1.1.3. Interactions of the GM plant with target organisms

The intended uses of cotton 281-24-236 x 3006-210-23 specifically exclude cultivation and the environmental exposure to cotton 281-24-236 x 3006-210-23 is limited to the accidental release of grains into environment during transportation and processing. The EFSA GMO Panel considers that it would need successful establishment and spread of high numbers of cotton 281-24-236 x 3006-210-23 to enable any significant interaction with target organisms, which is very unlikely.

6.1.1.4. Interactions of the GM plant with non-target organisms

Due to the intended uses of cotton 281-24-236 x 3006-210-23 which exclude cultivation and due to the low level of exposure to the environment, potential interactions of the GM cotton with non-target organisms were not considered an issue by the EFSA GMO Panel.

However the EFSA GMO Panel evaluated whether Cry proteins might potentially affect non-target organisms by entering the environment through manure and faeces from animals fed this GM cotton. Both Cry proteins are degraded by enzymatic activity in the gastrointestinal tract (see section 5.1.3.2b), meaning that only a very low amount of these proteins would remain intact to pass out in faeces (Accinelli *et al.*, 2008; Jiang *et al.*, 2008; Knox *et al.*, 2007; Shan *et al.*, 2008). It was demonstrated for Cry1Ab (Ahmad *et al.*, 2005; Einspanier *et al.*, 2004; Guertler *et al.*, 2008; Lutz *et al.*, 2006; Lutz *et al.*, 2005; Wiedemann *et al.*, 2006). There would subsequently be further degradation of these proteins in the manure and faeces due to microbiological proteolytic activity.

In addition there will be further degradation of Cry proteins in soil reducing the possibility for exposure of potentially sensitive non-target organisms. While Cry proteins may bind to clay minerals and humic substances in soil, thereby reducing their availability to microorganisms for degradation, there are no indication of persistence and accumulation of Cry proteins from GM crops in soil (reviewed by Icoz and Stotzky, 2008).

The EFSA GMO Panel is not aware of evidence of released Bt toxins or PAT protein causing significant negative effects on soil microorganisms.

6.1.1.5. Interactions with the abiotic environment and biogeochemical cycles

Considering the scope of the application and the intended uses of cotton 281-24-236 x 3006-210-23 and due to the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles were not considered an issue by the EFSA GMO Panel.

6.1.2. Post-market environmental monitoring

The objectives of a monitoring plan according to Annex VII of Directive 2001/18/EC are to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment are correct and to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment which were not anticipated in the environmental risk assessment.

Monitoring is also related to risk management, and thus a final adoption of the post-market monitoring plan falls outside the mandate of EFSA. However, the EFSA GMO Panel gives its opinion on the scientific quality of the monitoring plan provided by the applicant (EFSA, 2006a, 2006b). The only significant exposure of the environment to the genetically modified cotton would be related to accidental spillage. The EFSA GMO Panel is aware that, due to physical characteristics of cottonseed and methods of transportation, accidental spillage cannot be excluded. Therefore, the EFSA GMO Panel recommends that appropriate management systems are introduced to actively monitor the occurrence of feral cotton plants in areas where cottonseed spillage and plant establishment are likely to occur as proposed in the EFSA Guidance Document (EFSA, 2006a) and the scientific opinion of the EFSA GMO Panel on post-market environmental monitoring (EFSA, 2006b).

The scope of the monitoring plan provided by the applicant is in line with the intended uses for the GMO. Since the environmental risk assessment did not cover cultivation and identified no potential adverse environmental effects, no case-specific monitoring is necessary.

The general surveillance plan proposed by the applicant includes (1) the description of an approach involving operators (federations involved in cotton import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by EuropaBio for the collection of the information recorded by the various operators; and (3) the use of networks of existing surveillance systems (Lecoq *et al.*, 2007; Windels *et al.*, 2008). The applicant proposes a general surveillance report on an annual basis and a final report at the end of the consent.

The EFSA GMO Panel is of the opinion that the scope of the monitoring plan provided by the applicant is in line with the intended uses of cotton 281-24-236 x 3006-210-23 since the environmental risk assessment did not cover cultivation and identified no potential adverse environmental effects. The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the general surveillance plan.

6.2. Conclusion

Cotton 281-24-236 x 3006-210-23 is being assessed for food and feed uses, import and processing, thus there is no requirement for scientific information on environmental effects associated with cultivation. The EFSA GMO Panel addressed the environmental issues raised by Member States in Annex G of the EFSA overall opinion and concludes as follows: *G. hirsutum* L., which has no wild relatives in Europe, is a cultivated plant in Europe since the 19th century and occurs only occasionally as feral plants in Europe.

If accidental spillage and subsequent release into the environment of cotton 281-24-236 x 3006-210-23 seeds occur, cotton 281-24-236 x 3006-210-23 plants would have a selective advantage only under infestation of target pest species or in the presence of glufosinate-ammonium herbicides which are not commonly used on cultivated cotton or in most areas where the GM cotton might be spilled. Therefore, the EFSA GMO Panel is of the opinion that the likelihood of the establishment and spread of cotton 281-24-236 x 3006-210-23 is very low and that unintended environmental effects due to this GM cotton will be no different from that of other cotton varieties. Furthermore, the scope of the monitoring plan provided by the applicant is in line with the intended uses of cotton 281-24-236 x 3006-210-23 since this does not include cultivation.

