Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2016:10

Final health and environmental risk assessment of genetically modified LLcotton25

Scientific opinion on glufosinate-tolerant, genetically modified LLcotton25 from Bayer CropScience for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (Application EFSA/GMO/NL/2005/13)

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) 2016:10
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14.03.2016

ISBN: 978-82-8259-200-0
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Suggested citation: VKM (2016) Final health and environmental risk assessment of genetically modified LLcotton25. Scientific opinion on glufosinate-tolerant genetically modified LLcotton25 from Bayer CropScience for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (Application EFSA/GMO/NL/2005/13). Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety, ISBN: 978-82-8259-200-0, Oslo, Norway.

Final health and environment assessment of genetically modified LLcotton25 (EFSA/GMO/NL/2005/13)

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Assessed and approved

The opinion has been assessed and approved by Panel on Genetically Modified Organisms. Members of the panel are: Åshild Andreassen (chair), Per Brandtzæg, Knut Helkås Dahl, Knut Tomas Dalen, Olavi Junttila, Richard Meadow, Inger Elisabeth Måren, Kåre M. Nielsen, Monica Sanden, Hilde-Gunn Sorteberg, and Rose Vikse.

Acknowledgment

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 Mikaelsen, Anne Marie Bakke, Ville Erling Sipinen and Merethe Aasmo Finne.

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 LLcotton25 from Bayer CropScience expresses the *bar* gene from *Streptomyces hygroscopicus* ATCC21705 encoding the phosphinothricin-acetyl–transferase (PAT) enzyme, which confers tolerance to the active herbicide glufosinate-ammonium.

Updated bioinformatics analyses of the inserted DNA and flanking sequences in LLCotton25 have not indicated potential production of putatively harmful toxins or allergens caused by the genetic modification. Genomic stability of the functional insert and consistent expression of the *bar* gene have been shown over several generations of LLCotton25.

Data from field trials indicate that with the exception of the newly introduced trait, LLCotton25 is compositionally, phenotypically and agronomically equivalent to its conventional counterpart Coker 312 and other cotton cultivars.

A 33-day nutritional assessment trial with broilers has not revealed adverse effects of cottonseed meal from LLCotton25. Toxicity testing of the PAT protein in a repeated-dose dietary exposure test with rats did not indicate adverse effects. The PAT protein produced in LLCotton25 does not show amino acid sequence resemblance to known toxins or IgE-dependent allergens, nor has it been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the PAT protein will cause toxic or IgE-mediated allergic reactions to food or feed containing LLCotton25 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 LLCotton25 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterpart and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that LLCotton25 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-ammonium tolerant genetically modified LLCotton25 (Unique Identifier ACS-GHØØ1-3) from Bayer CropSciences is approved in EU under Regulation (EC) No 1829/2003 for food and feed uses, import and processing since 13th of April 2007 (Application EFSA/GMO/NL/2005/13, Commission Implementing Decision 2008/837/EC).

LLCotton25 has previously been assessed by the VKM GMO Panel commissioned by the NFSA related to the EFSAs public hearing of the application EFSA/GMO/NL/2005/13 in 2005 (VKM, 2005). LLCotton25 has been used as a component of the stacked GM event GHB614 x LLCotton25 (EFSA/GMO/NL/2010/77), which has been evaluated by EFSA (EFSA, 2014), but not by VKM.

The current food, feed and environmental risk assessment of the LLCotton25 is based on information provided in the application EFSA/GMO/NL/2005/13, relevant peer-reviewed scientific literature including scientific opinions and comments from EFSA (EFSA, 2006a), 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 LLCotton25 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, 2006b and 2011b), the environmental risk assessment of GM plants (EFSA, 2010a), 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 LLCotton25 includes molecular characterisation of the inserted DNA and expression of the novel protein, 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, included in 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 possible herbicide residues in food and feed from genetically modified plants specifically.

The event LLCotton25 was developed by *Agrobacterium tumefaciens* mediated transformation to express a modified *bar* gene from *Streptomyces hygroscopicus*. The gene encodes the enzyme phosphinothricin-acetyl-transferase (PAT) that acetylates L-glufosinate, which renders LLCotton25 tolerant to glufosinate ammonium based herbicides.

Molecular characterisation

The LLCotton25 genome has a complete, single integrated copy of the *bar*-expression cassette. Even though the PAT concentration is low, 0.21-0.35% of total crude protein in the leaves, it is highest in the plant parts exposed to herbicide treatment. This is consistent with the regulation by the inserted *35S* promoter, with highest activity in leaves and stems.

Out of 26 putative novel open reading frames (ORFs) identified in the GM cotton, only three short ORFs located in the 3' region of the insert were theoretically found to encode potential novel gene products. No relevant homologies were found between these theoretically predicted translation products and known toxins or allergens. 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 event.

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in LLCotton25 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 in the USA during 2000 and 2001 for compositional assessments of whole linted cottonseeds, cotton lint, and different processed cottonseed products. Field trials in USA, Brazil and Australia during numerous growing seasons were performed for agronomic and GM phenotype assessments. In all trials, LLCotton25 was compared to its conventional counterpart, parent line Coker 312. LLCotton25 was grown using conventional or glufosinate-based herbicide while cotton Coker 312 was grown using conventional herbicides.

With the exception of the changes caused by the introduced transgenic trait, data provided by the applicant revealed no biologically relevant differences between LLCotton25 and its conventional counterpart Coker 312. The few 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 PAT-protein, the VKM GMO Panel concludes that LLCotton25 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart and other cotton cultivars.

Food and feed risk assessment

A 33-day nutritional assessment trial with broilers did not reveal biologically relevant adverse effects or differences in the performance of animals fed diets containing cottonseed meal from LLCotton25 compared to conventional counterpart Coker 312 or another cotton cultivar. Bioinformatics analysis of the amino acid sequence of the PAT protein did not show sequence resemblance to known toxins or IgE-dependent allergens, nor has the protein been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the PAT protein will cause toxic or IgE-mediated allergic reactions to food or feed containing LLCotton25 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that LLCotton25 is nutritionally equivalent to and as safe as its conventional counterpart Coker 312 and other cotton cultivars.

Environmental assessment

Considering the intended uses of LLCotton25, 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 LLCotton25.

With the exception of the introduced tolerance to the herbicide glufosinate-ammonium, LLCotton25 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton cultivars, and there are no indications of an increased likelihood of spread and establishment of cotton plants in the case of accidental release of seeds from LLCotton25 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 LLCotton25 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 LLCotton25 does not represent an environmental risk in Norway.

Overall conclusion

Based on current knowledge and with the exception of the introduced trait, the VKM GMO Panel concludes that LLCotton25 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterpart and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that LLCotton25 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.), LLCotton25, unique identifier ACS-GHØØ1-3, EFSA/GMO/NL/2005/13, glufosinate tolerance, PAT protein, *bar* gene, food/feed safety, human and animal health, import and processing, Regulation (EC) No 1829/2003.

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 bomullssorten LLCotton25 (unik kode ACS-GØØ1-3) fra Bayer CropScience er fremkommet ved genmodifisering av bomullshybriden Coker 312. Hensikten med LLCotton25 er motstandsdyktighet mot ugressmidler som inneholder glufosinat-ammonium, f.eks. Basta, Buster, Finale, Ignite, Liberty og Rely.

LLCotton25 ble godkjent til import, videreforedling og til bruk som mat og fôr under forordning 1829/2003 den 29. oktober 2008 (Kommisjonsbeslutning 2008/837/EC). Søknaden og godkjenningen omfatter ikke dyrking.

LLCotton25 ble første gang vurdert av VKMs faggruppe for GMO i 2005 (VKM, 2005) i forbindelse med den offentlige høring av søknad EFSA/GMO/NL/2005/13 i 2005. EFSAs endelig vurdering ble publisert i 2006 (EFSA, 2006a). LLCotton25 har også blitt brukt som en komponent i bomullhybriden GHB614 x LLCotton25 (søknad EFSA/GMO/NL/2010/77), som har blitt vurdert av EFSA (EFSA, 2014), men ikke av VKM.

Risikovurderingen av den genmodifiserte bomullen er basert på søkers dokumentasjon som er gjort tilgjengelig på EFSAs GMO Extranet, og uavhengige vitenskapelige publikasjoner, inklusiv vitenskapelige vurderinger fra EFSA (EFSA, 2006a) 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, 2006a) 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, 2006b, 2010a, 2011a, 2011b og 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.

LLCotton25 er utviklet ved hjelp av *Agrobacterium*-mediert transformasjon til å uttrykke et modifisert *bar* -gen fra *Streptomyces hygroscopicus*. Genet koder for enzymet phosphinothricin-acetyl-transferase (PAT) som gir LLCotton25 en økt toleranse overfor glufosinat-ammonium baserte ugressmidler.

Molekylær karakterisering

Den molekylære karakteriseringen fra søker viser at det kun er inkorporert én intakt kopi av det transgene innskudds-DNAet (T-DNAet) bestående av en *35S* promoter og *bar*-genet som til sammen fører til uttrykk av proteinet PAT. Proteinmålinger utført på prøver av LLCotton25 viser at PAT proteinet er uttrykket og aktivt i blad, stengel, rot, pollen og bomullsfrø og hovedsakelig i blad. Det er identifisert 26 mulige nye åpne leserammer (ORFs), i og ved den innsatte T-DNA-sekvensen i plantens genom, hvorav kun tre er antatt å kunne føre til produksjon av korte genprodukt i LLCotton25. Databasesøk utført av søker viser derimot ingen likheter mellom de tre antatte genproduktene og 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 LLCotton25.

Ut i fra dagens kunnskap og informasjon fra søker, konkluderer VKMs faggruppe for GMO med at den molekylære karakteriseringen av de tilsiktede endringene i LLCotton25 er tilstrekkelig og at det ikke er identifisert utilsiktede endringer som krever spesifikk oppfølging i den videre vurderingen.