The EFSA GMO Panel is aware that, due to the physical characteristics of cottonseed and methods of transportation, accidental spillage cannot be excluded. Therefore the EFSA GMO Panel recommends that, within general surveillance, appropriate management systems are introduced to actively monitor the occurrence of feral cotton plants in areas where cottonseed spillage and plant establishment are likely to occur.

The EFSA GMO Panel also recommends that appropriate management systems should be in place to restrict seeds of cotton 281-24-236 x 3006-210-23 entering cultivation as the latter requires specific approval under Directive 2001/18/EC or Regulation (EC) No 1829/2003.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The EFSA GMO Panel was requested to carry out an evaluation of a scientific risk assessment of the cotton (*Gossypium hirsutum* L.) 281-24-236 x 3006-210-23 for food and feed uses, import and processing. Cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of single cotton events 281-24-236 and 3006-210-23, for food and feed uses, import and processing. In evaluating cotton 281-24-236 x 3006-210-23, the EFSA GMO Panel considered the application EFSA-GMO-NL-2005-16, additional information provided by the applicant, scientific comments submitted by Member States, and relevant scientific publications.

The EFSA GMO Panel is of the opinion that the molecular characterisation provided for the cotton event 281-24-236 x 3006-210-23 as well as for its respective single events is sufficient for the safety assessment. The bioinformatic analyses of the inserted DNA and flanking regions do not raise any safety concern. The expression of the genes introduced by genetic modifications has been sufficiently analysed, the stability of the genetic modification has been demonstrated over several generations in the single events, and the integrity of the inserts has been demonstrated in the stack 281-24-236 x 3006-210-23. The EFSA GMO Panel considers that the molecular characterization does not indicate any safety concern.

The results of the comparative analysis indicated that cotton 281-24-236 x 3006-210-23 and its respective single events assessed in this application are compositionally, agronomically and phenotypically equivalent to their conventional counterpart, except for the presence of newly expressed proteins Cry1Ac, Cry1F and PAT. Based on the evaluation of data available, including additional information provided by the applicant in response to requests from the EFSA GMO Panel on cotton 281-24-236 x 3006-210-23, the respective single events and their conventional counterpart, the EFSA GMO Panel is of the opinion that stacking of the single lines 281-24-236 and 3006-210-23

result in no interactions between the single cotton events which causes unintended compositional, agronomic or phenotypic changes.

Evidence has been provided that there is no safety concern regarding the newly expressed proteins in cotton 281-24-236 x 3006-210-23. Based on the mode of action of the Cry and PAT proteins and given all the information provided, the EFSA GMO Panel concludes that interactions between the newly expressed proteins that might impact on food and feed safety of cotton 281-24-236 x 3006-210-23 are unlikely; the nutritional properties of cotton 281-24-236 x 3006-210-23 would not be different from those of its conventional counterpart; and that it is unlikely that the overall allergenicity of the whole plant is changed. The EFSA GMO Panel concludes that cotton 281-24-236 x 3006-210-23 and its derived products obtained through seed processing are unlikely to have any adverse effects on human and animal health in the context of its intended uses.

Considering the scope of the application, there is no requirement for scientific information on possible environmental effects associated with the cultivation of cotton 281-24-236 x 3006-210-23. The EFSA GMO Panel is of the opinion that the likelihood of the spread and establishment of cotton 281-24-236 x 3006-210-23 is very low and that unintended environmental effects due to this cotton will be no different from that of other cotton varieties. The scope of the monitoring plan provided by the applicant and the reporting intervals are in line with the intended uses of cotton 281-24-236 x 3006-210-23. However, the EFSA GMO Panel is aware that, due to the physical characteristics of cottonseed and methods of transportation, accidental spillage cannot be excluded. Therefore the EFSA GMO Panel recommends that, within general surveillance, appropriate management systems are introduced to actively monitor the occurrence of feral cotton plants in areas where cottonseed spillage and plant establishment are likely to occur. The EFSA GMO Panel recommends that appropriate management systems should be in place to restrict seeds of cotton 281-24-236 x 3006-210-23 entering cultivation as the latter requires specific approval under Directive 2001/18/EC or Regulation (EC) No 1829/2003.

In conclusion, the EFSA GMO Panel considers that information available for cotton 281-24-236 x 3006-210-23 as well as for its respective single events addresses the scientific comments raised by Member States and concludes that the cotton 281-24-236 x 3006-210-23 assessed in this application is as safe as its conventional counterpart and other appropriate comparators.

The EFSA GMO Panel concludes that cotton 281-24-236 x 3006-210-23 is unlikely to have any adverse effect on human and animal health and the environment, in the context of its intended uses.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Competent Authority of The Netherlands, dated 28 June 2005, concerning a request for placing on the market of cotton 281-24-236 x 3006-210-23 in accordance with Regulation (EC) No 1829/2003.
2. Acknowledgement letter, dated 30 June 2005, from EFSA to the Competent Authority of The Netherlands (Ref. SR/SM/jq (2005)804).
3. Letter from EFSA to applicant, dated 8 August 2005, delivering the 'Statement of Validity' for application NL-2005-16, cotton 281-24-236 x 3006-210-23 submitted by Dow AgroSciences under Regulation (EC) No 1829/2003 (Ref. SR/SM/jq (2005)1002).
4. Letter from EFSA to applicant, dated 21 October 2005, requesting additional information and stopping the clock (1) (Ref. SR/SM/cz (2005)).
5. Letter from applicant to EFSA, dated 2 November 2005, providing the timeline for submission of response.

6. Letter from EFSA to applicant, dated 15 December 2005, requesting additional information and maintaining the clock stopped (2) (Ref. SR/SM/cz (2005)1447).
7. Letter from applicant to EFSA, dated 6 March 2006, providing additional information (requested by EFSA on 21 October 2005).
8. Letter from DG SANCO to applicant, dated 13 December 2005, requesting clarification of scope of the application EFSA-GMO-NL-2005-16 submitted under Regulation (EC) No 1829/2003 (Ref. SANCO D4/SG/cc-D(05)441004).
9. Letter from applicant to EFSA, dated 8 March 2006, providing clarification of scope and proposal to update the application package.
10. Letter from EFSA to applicant, dated 24 March 2006, accepting the proposal to update the application EFSA-GMO-NL-2005-16, based on the updated scope launching a second consultation with Member States for a period of 6 weeks (Ref. SR/KL/jp (2006) GMO/262).
11. Letter from EFSA to applicant, dated 7 July 2006, requesting additional information and clarifications on the additional information already provided, maintaining the clock stopped (3) (Ref. SR/SM/jq (2006) 1623208).
12. Letter from applicant to EFSA, dated 23 August 2006, providing clarifications requested on additional information (requested by EFSA on 7 July 2006).
13. Letter from EFSA to applicant, dated 14 November 2006, request for additional information and maintaining the clock stopped (4) (Ref. SR/CP/jq (2006)1832026).
14. Letter from applicant to EFSA, dated 27 November 2006, providing the timeline for submission of response.
15. Letter from EFSA to applicant, dated 8 November 2007, reminding the timeline for submission of response (Ref. SR/SM/shv (2007)2493539).
16. Letter from applicant to EFSA, dated 18 December 2007, providing new timeline for submission of response.
17. Letter from EFSA to applicant, dated 31 July 2008, reminding that the new timeline for submission of response was not met (Ref. SR/SM/Shv (2008)3205270).
18. Letter from applicant to EFSA, dated 29 August 2008, providing another new timeline for submission of response.
19. Letter from applicant to EFSA, dated 9 December 2008, providing additional information (requested by EFSA on 14 November 2006).
20. Letter from EFSA to applicant, dated 17 March 2009, request for additional information and maintaining the clock stopped (5) (Ref. PB/SM/md (2009)3810863).
21. Letter from applicant to EFSA, dated 29 April 2009, requesting clarifications on proposed approach for answering some questions, and providing the timeline for submission of response.
22. Letter from EFSA to applicant, dated 20 May 2009, providing clarifications to the proposed approach for answering some questions (Ref. PB/SM/md (2009)3978423).
23. Letter from applicant to EFSA, dated 29 May 2009, providing renamed reference files.
24. Letter from applicant to EFSA, dated 8 July 2009, providing two references with adjusted names.

25. Letter from applicant to EFSA, dated 29 September 2009, providing additional information (requested by EFSA on 17 March 2009).
26. Letter from EFSA to applicant, dated 21 October 2009, request for clarification concerning additional information and maintaining the clock stopped (6) (Ref. PB/KL/YL/lS (2009) 4371610).
27. Letter from applicant to EFSA, dated 25 January 2010, providing timeline for submission of response.
28. Letter from applicant to EFSA, dated 25 January 2010, providing new timeline for submission of response.
29. Letter from applicant to EFSA, dated 25 January 2010, providing another new timeline for submission of response.
30. Letter from applicant to EFSA, dated 12 March 2010, providing additional information (requested by EFSA on 21 October 2009).
31. Letter from EFSA to applicant, dated 30 March 2010, restarting the clock (Ref. PB/KL/YL/shv (2010)4767809).