Komparative analyser

Søker har utført flere feltforsøk i USA i 2000 og 2001 med påfølgende analyse av næringsstoffer, antinæringsstoffer og andre relevante, biologisk aktive stoffer i bomullsfrø, bomullsfrømel, urenset og renset bomullsfrøolje og øvrig prosessert plantemateriale. Registrering av agronomiske og fenotypiske egenskaper har blitt utført under feltstudier i USA, Australia og Brazil over flere år. For alle feltstudiene ble data fra LLCotton25 sammenlignet med konvensjonell kontroll Coker 312.

Tilgjengelig data fra søker viser at med unntak av den ønskede endringen, var det ingen biologisk relevante forskjeller i enkeltparametere mellom den genmodifiserte LLCotton25 og konvensjonell kontroll Coker 312. 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 det introduserte proteinet PAT, konkluderer VKMs faggruppe for GMO med at LLCotton25 er vesentlig lik konvensjonell kontroll og andre bomullssorter med hensyn til næringsstoffsammensetning og agronomiske og fenotypiske egenskaper.

Helserisiko

Et 33-dagers fôringsforsøk med broilere har blitt utført med bomullsfrømel fra LLCotton25, konvensjonell kontroll Coker 312 og en annen konvensjonell bomullssort. Studien viste ikke negative effekter eller andre relevante forskjeller hos broilere gitt fôr med frømel fra LLCotton25 sammenlignet med de konvensjonelle bomullssortene. Databasesøk viser ingen relevante sekvenslikheter mellom PAT-proteinet og kjente toksiner eller IgE-avhengige allergener, og er ikke rapportert å ha forårsaket IgE-medierte allergiske reaksjoner. Det foreligger derfor ikke data som tilsier at PAT proteinet vil føre til toksiske eller IgE-medierte allergiske reaksjoner fra mat og fôr som inneholder LLCotton25 sammenlignet med konvensjonelle bomullssorter.

Ut i fra dagens kunnskap og tiltenkt bruk, konkluderer VKMs faggruppe for GMO med at LLCotton25 er ernæringsmessig lik og like trygg som konvensjonell kontroll Coker 312 og andre bomullssorter.

Miljørisiko

Miljørisikovurderingen av LLCotton25 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 LLCotton25 i Norge.

Genmodifiseringen av LLCotton25 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 LLCotton25. Bomull dyrkes ikke i Norge, og arten har ikke viltvoksende populasjoner eller nærstående arter utenfor dyrking i Europa. Det er derfor ikke risiko for utkryssing med dyrkede sorter eller ville planter i Norge. Det er ingen indikasjoner for at nyinnsatte gener fra LLCotton25 vil kunne overføres horisontalt til mikroorganismer i mage-tarm trakt 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 LLCotton25 ikke vil medføre miljørisiko i Norge.

Samlet vurdering

Ut i fra dagens kunnskap, og med unntak av den introdusert egenskapen, konkluderer VKMs faggruppe for GMO med at LLCotton25 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 LLCotton25 ikke vil medføre miljørisiko i Norge.

Abbreviations and/or glossary

4ocsΔMas2	'Mannopine synthase promoter from *Agrobacterium tumefasiens* plasmid *pTi15955*
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	*Bacillus thuringiensis*
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
In planta	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
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 7 March 2005, the European Food Safety Authority (EFSA) received from the Dutch Competent Authority an application (Reference EFSA/GMO/NL/2005/13) for authorisation of the glufosinate-tolerant genetically modified LLCotton25 (Unique Identifier ACS-GHØØ1-3), submitted by Bayer CropScience 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/2005/13 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 5 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 December 2005 (VKM, 2005). EFSA published its scientific opinion 6 December 2006 (EFSA, 2006a), and LLcotton25 was approved for food and feed uses, import and processing 29 October 2008 (Commission Implementing Decision 2008/837/EC).

LLCotton25 has been used as a component of the stacked GM event GHB614 x LLCotton25 (EFSA/GMO/NL/2010/77), which has been evaluated by EFSA (EFSA, 2014), but not by VKM.

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 food and feed from the GM plants (EFSA, 2006b, 2010a, 2011b and 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 current food, feed and environmental risk assessment of the genetically modified LLcotton25 is assessed with reference to the intended use. The risk assessment is based on information provided by the applicant in the application EFSA/GMO/NL/2005/13, relevant peer-reviewed scientific literature, and scientific opinion and comments from VKM (VKM, 2005), EFSA (EFSA, 2006a) 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.

LLCotton25 has been used as a component of the stacked GM event GHB614 x LLCotton25 (EFSA/GMO/NL/2010/77), which has been evaluated by EFSA (EFSA, 2014), but not by VKM.

Genetically modified LLcotton25 (Unique Identifier ACS-GHØØ1-3) is derived from the cotton variety Coker 312 which was transformed by *Agrobacterium*-mediated gene transfer (Zambryski, 1992). LLCotton25 was genetically modified to express the *bar* gene. The *bar* gene encodes the enzyme phosphinothricin-acetyl-transferase (PAT), which confers tolerance to the herbicide glufosinate ammonium (commercial names Liberty®, Basta®).

Molecular analysis shows that LLCotton25 contains a single insert and does not retain backbone sequences from the vector. The purpose of the modification is to allow for effective weed control during the cultivation of LLCotton25. The genetic modification in LLCotton25 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.

The source of the *bar* gene is the bacterium *Streptomyces hygroscopius* strain ATCC21705 (Murkami et al., 1986).

Streptomyces hygroscopius belongs to the *Streptomyceta*, and is generally soil-borne, although it may be isolated from water. *Streptomyces* are not typically pathogenic to animals or humans, and few species have been shown to be phytopathogenic (Bradbury, 1986; Kutzner, 1981). The bacteria *S. hygroscopius*, also naturally produces the toxin bialaphos, which is an effective broad-spectrum herbicide. The PAT enzyme prevents autotoxicity in the bacterial organism and generates complete resistance towards high doses of PPT by acetylating the free amino group of PPT, bialaphos or the synthetically produced glufosinate-ammonium.

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 LLCotton25 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.

LLCotton25 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, 2006b and 2011b), the environmental risk assessment of GM plants (EFSA, 2010a), 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 VKM and EFSA GMO Panels (VKM, 2005, Appendix I; EFSA, 2006a, Appendix II) have previously assessed the molecular characterisation of the cotton event LLCotten25 (inserted *bar*-gene) with regards to the following:

1. The transformation system and vector construct
2. Characterisation of transgene insertion and construct
3. Information on the expression of the insert
4. Analyses of new open reading frame(s) (ORFs)
5. Inheritance and the stability of the inserted DNA

Both Panels concluded that the applicant had provided sufficient analyses for the molecular characterisation. Initially, cotton tissue from *Gossypium hirsutum*, variety Coker 312, was transformed by *Agrobacterium tumefaciens* mediated gene transfer with the binary plasmid vector pGSV71. The vector contained the T-DNA region, with the left and right borders (LB and RB) delimiting a gene construct for expression of a modified *bar* gene derived from *Streptomyces hygroscopicus*. The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT) that acetylates L-glufosinate-ammonium and provides tolerance to glufosinate-based herbicides. This makes event LLCotten25 tolerant to herbicides based upon glufosinate ammonium such as Basta®, Buster®, Finale®, Ignite®, Liberty® and Rely®. In addition to the *bar*-gene the inserted T-DNA sequence in LLCotten25 contains the 35S promoter from Cauliflower Mosaic Virus, and the 3'nos terminator sequence including the 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (nopaline Ti plasmid).

A number of molecular analyses, Southern and Northern hybridisations, PCR, BLAST searches and ELISA, have been performed by the applicant to determine the number of insertion sites, copy number, integrity of the insert, evaluation of the presence or absence of plasmid backbone sequences, expression levels of *bar,* and levels of PAT protein. The cotton variety Coker 312 was used as the negative control for these analyses.

The results show the presence of a single insertion site consisting of one copy of the *bar* gene construct in LLCotten25 of 2319 bp, equal to the original transgene cassette in vector pGSV71. No vector backbone sequences were detected in LLCotten25. The inserted *bar* gene contains two intended sequence alterations compared to the native *bar* gene derived from *Streptomyces hygroscopicus*. The GTG translation initiation codon has been changed to the ATG translation codon, and the second codon of the *bar* gene (AGC encoding serine) has been modified to GAC (encoding aspartic acid) to ensure correct translation initiation in plants.

PAT protein expression levels were measured by ELISA with samples from plants grown under greenhouse conditions. Stems, roots, seeds, leaves and pollen, from both glufosinate-treated and untreated LLCotton25 plants, were examined. The PAT protein was detected in all tissues tested. The level of PAT protein accumulation was measured as PAT protein content relative to total extractable protein. Leaves and stems contained more PAT than roots, and considerably more than seeds and pollen. The average level of PAT protein in four growth stages of the life cycle of the plant (2-4 leaf-stage, 4-6 leaf-stage, early and full flowering stages) ranged from approximately 58 to 98 µg PAT/g fresh weight (fw) in both, glufosinate-treated and untreated leaf samples. PAT protein content declined in the later growth stage in leaves of both treated and untreated LLCotton25. PAT protein comprised an average of 0.21-0.35% of the total crude protein in the leaves of LLCotton25.

Insertion of the gene cassette introduced 26 novel ORFs for putative peptides spanning the 5-prime upstream and 3-prime downstream junctions of the inserted DNA. According to the applicant, only three were found to potentially give rise to short putative peptides. Further bioinformatics analysis of these three ORFs revealed no relevant sequence homologies between the theoretically predicted translation products with known toxins or allergens.