REFERENCES

- Accinelli C, Koskinen WC, Beker JM, Sadowsky MJ, 2008. Mineralization of the *Bacillus thuringiensis* Cry1Ac endotoxin in soil. *Journal of Agricultural and Food Chemistry*, 56, 1025-1028.
- Addison SJ, Farrell T, Roberts GN and Rogers DJ, 2007. Roadside surveys support predictions of negligible naturalisation potential for cotton (*Gossypium hirsutum*) in north-east Australia. *Weed Research*, 47, 192-201.
- Ahmad A, Wilde GE and Zhu KY, 2005. Detectability of coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods. *Environmental Entomology*, 34, 385-394.
- Atkins FM, Wilson M and Bock SA, 1988. Cottonseed hypersensitivity: new concerns over an old problem. *Journal of Allergy and Clinical Immunology*, 82, 242-250.
- Bagavathiannan MV and Van Acker RC, 2008. Crop ferality: Implications for novel trait confinement. *Agriculture Ecosystems & Environment*, 127, 1-6.
- Barker RF, Idler KB, Thompson DV and Kemp JD, 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine TI plasmid PTI15955. *Plant Molecular Biology*, 2, 335-350.
- CAC, 2003. Codex principles and guidelines on foods derived from biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organisation: Rome.
- Christensen AH, Sharrock RA and Quail PH, 1992. Maize polyubiquitin genes - structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology*, 18, 675-689.
- Davies PH, 1967. *Flora of Turkey and the East Aegean Islands*. Vol.2. Editor. Edinburgh Univ. Press,
- Eastick RJ and Hearnden MN, 2006. Potential for weediness of Bt cotton in northern Australia. *Weed Science*, 54, 1142-1151.

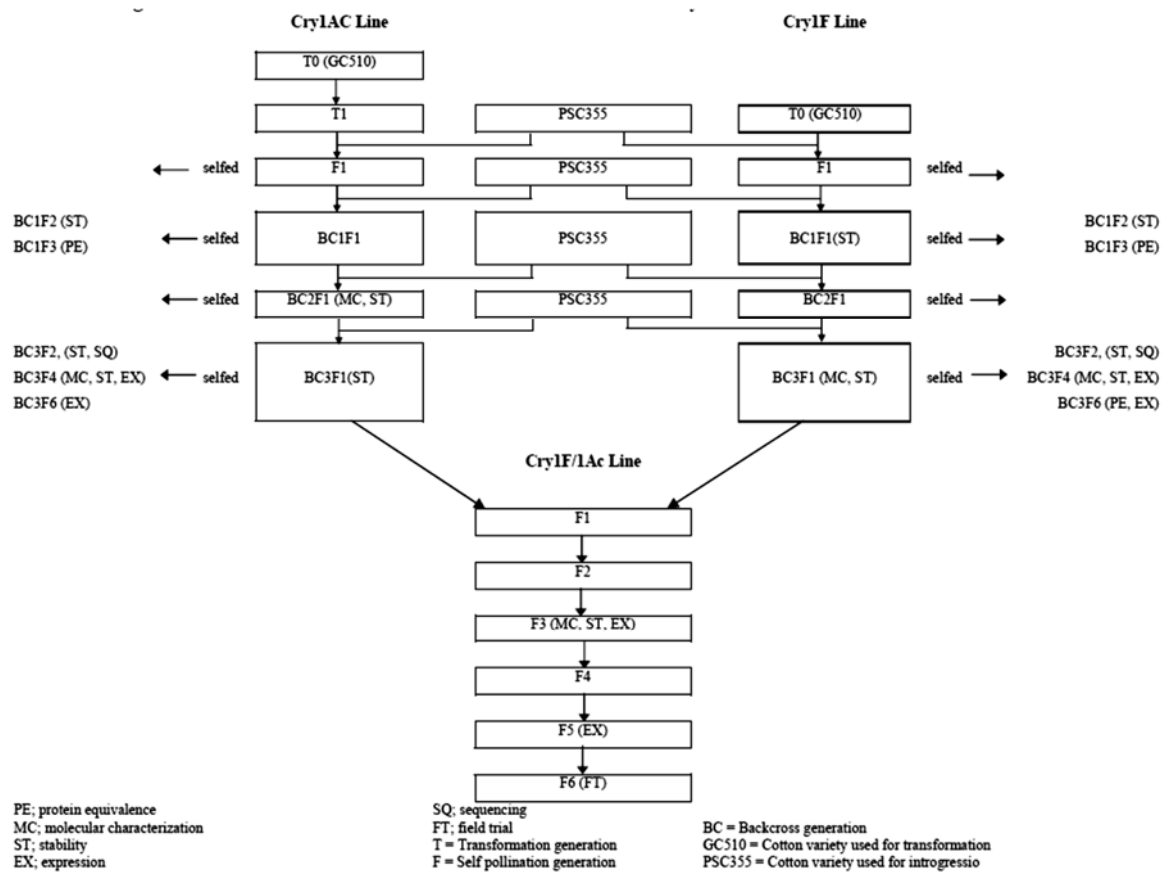
- EC, 2001. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities, L106, 1-38.
- EC, 2009. Regulation (EC) No 1107/2009 of the European Parliament and of Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union, L309, 1-50.
- EFSA, 2006a. Guidance Document of the Scientific Panel on Genetically Modified Organisms for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed The EFSA Journal, 99, 1-100.
- EFSA, 2006b. Opinion of the Scientific Panel on Genetically Modified Organisms (GMO) on the Post Market Environmental Monitoring (PMEM) of genetically modified plants. The EFSA Journal, 1-27.
- EFSA, 2006c. Opinion of the Scientific Panel on Genetically Modified Organisms (GMO) related on an application (Reference EFSA-GMO-NL-2005-13) for the placing on the market of glufosinate-tolerant genetically modified LLCotton25, for food and feed uses, and import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience. The EFSA Journal, 1-19.
- EFSA, 2007. Guidance Document for the risk assessment of genetically modified plants containing stacked transformation events by the Scientific Panel on Genetically Modified Organisms (GMO). The EFSA Journal, 1-5.
- EFSA, 2008. Scientific Opinion of the Panel on Contaminants in the Food Chain on Gossypol as undesirable substance in animal feed. The EFSA Journal, 2008, 1-55.
- Einspanier R, Lutz B, Rief S, Berezina O, Zverlov V, Schwarz W and Mayer J, 2004. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize. European Food Research and Technology, 218, 269-273.
- Ellis JG, Llewellyn DJ, Walker JC, Dennis ES and Peacock WJ, 1987. The OCS element - a 16 base pair palindrome essential for activity of the octopine synthase enhancer. Embo Journal, 6, 3203-3208.
- EPA, 2004. Products characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (Bt) cotton products. <http://www.epa.gov/scipoly/sap/meetings/2004/june/agenda.htm>
- Gao Y, Fencil KJ, Xu X, Schwedler DA, Gilbert JR and Herman RA, 2006. Purification and characterization of a chimeric Cry1F delta-endotoxin expressed in transgenic cotton plants. Journal of Agricultural and Food Chemistry, 54, 829-835.
- Guertler P, Lutz B, Kuehn R, Meyer HHD, Einspanier R, Killermann B and Albrecht C, 2008. Fate of recombinant DNA and Cry1Ab protein after ingestion and dispersal of genetically modified maize in comparison to rapeseed by fallow deer (*Dama dama*). European Journal of Wildlife Research, 54, 36-43.
- Herouet C, Esdaile DJ, Mallyon BA, Debruyne E, Schulz A, Currier T, Hendrickx D, van der Klis RJ and Rouan D, 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicology and Pharmacology, 41, 134-149.
- Hofs JL, Klein E, Pierre J, Chèvre AM and Hau B, 2007. GM cotton gene flow in small-scale farming systems: probable impact on organic cotton production in Africa. In: Book of abstracts of the third International Conference on Coexistence between Genetically Modified (GM) and non-GM-based Agricultural Supply Chains. AJ Stein, E Rodriguez-Cerezo. European Commission, pp. 87-90.
- IRMM, 2006. The certification of reference materials of cotton seed powder with different mass fractions of the cotton event 281-24-236 x 3006-210-23 Certification report EUR 22527 EN.

- https://irmm.jrc.ec.europa.eu/html/reference_materials_catalogue/catalogue/attachements/ERM-BF422_report.pdf
- Jiang LJ, Tian XL, Duan LS, Li ZH, 2008. The fate of Cry1Ac Bt toxin during oyster mushroom (*Pleurotus ostreatus*) cultivation on transgenic Bt cottonseed hulls. *Journal of the Science of Food and Agriculture*, 88, 214-217.
- Kareiva P, Morris W and Jacobi CM, 1994. Studying and managing the risk of cross-fertilization between transgenic crops and wild relatives. *Molecular Ecology*, 3, 15-21.
- Knox OGG, Gupta Vadakattu V.S.R, Roberts GN, Downes SJ, 2007. Improving Environmental Loading Assessments of Cry Protein from GM Plants Based on Experimentation in Cotton, *The Open Agriculture Journal*, 2, 105-112.
- Llewellyn D and Fitt G, 1996. Pollen dispersal from two field trials of transgenic cotton in the Namoi Valley, Australia. *Molecular Breeding*, 2, 157-166.
- Llewellyn D, Tyson C, Constable G, Duggan B, Beale S and Steel P, 2007. Containment of regulated genetically modified cotton in the field. *Agriculture Ecosystems & Environment*, 121, 419-429.
- Lutz B, Wiedemann S and Albrecht C, 2006. Degradation of transgenic Cry1Ab DNA and protein in Bt-176 maize during the ensiling process. *Journal of Animal Physiology and Animal Nutrition (Berl)*, 90, 116-123.
- Lutz B, Wiedemann S, Einspanier R, Mayer J and Albrecht C, 2005. Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. *Journal of Agricultural and Food Chemistry*, 53, 1453-1456.
- Malanin G and Kalimo K, 1988. Angioedema and urticaria caused by cottonseed protein in whole-grain bread. *Journal of Allergy and Clinical Immunology*, 82, 261-264.
- McGregor SE, 1959. Cotton-flower visitation and pollen distribution by honey bees. *American Association for the Advancement of Science. Science*, 129, 97-98.
- Mendelsohn M, Kough J, Vaituzis Z and Matthews K, 2003. Are Bt crops safe? *Nature Biotechnology*, 21, 1003-1009.
- Moffett JO and Stith LS, 1972. Honey bees as pollinators of hybrid cotton-D. *Environmental Entomology*, 1, 368-370.
- Moffett JO, Stith LS, Burkhart CC and Shipman CW, 1975. Honey Bee Hymenoptera-Apidae visits to Cotton Flowers. *Environmental Entomology*, 4, 203-206.
- OECD, 2004. Consensus Document on Compositional Considerations for New Varieties of Cotton (*Gossypium hirsutum* and *Gossypium barbadense*): key food and feed nutrients, Anti-Nutrients. *Series on the Safety of Novel Foods and Feeds*, 11,
- Shan GM, Embrey SK and Schafer BW, 2007. A highly specific enzyme-linked immunosorbent assay for the detection of Cry1Ac insecticidal crystal protein in transgenic wideStrike cotton. *Journal of Agricultural and Food Chemistry*, 55, 5974-5979.
- Shan G, Embrey SK, Herman RA, McCormick R, 2008. Cry1F Protein Not Detected in Soil After Three Years of Transgenic Bt Corn (1507 Corn) Use. *Environmental Entomology*, 37, 255-262.
- Todaro F, 1917. *Lezioni di Agricoltura*. Editor. Casa Editrice Fratelli Marescalchi, Casale Monferrato. Vol 2, pp. 317-319,
- Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM and Webb DA, 1992. *Flora Europaea*. Editor. Cambridge University Press, Cambridge. Vol 2, 5th reprint, pp. 469,
- Umbeck PF, Barton KA, Nordheim EV, McCarty JC, Parrott WL and Jenkins JN, 1991. Degree of pollen dispersal by insects from a field-test of genetically engineered cotton. *Journal of Economic Entomology*, 84, 1943-1950.