The stability of the insert in LLCotton25 plants was analysed by Southern hybridisation of leaf tissues over multiple generations. The expected integration pattern was present in all samples analysed. Phenotypic stability was demonstrated by Mendelian inheritance of the glufosinate-ammonium tolerance trait over multiple generations and field locations.

2.2 Conclusion

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in LLCotton25 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 LLCotton25 has previously been assessed as food and feed by the VKM GMO Panel (VKM, 2005; Appendix I) commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency related to the EFSAs public hearing of the application EFSA/GMO/NL/2005/13 in 2005 and in EFSA's final opinion (EFSA, 2006a; Appendix II). A brief summary from these reports are provided below.

3.1 Production of material for comparative assessment

For compositional studies, LLCotton25 was compared to its parent non-GM variety Coker 312, which is a commercial cotton variety grown in the Southern US since 1990. The comparison also included data from the scientific literature regarding the natural ranges of key compounds in various conventional cotton cultivars. Field trials were performed in year 2000 and 2001 in Arkansas, Georgia, Mississippi, Missouri, North Carolina and Texas, all belonging to the cotton growing regions of Southern United States. Each year trials were performed at 15 locations, three treatments at each location and three replications per treatment (except in one site where the sample plot was harvested three times per treatment). One site was excluded for the analysis of fatty acids as different methods had been used for the different treatment samples within the site. The three treatments consisted of: (a) non-GM cotton Coker 312 grown using conventional herbicide weed control, (b) GM LLcotton25 grown using conventional herbicide weed control, and (c) GM LLcotton25 grown with glufosinate-ammonium (Liberty®) herbicide weed control. Isolation distances of 12 m were maintained in order to avoid cross-pollination and herbicide treatment drift.

Compositional analysis was performed on whole, linted (fuzzy) cottonseed, delinted cottonseed, untoasted cottonseed meal, toasted cottonseed meal, cottonseed oil, both crude and refined, deodorised (proximates, fatty acids and tocopherol); linters (proximates only) and cotton seed hulls (proximates only) obtained from LLCotton25 and the parent line Coker 312 from the field trials conducted in the USA in 2000 and 2001. For the whole, linted cottonseeds, all material from all 15 sites in both 2000 and 2001 were analysed. For the other cottonseed products, cottonseeds from two sites for each year were processed to provide samples. Unless otherwise specified (see above), the samples were analysed for the components of importance for cotton and its various products used as food/feed as defined by the OECD consensus document for cotton (OECD, 2004), including proximates, amino acids, fatty acids, vitamin E, minerals, and the anti-nutrients gossypol, cyclopropenoid fatty acids and phytic acid, a total of 52 components. The statistical analysis of the data was carried out using a commercially available statistical package (SAS version 6.12).

The applicant also provided information on agronomic performance and phenotypic characteristics derived from several field trials in the USA, Australia and Brazil during multiple seasons. The characteristics that were analysed in these studies included parameters related

to plant morphology, seeds and plant development, reproductive traits, disease and pest susceptibility, weediness, weed control, volunteers, yields, cotton seed and fibre quality.

3.2 Compositional analysis

For data on whole, linted cottonseeds pooled from the 15 sites analysed over two years, statistically significant differences were observed for calcium and the cyclopropenoid fatty acid dihydrosterculic acid content between conventional counterpart Coker 312 and LLCotton25 treated with conventional herbicides. No differences were found between Coker 312 treated with conventional herbicides and LLCotton25 treated with Liberty herbicide. However, the differences were small and the concentrations of both analytes were within the range of values reported for other conventional cotton cultivars. Statistical comparison between the composition of LLCotton25 treated with conventional herbicides and LLCotton25 treated with Liberty were not provided by the applicant, but differences in data provided for these two groups were consistently small. Values for nearly all analytes provided for Coker 312 and conventionally and Liberty-treated LLCotton25 were within or close to the range of values reported for other conventional cotton cultivars, with the notable exception of free gossypol, which were higher in all three tested groups compared to the reported range of values. However, no statistically significant differences in total or free gossypol levels were observed in LLcotton25, treated either with conventional herbicides or Liberty, compared to the conventional counterpart Coker 312. Furthermore, the applicant pointed out that gossypol levels in the three tested groups were within the range reported by the International Life Science Institute's (ILSI) crop composition database (www.cropcomposition.org).

For the other cotton products analysed, statistically significant differences between Coker 312 and LLCotton25 were identified for a number of analytes for each product, but most of these were not considered biologically relevant as they were generally within or close to the range of values available for other conventional cotton cultivars. Possible exceptions were identified for vitamin E and zinc: observed vitamin E levels in delinted cottonseeds were variable and highest in Coker 312, and zinc levels in delinted cottonseeds and untoasted and toasted cottonseed meals from material harvested in 2000 were higher than literature values, especially for LLCotton25 treated with Liberty. The high zinc levels were attributed by the applicant to environmental conditions and/or contamination.

3.3 Agronomic traits and GM phenotype

The data from the field trials conducted in USA, Australia and Brazil showed that LLCotton25 did not differ significantly in terms of plant morphology, growth, agronomic performance, and susceptibility to diseases and pests from the non-transformed parent Coker 312. LLCotton25 did not exhibit any increased tendency towards weediness, compared to the unmodified parental line.

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 new protein, no biologically relevant differences were observed between LLCotton25, the conventional counterpart Coker 312, and other cotton cultivars. The few 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 PAT-protein, the VKM GMO Panel concludes that LLCotton25 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart 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. Greece's MY (Marketing Year) 2013/14 cotton production was 200,000 MT (Metric Tons) (Gain Report, 2014a), and Spain's MY 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 has ceased in Italy in 1991 and in Portugal in 1996.

LLCotton25, Trade Name: Fibermax™ Liberty Link™ was first cultivated in the USA in 2003 (food, feed, cultivation).

4.1 Previous evaluations by the VKM and EFSA GMO panels

LLCotton25 has previously been assessed as food and feed by the VKM GMO Panel commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency related to the EFSAs public hearings of the application EFSA/GMO/NL/2005/13 in 2005 (VKM, 2005; Appendix I). EFSA has also published a final opinion on LLcotton25 (EFSA, 2006a; Appendix II). The VKM GMO Panel and EFSA concluded that LLCotton25 was nutritionally equivalent to conventional cotton cultivars and it was unlikely that the inserted protein would cause toxic or allergic reaction to food or feed containing LLCotton25 compared to conventional cotton.

4.2 Product description and intended uses

According to the applicant, the genetic modification in LLCotton25 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 III.

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 III.

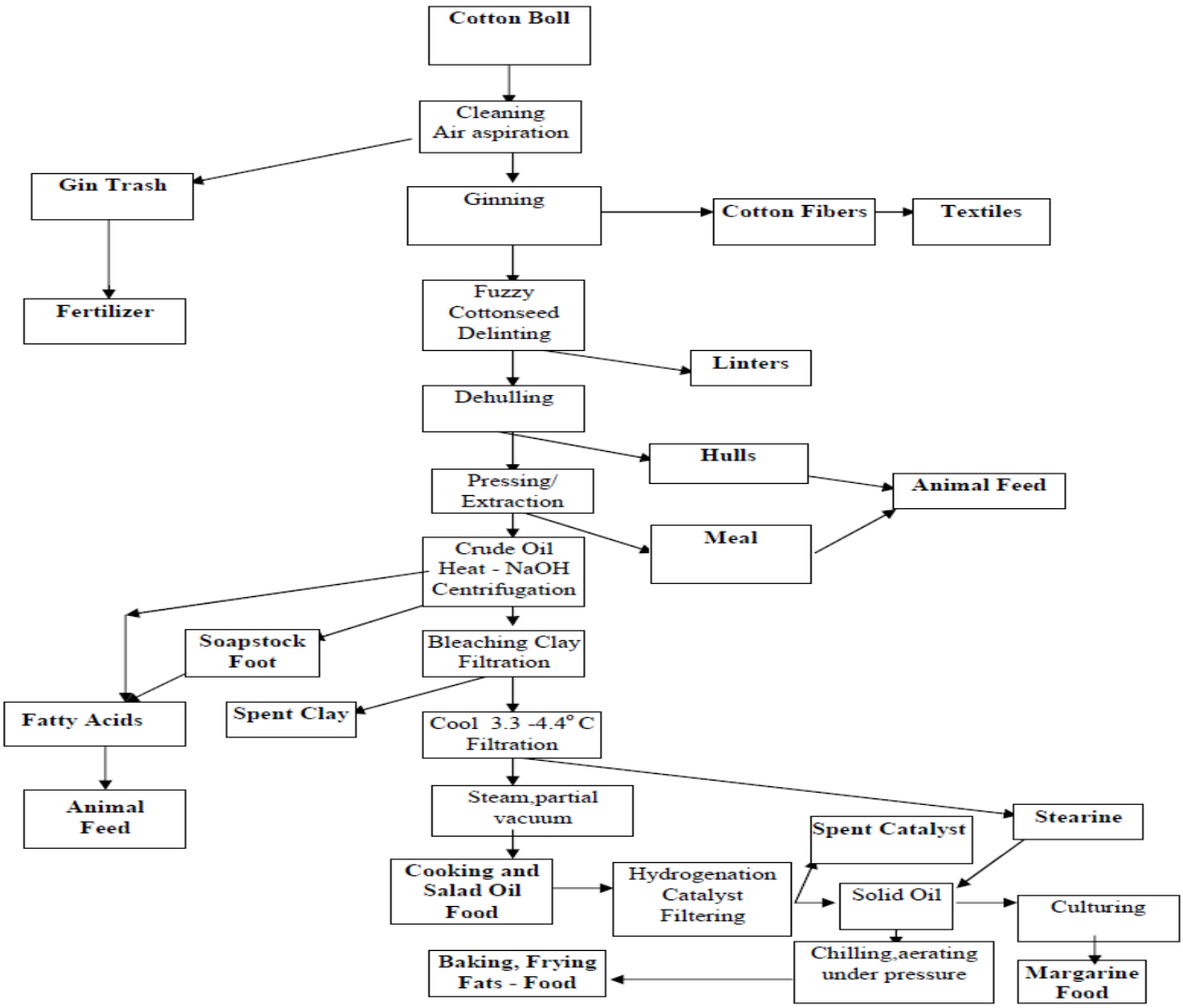

Figure 4.2-1: Processing of cotton boll, adapted from OECD (2004)

4.3 Effects of processing

According to the applicant, the commercial experiences have confirmed that the production and processing of LLCotton25 do not differ from the production and processing of the equivalent foods and feeds 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 (steamed), cooled and ground. Roasting (baking; dry heat), 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 III.