- Vaissiere BE and Vinson SB, 1994. Pollen morphology and its effect on pollen collection by honeybees, *Apis mellifera* L. (Hymenoptera: Apidae), with special reference to upland cotton, *Gossypium hirsutum* L. (Malvaceae). *Grana*, 33, 128-138.
- Van Deynze AE, Sundstrom FJ and Bradford KJ, 2005. Pollen-mediated gene flow in California cotton depends on pollinator activity. *Crop Science*, 45, 1565-1570.
- Wiedemann S, Lutz B, Kurtz H, Schwarz FJ and Albrecht C, 2006. In situ studies on the time-dependent degradation of recombinant corn DNA and protein in the bovine rumen. *Journal of Animal Science*, 84, 135-144.
- Xanthopoulos FP and Kechagia UE, 2000. Natural crossing in cotton (*Gossypium hirsutum* L.). *Australian Journal of Agricultural Research*, 51, 979-983.
- Zangheri P, 1976. *Flora Italica*. Editor. Padova,
- Zhang BH, Pan XP, Guo TL, Wang QL and Anderson TA, 2005. Measuring gene flow in the cultivation of transgenic cotton (*Gossypium hirsutum* L.). *Molecular Biotechnology*, 31, 11-20.
- Zohary D and Hopf M, 2000. Domestication of plants in the Old World: the origin and spread of cultivated plants in West Asia, Europe and the Nile Valley. *Domestication of plants in the Old World: the origin and spread of cultivated plants in West Asia, Europe and the Nile Valley*, xi + 316 pp.

Appendix III

Figure 1. Breeding scheme for cotton MXB-13



Appendix IV

COTTON

General information

Cotton is mainly grown for its commodity product the cotton boll. The fibres on the cotton boll are separated from the cottonseeds by a cotton gin machine. The fibres, which consist almost completely of cellulose, are primarily used for textiles, but also have some application for food or feed (see figure 4.2-1). Especially the fibres that are too short to be spun into textiles, known as linters, can be used as food additives. Cellulose and methylcellulose can be used as thickeners, stabilisers, emulsifiers, or fillers. The protein- and oil-rich whole cottonseeds (WCS) are used for oil extraction and cottonseed oil is used in food and feed. Following oil extraction, the cottonseed can be processed into various other side-products that are also used in food and feed, such as cottonseed meal, various protein preparations, and cottonseed milk. Protein-rich cottonseed meal is mostly used as an animal feed ingredient. Another major processed product derived from cottonseed are fibre-rich hulls, which may also be used in animal feeds (Figure 4.2-1).

Processing for food and feed uses

Cottonseed

Fuzzy cottonseed may be dehulled, cooked, cracked, flaked and is processed into four major products: oil, meal, hulls, and linters, see Figure 4.2-1. Typical processing yields of fuzzy cottonseed is 45% meal, 26% hulls, 16% oil, 9% linters and 4% lost in processing (OECD, 2004). WCS contains high quality protein and oil. The processing steps which are used to produce the various cotton products are shown in figure 4.2-1. The processing of WCS may include delinting, dehulling, crushing, flaking, extruding, extracting, roasting, bleaching and deodorizing. WCS are first cracked and de-hulled, then heated to approximately 60°C, ground to flakes with rollers, and are then treated with solvent to remove the oil. The flakes are toasted, cooled and grounded. Roasting, extruding, and cracking whole cottonseed has improved digestibility in some trials but under some conditional may also has increased the availability of free gossypol.

By-products of processing can be included in human diet, such as linters and oil, or in animal diet such as hulls and meal. The two main soluble proteins in cottonseed are albumin and globulin. The amounts of these proteins are three times higher than the fractions of insoluble proteins (prolamine and glutelin; Arieli, 1998). The rumen protein degradability values are usually over 70% in dairy cattle (Arieli, 1998).