Cottonseed from LLCotton25 contains comparable levels of the naturally occurring toxicants gossypol and cyclopropenoid fatty acids relative to its conventional cotton counterpart and other conventional 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 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 protein to very low or non-detectable levels in hulls and cottonseed meal, and were not detectable in refined oil.

4.4 Toxicological assessment of LLCotton25

4.4.1 Toxicological assessment of the expressed novel protein

The PAT protein expressed in LLCotton25 is also expressed in numerous other genetically modified plants that have been assessed and considered safe by both VKM and EFSA, and has also been reviewed by others (OECD, 1999; Herouet et al., 2005). The toxicological evaluation of PAT protein produced by *E. coli* was conducted by Pfister et al. (1999), 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 LLCotton25:

- 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, 2006a)

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

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

4.4.1.1 *Acute toxicity testing of novel protein PAT*

The applicant provided data on an acute toxicity study in mice with a PAT protein encoded by the *bar* gene generated in *E. coli*. Because of the expected efficient proteolytic degradation in digestive environments, the potential toxicity of the protein was studied after intravenous injection at the dose levels of 1 and 10 mg/kg body weight. At 10 mg/kg body weight, no signs of systemic toxicity were observed.

The VKM GMO panel agrees with the 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 recognizes 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 protein PAT expressed in LLCotton25. However, a trial has been conducted with this protein and used in the assessment of numerous other transgenic crops with the same inserted *pat* genes. This is summarised below.

A repeated dose feeding study of reduced duration (14-day) relative to prevailing guidelines (OECD guideline 407; 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 body weight/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, unsupplemented group (0 PAT protein), they were not regarded as toxicologically relevant.

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.

4.4.2 Toxicological assessment of the whole GM food/feed

4.4.2.1 *90-day sub-chronic toxicity study of whole GM food/feed*

No 90-day sub-chronic oral toxicity study with LLCotton25 has been performed by the applicant. Since the compositional studies indicated that LLCotton25 was compositionally equivalent to its conventional counterpart Coker 312 and other cotton cultivars, and the molecular and compositional analyses did not indicate any unintended effects of the genetic modification, EFSA concluded that further toxicity studies with laboratory animals were not needed (EFSA, 2006a).

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, 2006b and 2010b).

4.4.3.1 *Assessment of allergenicity of the newly expressed protein*

In order to assess the potential for introduced IgE-dependent allergens in LLCotton25 sequence evaluation schemes were used to assess the similarity of the PAT protein 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 PAT protein, no immunologically significant sequence identity was detected, indicating no homology to known IgE-dependent allergens, based on amino acid sequences in PAT.

In vitro simulated gastric fluid (SGF) digestibility studies were also conducted on the protein. According to the applicant PAT was rapidly digested and no longer detectable by SDS-PAGE or western blot analysis within one minute of exposure to SGF. Thermolability results for PAT protein also indicated that the protein was not biologically active following exposure to elevated temperature (>75°C).

The results of these studies indicate that the PAT protein does not exhibit characteristics commonly attributed to an IgE-dependent allergenic protein.

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 LLCotton25 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.

LLCotton25 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. 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 this protein 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.

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. However, 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 is used 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 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 (CPFA) 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 produce gossypol so the resulting effects have been attributed to CPFA (OTGR, 2008).

Analysis of cotton products derived from LLCotton25 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 cottonseed for use as food or feed in 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

Data from a 33-day broiler chicken nutritional assessment study with LLCotton25 was submitted by the applicant (Stafford, 2004). A total of 560 Ross #508 1 day-old chicks distributed in 56 pens with 10 birds/pen, 5 males and 5 females per pen, 14 replicate pens for each diet group, were given the same feed supplemented with 10% cottonseed meal from four different cotton plant sources: FiberMax (a current commercial non-transgenic variety), Coker 312 (non-transgenic isogenic control), as well as Liberty (glufosinate)-unsprayed and sprayed transgenic LLCotton25.

The nutritional assessment study was not conducted according to the latest EFSA guidelines (EFSA, 2011b), but the VKM GMO Panel recognizes that the applicant submitted the application prior to the last guidance document. The formulated experimental diets were analysed for proximates, amino acids, fibres, minerals, tocopherols, pesticides, PCBs and toxic metals, but not for the antinutrients free or total gossypol, cyclopropenoid fatty acids or phytic acid. The duration of the feeding trial was 33 days, which is shorter than the usually practiced study period of 42 days (ILSI, 2003 and 2007). According to the applicant, the feeding trial was shortened due to a shortage of feed. The VKM GMO Panel recognises that 33 days is within the range of regularly practiced production periods for broilers and includes the especially sensitive first 21-days of life when an approximate 15-fold increase in body weight is observed. The data reported from the 33-day broiler study can therefore be considered valid for the nutritional and toxic assessment of LLCotton25.

The data and report were generally produced in compliance with US EPA Good laboratory practice regulations (40 CFR, Part 160), OECD Principle of Good Laboratory Practice (ENV/MC/CHEM (98) 17) and Japan MAFF (59 NouSan, Notification No. 3850, Agriculture Bureau), with the exception of the routine water analyses and feed contaminant screening for pesticides, PCBs and toxic metals, which were conducted by external laboratories using standard U.S. EPA procedures, but who could not claim compliance to GLP procedures (e.g. no distinct protocol).

No statistically significant differences in total feed consumption, total weight gain, feed conversion to body weight (Table 4.6.2-1), survival, or mean chilled carcass weight among the cottonseed meal types tested were observed.

Table 4.5.2-1 Feed consumption, weight gain and feed conversion for broilers fed diets containing cottonseed meal from different sources

Cottonseed meal source	Mean feed consumption (g)						Mean total weight gain[1](g)	Feed conver-sion[2]
	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Total		
FiberMax™	101.5 ± 5.0	282.8 ± 15.8	552.6 ± 75.9	666.5 ± 49.6	356.5 ± 27.6	1963.6 ± 144.0	1114.0 ± 45.3	1.8
Coker 312	90.6 ± 6.8	280.0 ± 10.1	550.4 ± 77.6	688.0 ± 50.1	397.1 ± 50.0	2006.1 ± 119.0	1087.2 ± 93.3	1.9
LLCotton25 - unsprayed	104.6 ± 105.0	295.2 ± 295.0	587.0 ± 73.6	649.1 ± 48.2	364.9 ± 17.4	2000.7 ± 94.0	1071.5 ± 64.3	1.9
LLCotton25 - sprayed	99.8 ± 4.7	281.7 ± 12.1	533.8 ± 47.9	644.0 ± 33.4	360.4 ± 24.3	1919.8 ± 79.0	1097.7 ± 50.4	1.8

[1] Mean total weight gain = live weight on day 33 – live weight on day 0

[2] Feed conversion calculated as (total feed consumption)/(total weight gain)

However, statistically significant differences between treatment groups were observed for some carcass characteristics. For broilers fed the unsprayed LLCotton25, the mean breast weight was on average 8.9% lower than that of those fed the commercial variety FiberMax™, and thigh-weight was on average 7.3% lower than those fed either the commercial or isogenic control varieties. For broilers fed the Liberty-treated LLCotton25, however, no significant differences were observed in these variables compared to broilers fed the other three diets.

These results indicate that the cottonseed meal derived from LLCotton25 is nutritionally comparable with its near isogenic non-GM counterpart Coker 312 and the other conventional cultivars included in the study.

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

4.6 Conclusions

A 33-day nutritional assessment trial with broilers did not reveal biologically relevant adverse effects or differences in the performance of animals fed diets containing cottonseed meal from LLCotton25 compared to conventional counterpart Coker 312 or another cotton cultivar. Bioinformatics analysis of the amino acid sequence of the PAT protein did not show sequence resemblance to known toxins or IgE-dependent allergens, nor has the protein been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the PAT protein will cause toxic or IgE-mediated allergic reactions to food or feed containing LLCotton25 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that LLCotton25 is nutritionally equivalent to and as safe as its conventional counterpart Coker 312 and other cotton cultivars.

5 Environmental risk assessment

5.1 Introduction

Considering the scope of the application for the LLCotton25, 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 LLCotton25 line has tolerance to the glufosinate-ammonium herbicides. Use of glufosinate-ammonium is forbidden in Norway.

Genus *Gossypium* (Malvaceae) contains about 50 diploid or allotetrapleois 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 into 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 LLCotton25 is similar to those of conventional, non-transformed cotton. Thus, under Norwegian conditions, it is highly unlikely that the seeds of the GM line 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 will have selective advantages only if exposed to glufosinate-ammonium. Consequently, the establishment of feral population of LLCotton25 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; Bensasson et al., 2004; reviewed in EFSA, 2004 and 2009).

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 feces 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 the 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 the 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 LLCotton25, 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 LLCotton25, 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 LLCotton25.

With the exception of the introduced tolerance to the herbicide glufosinate-ammonium, LLCotton25 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton cultivars, and there are no indications of an increased likelihood of spread and establishment of cotton plants in the case of accidental release of seeds from LLCotton25 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 LLCotton25 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 LLCotton25 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

The LLCotton25 genome has a complete, single integrated copy of the *bar*-expression cassette. Even though the PAT concentration is low, 0.21-0.35% of total crude protein in the leaves, it is highest in the plant parts exposed to herbicide treatment. This is consistent with the regulation by the inserted *35S* promoter, with highest activity in leaves and stems.