WCS typically contains 1.5-2.0% gossypol, all in the unbound form, but levels can vary to as low as 0.4% in some commercial cultivars (Calhoun et al., 1995). The presence of gossypol and cyclopropenoid fatty acids (CPFA) in cottonseed limits its use as a protein supplement in animal feed, except for cattle, who are unaffected by these components because they are detoxified by digestion in the rumen.

Cottonseed oil

Several methods are used to extract cottonseed oil either by mechanical pressing, solvent (usually n-hexane) extraction or supercritical fluid extraction (Saxena et al., 2011). The various steps in refining the oil are alkali refining (removes free fatty acids, glycerol, metals, proteins), bleaching (removes metals and colour), winterization (low temperature causes stearin to precipitate), hydrogenation (hydrogenate carbon-carbon double bonds) and deodorization (removes volatile compounds e.g. free fatty acids and peroxide). Processing of the oil removes most of the gossypol and CPFAs. Cottonseed oil consists of 70% unsaturated fatty acids including 18% oleic acid, 52% linoleic acid, and 26% saturated fatty acids (primarily palmitic and stearic acids). The main fatty acid composition of refined cottonseed oil (in % of total fatty acids) is 16:0 palmitic acid (range 21.1-28.1%), 18:0 stearic acid (2.1-3.1%), 18:1 oleic acid (12.9-20.1%) and 18:2 linoleic acid (46.0-58.2) (OECD, 2004). Cottonseed oil is a high-value cooking or frying oil and is sometimes used to make margarine. The oil is also a source of vitamin E.

Cottonseed meal (CSM)

The cottonseed meal is the by-product of cottonseed oil extraction and is a protein-rich feed ingredient. The presence of gossypol and cyclopropenoid fatty acids (CPFA) in cottonseed limits its use as a protein supplement in animal feed, except for cattle, who are unaffected by these components because they are detoxified by digestion in the rumen. The rumen protein degradability values are usually over 70% in dairy cattle (Arieli, 1998). Calves, however, are susceptible to gossypol toxicity because of their incomplete rumen development.

Inactivation or removal of gossypol and CPFA during processing enables the use of low levels of cottonseed meal in feeds for fish, poultry, rabbit and swine (Heuzé and Tran, 2015).

Cottonseed hulls

Cottonseed hulls (CSH) are the by-product of the dehulling step of cottonseed oil extraction. The hull is mainly hemicellulose and lignin compounds with a nearly pure cellulose linter fibre attached. No pigment glands have been reported on the hull fibre or linter fibre fractions after processing. Hulls have less than 0.049 % free gossypol content (Forster and Calhoun, 1995).

Cottonseed hulls also contain condensed tannins, which are mainly bound to fibre and protein (Yu et al., 1996). Condensed tannins can have an anti-nutritional factor effect on ruminants, but at low concentrations they can improve efficiency of protein digestion by forming hydrogen-bonded complexes with proteins in the rumen (Yu et al., 1995).

Linters

The linted cottonseed remaining after the ginning process is called fuzzy or whole cottonseed, and the short fibers still adhering to the cotton seed after the ginning process are called linters. Unprocessed fuzzy cottonseeds are not suitable for food.

Cotton linters are short fibre removed from cottonseed during processing. Linters, like raw cotton, are 90-95% cellulose, with no lignin, and only a small amount of waxes, pectin, organic acids, and ash-producing inorganic substances. Linters are a major source of cellulose for both chemical and food uses. When linters are used in food products, they undergo processing (for example, alkaline washing at high temperatures), which would effectively denature and/or remove any protein present.

Linters are also used in absorbent cotton, medical pads, gauze, twine, wicks, carpet yarns, surgical, paper, and packing products; second-cut linters, in chemical cellulose for preparation of regenerated s, films, lacquers, explosives, plastics, and papers; and mill-run linters in chemical cellulose and padding products.

Endogenous toxin gossypol

Gossypol is a terpenoid phytoalexin pigment found naturally in many *Gossypium* species and is located in glands throughout the plant. Gossypol (Chemical Abstracts Service CAS Registry Number 303-45-7) is crystalline, intensely yellow, insoluble in water and soluble in organic solvents and fats. Free gossypol will covalently bind to cottonseed protein and reduce the protein quality due to binding to lysine. The availability of lysine is reduced when meal is fed to non-ruminants (OECD, 2004; EFSA, 2008).

Animal sensitivity to gossypol differs considerably between species and classes of animals. It is particularly toxic to non-ruminants. Acute toxicity has been shown in the heart, lung, liver, and blood cells, resulting in increased erythrocyte fragility (EFSA, 2008). Reproductive toxicity is seen particularly in males, where gossypol affects sperm motility and inhibits spermatogenesis. In females gossypol disrupts the oestrus cycles (EFSA, 2008).