Out of 26 putative novel open reading frames (ORFs) identified in the GM cotton, only three short ORFs located in the 3' region of the insert were theoretically found to encode potential novel gene products. No relevant homologies were found between these theoretically predicted translation products and known toxins or allergens. 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 event.

Based on current knowledge and information provided by the applicant, the VKM GMO panel concludes that the intended changes in LLCotton25 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 in the USA during 2000 and 2001 for compositional assessments of whole linted cottonseeds, cotton lint, and different processed cottonseed products. Field trials in USA, Brazil and Australia during numerous growing seasons were performed for agronomic and GM phenotype assessments. In all trials, LLCotton25 was compared to its conventional counterpart, parent line Coker 312. LLCotton25 was grown using conventional or glufosinate-based herbicide while cotton Coker 312 was grown using conventional herbicides.

With the exception of the changes caused by the introduced transgenic trait, data provided by the applicant revealed no biologically relevant differences between LLCotton25 and its conventional counterpart Coker 312. The few 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 PAT-protein, the VKM GMO Panel concludes that LLCotton25 is compositionally, agronomically and phenotypically equivalent to its conventional counterpart and other cotton cultivars.

Food and feed risk assessment

A 33-day nutritional assessment trial with broilers did not reveal biologically relevant adverse effects or differences in the performance of animals fed diets containing cottonseed meal from LLCotton25 compared to conventional counterpart Coker 312 or another cotton cultivar. Bioinformatics analysis of the amino acid sequence of the PAT protein did not show sequence resemblance to known toxins or IgE-dependent allergens, nor has the protein been reported to cause IgE-mediated allergic reactions. It is therefore unlikely that the PAT protein will cause toxic or IgE-mediated allergic reactions to food or feed containing LLCotton25 compared to conventional cotton cultivars.

Based on current knowledge, and considering the intended uses, the VKM GMO Panel concludes that LLCotton25 is nutritionally equivalent to and as safe as its conventional counterpart Coker 312 and other cotton cultivars.

Environmental assessment

Considering the intended uses of LLCotton25, 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 LLCotton25.

With the exception of the introduced tolerance to the herbicide glufosinate-ammonium, LLCotton25 has no altered survival, multiplication or dissemination characteristics compared to conventional cotton cultivars, and there are no indications of an increased likelihood of spread and establishment of cotton plants in the case of accidental release of seeds from LLCotton25 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 LLCotton25 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 LLCotton25 does not represent an environmental risk in Norway.

Overall conclusion

Based on current knowledge and with the exception of the introduced trait, the VKM GMO Panel concludes that LLCotton25 is nutritionally, compositionally, phenotypically and agronomically equivalent to and as safe as its conventional counterpart and other cotton cultivars.

Considering the intended uses, which exclude cultivation, the VKM GMO Panel concludes that LLCotton25 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 of genetically modified plants as a result of the application of plant protection products fall outside the remit of the VKM GMO Panel.

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Appendix I

UTTALELSE OM BAYER CROPSCIENCE GENMODIFISERTE BOMULL LLCOTTON25 (EFSA/GMO/NL/2005/13)

Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

UTTALELSE OM BAYER CROPSCIENCE GENMODIFISERTE BOMULL LLCOTTON25 (EFSA/GMO/NL/2005/13)

Vurdert og godkjent av Faggruppe for genmodifiserte organismer

DATO: 2.12.05

SAMMENDRAG

Vurderingen av den genmodifiserte herbicidresistente og insektstolerante bomullslinjen LLCOTTON25 fra Bayer CropScience er utført av Faggruppe for genmodifiserte organismer under Vitenskapskomiteen for mattrygghet. Mattilsynet ber Vitenskapskomiteen for mattrygghet om å vurdere den genmodifiserte bomullslinjen LLCOTTON25 til bruk i næringsmidler og fôrvarer.

Hybriden LLCOTTON25 er fremkommet ved genmodifisering av bomullshybriden Cocker312. Hensikten med LLCOTTON25 er motstandsdyktighet mot sprøytemidlene Basta, Buster, Finale, Ignite, Liberty og Rely.

Vurdering av den genmodifiserte bomullen er basert på den dokumentasjonen som er gjort tilgjengelig på EFSAs nettside GMO EFSAnet. LLCOTTON25 er vurdert i henhold til tiltenkt bruk og de prinsipper som er lagt til grunn i EFSAs 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 hybriden LLCOTTON25 til bruk som mat og fôr.

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

Faggruppen konkluderer med at bomullsolje og fôrvarer fra LLCOTTON25 er vesentlig lik olje og fôrvarer fra umodifiserte bomullsfrø, og finner ikke at bruk av olje og fôrvarer fra LLCOTTON25 utgjør noen større helserisiko enn kommersiell olje og fôrvarer fra umodifiserte bomullsplanter.

NØKKELORD

Genmodifisert bomull, LLCOTTON25, herbicidtoleranse, *bar*, PAT, 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 (LLCOTTON25) til bruk i næringsmidler og fôrvarer. Vurdering av den genmodifiserte bomullen er basert på den dokumentasjonen som er gjort tilgjengelig på EFSAs nettside GMO EFSAnet. LLCOTTON25 er vurdert i henhold til tiltenkt bruk og de prinsipper som er lagt til grunn i EFSAs 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 parametere 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 maisen. Bruksområdet som søknaden gjelder for er import, prosessering, næringsmidler og fôrvarer i henhold til EUs Forordning (EC) nr. 1829/2003, artiklene 3(1)(c) og 15(1)(c). 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 EFSAnet, og sende kopi av vurderingen til Mattilsynet.

Linjen er fremkommet ved genmodifisering av den tradisjonelle bomullslinjen Coker312.

Produktet som ønskes vurdert, er:

Genmodifisert bomull, EFSA/GMO/NL/2005/13 (LLCOTTON25). Unik kode er. ACS-GHØØ1-3

Status i EU: Søknad under 1829/2003/EF. EFSAs frist for innspill er 2.12.05.

RISIKOVURDERING

Innledning

Den genmodifiserte bomullshybriden LLCOTTON25 ble vurdert ut fra Mattilsynets oppdrag. I henhold til Bayer CropScience 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 bomullsolje produksjonen brukes til dyrefôr.

Faggruppe for genmodifiserte organismer har på faggruppemøtet 02.02.05 vedtatt å bruke EFSAs retningslinjer som gruppens retningslinjer for vurdering av genmodifiserte planter. Prinsippene som er lagt til grunn for vurderingen, er derfor hentet fra EFSAs 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/2005/13) til bruk i næringsmidler og fôrvarer under forordning 1829/2003.

Bakgrunnsinformasjon

Genmodifisering av bomullshybriden Coker312.

Bomullslinjen Coker312 har ved hjelp av en *Agrobacterium*-mediert transformasjon med plasmidet pGSV71 fått innsatt et rekombinant DNA-fragment (ekspresjonskassett) med genet *bar* fra den gram-positive jordbakterien *Streptomyces hygroscopicus*. Det inneholder ikke markørgener for antibiotikaresistens. Celler som hadde tatt opp fragmentet ble selektert på medium med phosphinotricin (glufosinat) og utviklet videre til kallus (udifferensierte celler). Deretter regenererte man skudd og røtter og fikk grønne planter. Det kommersielle navnet til den genmodifiserte planten som er produsert av Bayer CropScience er LibertyLink cotton25, eller LLCOTTON25. Tidligere forskningsrapporter viser at CaMV35S-promoteren innsatt i bomull særlig styrer genuttrykk i vaskulært vev, blant annet i blad, rot og blomsterorganer.

Beskrivelse av de innsatte genene

Den molekylærbiologiske karakteriseringen viser at det er satt inn ett rekombinant DNA-fragment i bomullen. Fragmentet inneholder en ekspresjonskassett.

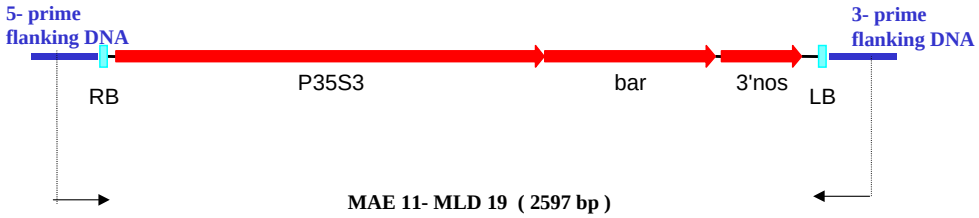
bar-ekspresjonskassetten inneholder (se figur):

a) RB-høyre grense fra plasmidet pTIB6S3, med en polylinker sekvens

b) *P35S3*-promoter fra blomkål mosaikkvirus

c) *bar* – syntetisk versjon av glufosinat resistensgenet b*ar* fra den gram-positive jordbakterien *Streptomyces viridochromogenes*. Genet *bar* uttrykker proteinet PAT. De to N-terminale kodonene i villtype genet er endret til ATG og GAC, for å garantere korrekt translasjon i planter. Sekvensen i PAT-proteinet som uttrykkes i planten er endret i forhold til villtypeproteinet med en aminosyre, fra asparginsyre til serin.

d) *LB* – terminator sekvens fra plasmidet pTiB6S3



Figur: Rekombinant T-DNA fragment med flankerende genomiske sekvenser.