According to EFSA (2008), the potential exposure to free gossypol, based on the maximum permitted concentration in cottonseed meal and recommended maximum inclusion rates in complete feed, would not be expected to result in adverse effects in ruminants, poultry or fish. However, not all monogastric livestock animals, e.g. pigs, have been fully investigated for potential reproductive effects occurring at low doses.

The current EU regulations (Annex I of Council Directive 2002/32/EC; as reported in EFSA, 2008) specifies maximum levels of free gossypol in various feed commodities and animal feeds with a moisture content of 12%:

- 5000 mg/kg in cottonseed
- 1200 mg/kg in cottonseed cake and cottonseed meal
- 20 mg/kg in complete formulated feeds for most monogastric animals, including piglets, fish and laying hens

- 500 mg/kg in complete formulated feeds for ruminants (cattle, sheep and goats)
- 100 mg/kg in complete formulated feeds for poultry (other than laying hens) and calves
- 60 mg/kg in complete formulated feeds for rabbits and pigs (except piglets)

The toxicity of the (–) enantiomer was the more toxic isomer in a study with broiler (Gamboa et al., 1997). There is also a relative good relationship between dietary free gossypol and tissue accumulation of gossypol enantiomers (Gamboa et al., 2001). Accumulation of total gossypol occurs at a faster rate in liver than in plasma or any other tissue. In this feeding study by Gamboa et al. (2001), one-day-old broilers were fed 0, 7, 14, 21 and 28 % cottonseed meal in their diets, corresponding to 0, 0.13, 0.26, 0.39 and 0.53 g/kg diet of free gossypol, for 21 days. An increment of 1 µg/g of dietary free gossypol resulted in an increment of 0.568 µg/g dry matter (DM) in liver, 0.065 µg/g DM in kidney, 0.018 µg/g DM in muscle, and 0.026 µg/mL in plasma. The proportion of (–)- gossypol was higher in plasma (26.7%) and kidney (25.6%) when compared to muscle (19.1%) and liver (16.0%).

The toxicity of (±) gossypol acetic acid has also been studied in Cynomolgus monkeys (Heywood, 1988). They were administered 25 mg (±) gossypol/kg bw per day for thirteen weeks. At this gossypol concentration gossypol induced death, a variety of clinical signs, extensive biochemical changes and pathology in the heart, liver, kidney and testes. The toxicity of the enantiomeric form (–) gossypol was investigated in male Cynomolgus monkeys at dosages of 1.5, 4 or 5 mg/kg/day for 4 weeks. No animals died. Clinical signs involving the gastrointestinal tract, adverse effects on body weight gain, consistent biochemical changes in serum proteins, calcium, inorganic phosphorus and serum cholesterol were recorded at 4 mg/kg per day and above. Morphological change was not induced (Heywood, 1988).

Gossypol is less toxic to ruminants, but inhibition of spermatogenesis, embryo development and increased erythrocyte fragility occurred at doses of 6-18 mg/kg bw per day in cattle and cardiomyopathy in lambs at 2-3 mg/kg bw per day (EFSA, 2004).

Gossypol levels in the reported feeding trials (sections 4.4.2 and 4.5.2)

90-day subchronic toxicity trial with Crl:CD(SD) rats (see section 4.4.2)

The reported levels of free gossypol content (mg/kg DW) in cottonseed from MXB-13 and controls were as follows: 2330 in cotton PSC355, 1220 in PHY72, 92 in PHY78, 2930 in 98M-2983, and 1180 mg/kg in MXB-13. The levels of free gossypol in PSC355, PHY72 and 98M-2983 exceeded the MPL (maximum permitted level) of 1200 mg/kg in cottonseed meal but are within the MPL of 5000 mg/kg for cottonseed (EFSA, 2008; see section 3.2).

According to the applicant, the analysed levels of free gossypol in dry weight of the complete formulated diets given to the rats with an inclusion level of 10% cottonseed meal were 180 (PSC355), 140 (PHY72), 100 (PHY78), 110 (98M-2983), and 60 (MXB-13) mg/kg. Thus the levels of free gossypol in all diets exceeded the applicant's goal of 40 mg/kg feed and were

also above the MPL for complete formulated diets for most monogastric animals set at 20 mg/kg (EFSA, 2008).

According to the applicant these levels did not greatly exceed the MPL recommended for pigs of 60 mg/kg of feed, corresponding to a No-Observed-Adverse-Effect level (NOAEL) of 3 mg/kg bw/day; nor for broilers of 100 mg/kg diet, corresponding to an NOAEL of 200 mg free gossypol/kg feed or 20-30 mg/kg bw per day (EFSA, 2008; see section 3.2).

42-day nutritional assessment trial with broilers (see section 4.5.2)

According to the applicant the free gossypol content in cottonseed (in mg/kg fresh weight) were 480 in PSC355, 1120 in Pima, 510 in Acala, and 490 in MXB-13.

The free gossypol content in the complete formulated starter diets (in mg/kg fresh weight) were analysed and found to contain: 110 (PSC355), 120 (Pima), 120 (Acala), and 90 (MXB-13).

In the grower diets, analysis revealed gossypol levels of 40 mg/kg (PSC355), 90 (Pima), 60 (Acala), and 40 mg/kg (MXB-13).