Karakterisering av geninnsettingen

Analyser av genomisk DNA fra LibertyLink med Southern- og Northern blot, ELISA og PCR viser at DNA-fragmentet i LLCOTTON25 er stabilt inkorporert i plantens genom, og at *bar*-genet er aktivt i blad, stengel, rot, pollen og bomullsfrø. Det rekombinante DNA fragmentet som er satt inn i planten inneholder som vist på figuren ett fullengde *bar* gen. MAE11 og MLD19 er primere som ble benyttet til karakterisering av det rekombinante DNA fragmentet.

Molekylærbiologiske analyser viser at det rekombinante fragmentet i planten inneholder det samme genet og genelementer som er på det tilsvarende fragmentet i bakterien. Genet på det rekombinante DNA-fragmentet i LLCOTTON25 uttrykker PAT protein som er med unntak av to kodoner, identisk med proteinet som uttrykkes i bakterien. Det rekombinante fragmentet sitter ikke inne i et kodingsområde og inaktiverer heller ikke områder med regulatoriske sekvenser. Analysene viser også at det er fjernet 38 bp ved innsettingsstedet. Det var ingen åpne leserammer eller regulatoriske sekvenser i de 38 bp som ble fjernet. Undersøkelse av 5-prime flankesekvenser fra innsettingsstedet viser at *bar* kassetten ikke er integrert i kodingsområde i genomet. Northern blot med hybridiseringsprober for å plukker ut spesifikke transkripsjonsenheter fra flankeområdene ved innsettingsstedet, viser ikke uttrykk av eventuell kryptisk ekspresjon i blad, rot, stilk eller frø. Genomet til LibertyLink25 bomull inneholder én kopi av det innsatte rekombinante DNA-fragmentet, og dette genfragmentet er ikke rearrangert i planten. Det rekombinante DNA fragmentet er stabilt over minst seks generasjoner, under forskjellige vekstmiljøer og i krysninger med forskjellige bomullssorter (Fibermax966, . Fibermax832, Fibermax989, HS26 og AVS9023).

Påvisning av åpne leserammer (ORF)

Det gjort studier for å påvise åpne leserammer. Det ble påvist 26 antatte åpne leserammer. Tre åpne leserammer, ORF6, ORF7 og ORF8 ble funnet 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 sekvensdatabasene EST-other, genEMBL, GenBank, NRL_3D, PIR, GeneSeq AA, GenPept, SWISS-PROT og/eller trEML for homologi til proteiner. ORF-6 og ORF-8 har sekvenser som har likhet til en ris ”ragged stunt” virus (RRSV) polymerase. Likhet mellom ORF sekvensene og polymerasen var begrenset til et lite antall aminosyrer:

- 1,8 % (24/1357) av lengden til polymerasen for ORF-6

- 1,4 % (19/1357) av lengden til polymerasen for ORF-8

ORF-7 sekvensen har likhet med sekvensen til en serine/treonin kinase fra *Phytophthora capsici*. Likhet mellom ORF sekvensen og kinasen var begrenset til et lite antall aminosyrer: 5,3 % (22/413) av lengden til kinasen.

Genetes funksjon:

bar-genet:
Sprøytemidler basert på glufosinat-ammonium gir en irreversibel hemming av planters eget enzym glutamin syntetase. Glutamin syntetase 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.

Mengde PAT protein i frø, uten bomullsfiber, og pollen for vekstsesongen 2001 er henholdsvis 69,9 ± 6,0 µg/g ferskvekt (Range = 61,3-74,1) og 19,3 ± 39,2 µg/g ferskvekt (Range = 0,11-170). Verdiene er et gjennomsnitt av seks forsøksfelt. Forsøksfeltene var lokaliserte på områder som representerer forskjellige vekstmiljøer for bomull. Det er også målt mengde PAT protein i rot, stilk og blad. Forsøkene er utført i drivhus i 2001. Mengde PAT i rot, stilk og blad er henholdsvis 7,97 ± 1,86 µg/g ferskvekt (Range = 5,63-10,1), 36,8 ± 6,7 µg/g ferskvekt (Range = 34,3-44,5) og 52,9 ± 6,0 µg/g ferskvekt (Range = 45,1-57,3). Det er også utført drivhusforsøk i 2001 for å se på uttrykket av PAT-protein i blad under livssyklusen til planten. Northern blot analyse av *bar* RNA transkript i blad, rot, stilk og frø ved bruk av med sens og antisens probe viser uttrykk i alle vevene.

Faggruppen finner at karakteriseringen av det rekombinante innskuddet i LLCOTTON25 er tilfredsstillende, og mener at grunnlaget er tilstrekkelig for å risikovurdere bomullen. Faggruppen oppfordrer Bayer CropScience til å foreta målinger av sammensetning og agronomiske karakteristika over flere generasjoner slik EFSA-dokumentet legger opp til.

Dokumentasjon av ”vesentlig likhet”

Søkerens hoveddokument er utarbeidet etter EFSAs retningslinjer for risikovurdering av genmodifiserte planter og forordning 1829/2003. Analyser av sammensetning i bomullsfrø er fra bomullslinjene LLCOTTON25 og Coker312 (umodifisert kontrollhybrid). Prøvene som er analysert, stammer fra seks feltforsøk utført i 2000 på seks forskjellige dyrkningsområder og ni feltforsøk utført i 2001 på ni forskjellige dyrkningsområder. Alle forsøkene er utført i USA. Søker har en rimelig god beskrivelse av forsøksfeltoppsettet og hvordan prøvene er samlet inn. Dyrkningsområdene representerer forskjellige vekstmiljø for bomull. I hvert av de femten forsøksfeltene ble tre blokker med Coker312 og seks blokker med LLCOTTON25 plantet. Alle blokkene i hvert felt ble plantet og kultivert under samme forhold, med unntak av tre blokker med LLCOTTON25 som ble sprøytet med herbicidet Liberty. De tre blokkene ble sprøytet med 0,58 kg aktiv ingrediens/ha. Det er tatt ut 135 bomullsfrøprøver fra de femten forsøksfelter, og det er analysert for 52 komponenter. Dette er i henhold til EFSAs retningslinjer, se kapittel 7.1. Det også dokumentert analyser av andre agronomiske karakterer fra fjorten områder i USA i 2000 og 2001. Det også dokumenterte analyser av agronomiske trekk fra Brasil for årene 2000 til 2003.

I hoveddoumentet er resultatene fra de forskjellige analysene sammenfattet i elleve tabeller. Søker har i tabellene laget et sammendrag over analyser av utvalgte komponenter. I disse

tabellene er det i kolonnene for naturlig variasjon forskjeller med hensyn på variasjonen for flere komponenter.

Hovedkomponenter i bomullsfrø:

Søker har for LLCOTTON25 og umodifisert kontrollhybrid gitt uttrykk for at valget av analyseparametere er gjort i henhold til aksepterte internasjonale standarder og henviser til 27 standardtabeller over næringsinnhild i bomull. Henvisningene omfatter også utkastet til OECDs konsensusdokument for bomull (OECD 2004). Det er foretatt analyser av hovedkomponenter i produkter som benyttes til mat og fôr, dvs. avlede produkter fra frø (hele (fuzzy) -, linted - og delinted frø, samt "linters"). Produkter som benyttes til mat er olje og cellulose fra linters (brukes som fortykningsmiddel) og til fôr er frøskall, frøkake og mel.

Det ble analysert for aske, fett, protein, vann, karbohydrater, total fiber, kalorier, syrestabil fiber (ADF), nøytralstabil fiber (NDF), aminosyrer, fettsyrer, total vitamin E og tokoferoler (alfa, gamma, delta), fosfor, jern, kalium, kalsium, magnesium, sink, anti-næringsmidlene gossypol (totalt og fritt), fytinsyre og cyklopropenoid fettsyrer (sterkul-, malval- og dihydrosterkulsyre). Analysene ble utført under god laboratoriepraksis (GLP).

For hovedkomponentene vann (2001) og fiber (2001) er det funnet statistiske forskjeller, men disse er mindre enn 20 %.

Fettsyresammensetning i bomullsfrø:

Fettsyresammensetningen i hele linted - og delinted frø, samt u- og raffinert olje fra LLCOTTON25 og umodifisert kontrollhybrid er målt i henhold til OECDs konsensusdokument for bomull. Det ble analysert for 10 fettsyrer. Det er ikke funnet statistiske forskjeller.

Aminosyrer i bomullsfrø:

Både essensielle og ikke-essensielle aminosyrer ble analysert i hele linted - og delinted frø, ubehandlet og varmebehandlet mel. De aminosyrer som er målt er i henhold til OECD dokumentet. Det er ikke funnet store statistiske forskjeller over forsøksfeltene. Verdiene avviker ikke utover 20 %, og for alle aminosyrene ligger verdiene innenfor de typiske verdiene som er rapportert i litteraturen.

Vitaminer:

Vitamin som det i henhold til OECDs konsensusdokument for bomullsolje og -frø bør undersøkes for, er vitamin E. Det er målt for totalinnhold av vitamin E i hele -, linted - og delinted frø. Det er også målt totalinnhold av vitamin E, alfa-, delta og gamma tokoferol i uraffinert og raffinert olje. Det er ikke funnet store statistiske forskjeller for de fleste produktene, imidlertid er standardavviket for genmodifisert delinted frø stort, ca 50 %. For bomullsfrø lister OECD opp i en tabell analyser for vitaminene A, B_1, B_2, B_6, C, E, folat og niacin. OECD mener at slike analyser ikke er nødvendige for fôr.

Mineraler:

Mineralene som er målt for er fosfor, jern, kalium, kalsium, magnesium og sink. I OECDs konsensusdokument for bomull er kobber og natrium også listet opp. Det er ikke funnet store statistiske forskjeller for mineralene.

Antinæringsstoffer:

Det er for linted frø funnet statistiske forskjeller for antinæringsstoffene over flertallet av forsøksfeltene. Det er funnet relative store statistiske forskjeller for fytinsyre og dihydrosterul syre. Søker hevder at for de andre antinæringsstoffene er det for umodifiserte, sprøytet og usprøytet modifiserte planter ingen store statistiske forskjeller, p-verdi > 0,05. For fytinsyre hevder søker at det er likhet med umodifisert, men at p-verdien er < 0,05. For dihydrosterul syre er det statistiske forskjeller, men forskjellene er små, 22,2 % av gjennomsnittsverdien til Coker312. For uraffinert olje er det for total gossypol og dihydrosterulsyre funnet statistiske forskjeller mellom umodifisert og modifisert som er større enn 20 %. Slike forskjeller er ikke funnet i raffinert olje.

Toksiner og allergener.

Det er ikke målt for aflatoksiner.

Det er undersøkt for aminosyresekvenshomologi for PAT-proteinet til kjente toksiner i offentlig tilgjengelige databaser. Kriterier som er benyttet er 35 % homologi og et vindu på 80 aminosyrer. Det er ikke funnet homologe sekvenser med kjente toksiner.

Det er foretatt søk i offentlige tilgjengelige databaser for epitopsekvenshomologi for PAT proteinet med kjente allergener. Analysene er gjort i henhold til FAO/WHO sine retningslinjer (FAO/WHO 2001). Kriterier som er benyttet er oppdeling i overlappende blokker på 8 aminosyrer. Det ble ikke funnet sekvenshomologi til epitoper til kjente allergener. Det er også foretatt undersøkelser for potensielle O- og N-glykosyleringsseter siden disse ofte finnes i allergener. Det ble ikke funnet potensielle glykosyleringsseter i PAT-proteinet.

Analyse av protein og DNA i raffinert bomullsolje.

Bayer CropScience har analysert raffinert bomullsolje for protein og DNA. Hverken PAT-protein eller DNA er påvist over deteksjonsgrensen i raffinert olje. Deteksjonsgrense for DNA i olje er 0,1 µg/ml olje.

Konklusjon

Det er funnet statistiske forskjeller i enkeltparametre. Enkelte av verdiene for noen av komponentene viser det er statistiske forskjeller for enkelte forsøksfelt, men ikke for alle feltene. Imidlertid er forskjellene for alle komponenter, med unntak for dehydrosterulsyre, mellom genmodifisert bomull og umodifisert kontrollhybrid mindre enn 20 %. Faggruppen anser derfor at de forskjellene som er påvist ikke har noen helsemessig betydning.

Dokumentasjon av toksisitet og allergenisitet

Toksisitet:

PAT-protein

Søknaden inneholder dokumentasjon på fôringsforsøk med rotter og akutt intravenøs eksponering av mus med renfremstilt PAT-protein fra bakterier. Det er også utført studier med henholdsvis simulert magesaft (pepsin) (SGF) og simulert tarmsaft (pankreatin) (SIF) for å se på fordøyelighet av PAT-proteinet.

Fôringsforsøkene med renfremstilte protein er gjort i henhold til OECDs retningslinjer "OECD guidelines for testing of chemicals no. 407, Repeated dose 28-days oral toxicity studies in rodents" 1995. Det er ikke funnet noen testrelaterte endringer hos rottene ved fôring med henholdsvis 7619 og 7965 mg/kg kroppsvekt/dag for hann og hunnrotter. PAT-proteinet er heller ikke akutt-toksisk for mus ved intravenøs eksponering.

Nedbrytning av PAT i SGF (pH 2) er hurtig. PAT-proteinet degraderer fullstendig innen 30 sekunder. I SIF (pH 7,5) ble PAT fragmentert i løpet av sekunder. Fragmentene var fullstendig degradert innen 5 minutter. Påvisningen av PAT-protein og fragmenter fra proteinet er utført med Western-blot ved bruk av antistoff mot proteinet.

Fôringsforsøk på broiler:

Søknaden inneholder dokumentasjon fra 42-dagers fôringsforsøk på broilere, 560 dyr, fordelt i fire grupper som ble fôret med henholdsvis bomullsmel fra LLCOTTON25 (sprøytet og usprøytet planter), en umodifisert kontrollhybrid (Coker312) og den kommersielle umodifiserte referansehybriden (FiberMax). Det ble ikke påvist testrelaterte endringer for noen av gruppene. 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.

Allergenisitet:

Det er foretatt undersøkelse av glykosylering av PAT-proteinet. PAT-proteinet er renfremstilt fra blad fra den genmodifiserte bomullsplanten. Analyse av eventuelle bundne sukkermolekyler på PAT proteinet ble foretatt med GlycoProfile^{TM}III fluorescent detection kit. Det ble ikke påvist sukkermolekyler på PAT proteinet.

KONKLUSJON

Det er funnet statistiske forskjeller i enkeltparametere. Faggruppen finner, med unntak for fytin- og dihydrosterulsyre, at disse forskjellene er små. Faggruppen anser at de statistiske forskjellene som er påvist ikke har noen helsemessig signifikans. Da det ikke er funnet store statistiske forskjeller mellom genmodifisert – og umodifisert kontrollhybrid i enkeltparametre 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 umodifiserte bomullsplanter.

Flere studier viser at proteinet PAT ikke er akutt toksisk. Bayer CropScience har utført akuttstudier med mus for dette proteinet. Disse studiene viser at dette proteinet ikke er akutt

toksisk og fører ikke til påvisbare helseeffekter på dyrene. Bayer CropScience har foretatt fôringsforsøk med broilere, og utført sub-kroniske studier med rotter med fôr fra LLCOTTON25. Det er ikke påvist glykosylering av PAT-proteinet. 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 konkluderer med at bomullsolje fra LLCOTTON25 er vesentlig lik olje fra umodifiserte bomullsfrø, og finner ikke at bruk av olje fra LLCOTTON25 utgjør noen større helserisiko enn kommersiell olje fra umodifiserte bomullsplanter.

VURDERT AV

Faggruppe for genmodifiserte organismer:

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Koordinator fra sekretariatet: Arne Mikalsen

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Appendix II

The EFSA Journal (2006) 429,1-19

Opinion of the Scientific Panel on Genetically Modified Organisms 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[1]

(Question No EFSA-Q-2005-047)

Opinion adopted on 6 December 2006

SUMMARY

This document provides an opinion of the Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) on genetically modified LLCotton25 (Unique Identifier ACS-GHØØ1-3) developed to provide tolerance to glufosinate-containing herbicides.

In delivering its opinion the GMO Panel considered the application EFSA-GMO-NL-2005-13, additional information provided by the applicant (Bayer CropScience) and the scientific comments submitted by the Member States. The application EFSA-GMO-NL-2005-13 covers the import and processing of LLCotton25 seeds and its derived products for use as food (e.g. oil, linters) and/or feed (e.g. meal, hulls, oil). The GMO Panel assessed LLCotton25 with reference to the intended uses and the appropriate principles described in the Guidance document of the Scientific Panel on Genetically Modified Organisms for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed. The scientific assessment included molecular characterization of the inserted DNA and expression of the target protein. A comparative analysis of agronomic traits and composition was undertaken and the safety of the new protein and the whole food/feed was evaluated with respect to potential toxicity and allergenicity. Both a nutritional and an environmental assessment, including a monitoring plan, were undertaken.

LLCotton25 is derived from the cotton variety Coker312 which was transformed by *Agrobacterium*-mediated gene transfer technology. LLCotton25 expresses the *bar* gene leading to the production of the enzyme, phosphinothricin acetyl-transferase (PAT) that acetylates L-glufosinate-ammonium. The PAT enzyme confers tolerance to glufosinate-containing herbicides (trade names: Liberty®, Basta®).

Molecular analysis shows that LLCotton25 contains a single insert and does not retain backbone sequences from the vector. The GMO Panel is of the opinion that bioinformatic analysis of the DNA insert and flanking regions indicates no cause for concern, and that sufficient evidence for the stability of the insert structure was provided.

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[1] For citation purposes: Opinion of the Scientific Panel on Genetically Modified Organisms 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* (2006) 429, 1-19.

Compositional and agronomic analyses indicate that the LLCotton25 was compositionally and agronomically equivalent to other tested conventional cotton lines, except for the introduced transgenic trait. The comparative analysis of LLCotton25 therefore provides no indication for unintended effects resulting from the genetic modification. The GMO Panel is therefore of the opinion that the LLCotton25 is as safe as its non genetically modified counterparts.

The application EFSA-GMO-NL-2005-13 concerns import, processing and food/feed uses. There is therefore no requirement for scientific information on possible environmental effects associated with the cultivation of LLCotton25. The GMO Panel agrees that unintended environmental effects due to the establishment and spread of LLCotton25 will not be different from that of conventionally bred cotton.

Considering the intended uses of LLCotton25, the monitoring plan provided by the applicant is in line with the EFSA Guidance document and the opinion of the GMO Panel on post-market environmental monitoring. However the GMO Panel is aware that, due to the physical characteristics of cotton seeds and methods of transportation, accidental spillage is unavoidable. Therefore the GMO Panel recommends that specific measures are introduced to actively monitor the occurrence of feral cotton plants in areas where seed spillage is likely to occur.

In conclusion, the GMO Panel considers that the information available for LLCotton25 addresses the scientific comments raised by the Member States and that the GM LLCotton25 is as safe as its non genetically modified counterparts with respect to potential effects on human and animal health or the environment. Therefore the GMO Panel concludes that LLCotton25 is unlikely to have any adverse effect on human and animal health or on the environment in the context of its intended uses.

Key words: GMO, cotton, LLCotton25, glufosinate tolerance, food/feed safety, PAT protein, *bar* gene, PAT protein, ACS-GHØØ1-3, human and animal health, environment, import, Regulation (EC) No 1829/2003.

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BACKGROUND

On 7 March 2005 EFSA received from the Dutch Competent Authority an application (Reference EFSA-GMO-NL-2005-13), for authorisation of LLCotton25 (Unique Identifier ACS-GHØØ1-3), submitted by Bayer CropScience within the framework of Regulation (EC) No 1829/2003 on genetically modified food and feed (EC, 2003).

After receiving the application EFSA-GMO-NL-2005-13 and in accordance with Articles 5(2)(b) and 17(2)b of Regulation (EC) No 1829/2003, EFSA informed the Member States and the European Commission and made the summary of the dossier available to the public 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 5 August 2005 EFSA received additional information (requested on 14 July 2005) and declared the application as formally valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003 on 2 September 2005.

EFSA made the valid application available to Member States and the European Commission and consulted nominated risk assessment bodies of the Member States, including the national 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. The Member State bodies had three months after the date of receipt of the valid application (until 2 December 2005) within which to make their opinion known.

On 26 January 2006 the GMO Panel asked for additional data on the compositional analysis of LLCotton25. The applicant provided the complete requested information on 18 May 2006. After receipt and assessment of the full data package, the GMO Panel finalized its risk assessment of LLCotton25.

The GMO Panel carried out a scientific assessment of the genetically modified (GM) cotton LLCotton25 for food and feed uses and import and processing, in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003, taking into consideration the scientific comments of the Member States and the additional information provided by the applicant.

In giving its opinion on LLCotton25 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 receipt of the valid application. As additional information was requested by the EFSA GMO Panel, the time-limit of 6 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, the EFSA opinion shall include a report describing the assessment of the food and feed and stating the reasons for its opinion and the information on which its opinion is based. This document 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 overall opinion in accordance with Articles 6(5) and 18(5).

TERMS OF REFERENCE

The GMO Panel was requested, in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003, to carry out a scientific assessment of the genetically modified LLCotton25 for import, processing and food/feed uses.

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 GMO Panel was not requested to give an opinion on information required under Annex II to the Cartagena Protocol. The 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) LLCotton25 (Unique Identifier ACS-GHØØ1-3) was assessed with reference to its intended uses, taking account of the appropriate principles described in the Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a).

2. Molecular characterisation

2.1. Issues raised by the Member States

Questions were raised regarding (1) the putative deletions of plant DNA sequences which occurred as a consequence of the insertion and (2) the need for further data regarding such deletions (e.g. further transcriptional analysis).

Comments raised by the Member States on specific molecular detection methodologies as well as on their validation are not in the remit of the GMO Panel.

2.2. Evaluation of relevant scientific data

2.2.1. Transformation process and vector constructs

Explants of *Gossypium hirsutum* from variety Coker312 were transformed by the vector plasmid pGSV71 using *Agrobacterium tumefaciens* disarmed strain C58C1^{Rif}. The vector pGSV71 is derived from pGSC1700 and contains the origin of replication (ColE1) from pBR322 for replication in *E. coli*, the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens*, the *aadA* gene conferring resistance to streptomycin

and spectinomycin, and a T-DNA region containing a multiple cloning site and the right and left border sequences from pTib6S3.

An EcoRI/HindIII fragment inserted into the multiple cloning site comprises the following elements: the P35S3 region containing the Cauliflower Mosaic Virus 35S promoter, the *bar* gene from *Streptomyces hygroscopicus* ATCC21705 coding for glufosinate-ammonium tolerance, and the 3'nos terminator sequence including the 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37. Although the *bar* gene, commonly present in the nature/microorganisms, starts with a GTG initiation codon, the N-terminus of the *bar* coding region in LLCotton25 was modified to obtain an ATG initiation codon, thereby ensuring correct translation initiation in plants. Additionally, the second codon of the *bar* gene (AGC encoding serine) has been modified to GAC (encoding aspartic acid) prior to transformation.

The expression of the *bar* gene leads to the production of the enzyme, phosphinothricin acetyl-transferase (PAT) that acetylates L-glufosinate-ammonium and thereby confers tolerance to glufosinate-containing herbicides (trade names, Liberty®, Basta®).

2.2.2. Transgenic constructs in the genetically modified plant

Southern analysis of genomic DNA digested with five different restrictions enzymes using the entire T-DNA as a probe showed the presence of a single insertion locus. The absence of vector backbone sequences in LLCotton25 plants has been confirmed by Southern analysis using four overlapping probes that cover the entire vector backbone. Thereby it was confirmed that the *aadA* gene has not been transferred to LLCotton25.

The nucleotide sequence of the insert introduced into LLCotton25 has been determined in its entirety. The DNA sequence of the LLCotton25 insert has been proven to be identical to the corresponding transforming plasmid pGSV71 sequences. PCR analysis of the terminal repeats of the vector plasmid confirmed that the right border (RB) terminal repeat is not completely integrated in LLCotton25 as 23 bp are missing. The left border (LB) terminal repeat sequence displays a deletion of 4 bp. The sequences of the plant genome adjacent to the 3' and 5' sequences of the insert were determined using TAIL-PCR. Comparison of the flanking sequences to the respective wild type target site revealed that upon integration of the T-DNA into the genomic DNA a 38 bp fragment of genomic DNA at the target site was deleted. There was no indication that the insert is integrated in a coding region or that the insert disrupts gene regulatory sequences. These data presented proof that the insert has been integrated in a single chromosome at a single locus as intended.

2.2.3. Information on the expression of the insert

2.2.3.1. Expression of the introduced genes

Transcription of the bar gene was analysed by Northern analysis and detected in leaves, stems, roots and seeds. Pollen was not analysed. Analysis of PAT protein expression was carried out by ELISA using plants grown under greenhouse conditions. The tissues and plant samples examined were stems, roots, seeds, leaves and pollen from glufosinate-treated and untreated LLCotton25 plants. The PAT protein could be detected in all transgenic tissues mentioned. The level of PAT protein accumulation was measured as PAT protein content of total extractable protein in the following order for different tissues: leaves and stems more than roots much more than seeds and pollen.

[The average amount of PAT protein in four growth stages of the life cycle of the plant (2-4 leaf-stage, the 4-6 leaf-stage, beginning of bloom and full bloom stages) ranged from about 58 to 98 µg PAT/g fresh weight in both, the glufosinate-treated and untreated GM leaf samples. The PAT protein content declines in the latter growth stage in leaves of both treated and untreated LLCotton25. PAT protein comprised an average of 0.21-0.35% of the total crude protein in the leaves of LLCotton25.

Furthermore, field trials at different locations showed that the expression levels of the PAT protein in cotton seeds was of the same order of magnitude as found in leaves.

2.2.3.2. Putative cryptic open reading frames (ORF) in LLCotton25

Bioinformatic analysis (BLAST searches) of the LLCotton25 insert sequence indicates the presence of 26 putative open reading frames (ORFs for putative peptides of a size of 4 to 93 aa) spanning the junctions between the DNA of the nuclear cotton genome and the inserted DNA. This raises the possibility that new putative fusion proteins could be produced. Further analysis revealed that three ORF's were found at the 3' junction region of the insert that potentially could give rise to putative peptides. Bioinformatic analysis of these three ORF-sequences showed no sequence homology with known toxins or allergens. These results do not raise any safety concerns.

2.2.4. Inheritance and stability of inserted DNA

LLCotton25 was developed from cotton line Coker312 by *Agrobacterium*-mediated gene transfer technology. The inheritance of the introduced trait follows a Mendelian pattern. The LLCotton25 event has also been introduced into different genetic backgrounds (FiberMax966, FiberMax832 and FiberMax989, picker varieties; HS26 and AVS9023, stripper varieties). Such seeds with the LLCotton25 event in different genetic backgrounds were grown under greenhouse conditions and treated by a standard spray test using the herbicidal agent glufosinate-ammonium. The results confirmed phenotypically the presence of the herbicide-tolerant trait and indicated the presence of the functional PAT protein. DNA from individual plants of both LLCotton25 in different genetic backgrounds and its non GM counterparts was subjected to Southern analysis with a probe specific for the insert. Interpretation of the banding patterns from various restriction enzyme digests of the DNA of LLCotton25 in different genetic backgrounds demonstrated the stability at the genetic level over multiple generations.

The same kind of analysis was performed with genomic DNA isolated from plants of generation T6 grown at 11 different locations (i.e. different environmental conditions). The DNA was isolated and digested by a restriction enzyme (NcoI) recognizing two restriction sites within the inserted DNA. The entire T-DNA employed as probe for Southern analysis detected the expected banding pattern in all samples analysed including the two bands representing the junctions between the inserted DNA and the genomic plant DNA. These findings demonstrate the molecular stability of the transformation event LLCotton25 under different environmental conditions.

These results indicated phenotypic, genetic and molecular stability of the insert present in the LLCotton25 event in different genetic backgrounds, over several generations and under different environmental conditions.

2.3. Conclusion

The molecular characterisation data establish that LLCotton25 contains a single insert. The insert in LLCotton25 is constituted by the predicted and verified genetic elements present in the T-DNA in the transformation vector and does not contain genes from the vector backbone sequences. In addition analysis of ORFs spanning the two junction regions in the genetically modified cotton was performed by bioinformatic analysis. Bioinformatic analysis showed that, in the event that the three putative ORFs in the 3' region are expressed, any resulting polypeptides would show no significant sequence homology with known toxins or allergens.

The GMO Panel is of the opinion that the molecular characterisation of the DNA insert and flanking regions of LLCotton25 does not raise any safety concerns, and that sufficient evidence for the stability of the insert structure was provided.

3. Comparative analysis

3.1. Issues raised by Member States

Questions were raised regarding (1) the validity of the statistical analysis of the compositional data, (2) including the need for a statistical analysis of compositional data separately for each growing season and location.

3.2. Evaluation of relevant scientific data

Having considered the information provided in the application and the Member States comments, the GMO Panel requested from the applicant further data with respect to the statistical analysis of the compositional data as well as on the range of the gossypol content. The applicant provided an additional statistical analysis that the GMO Panel found adequate.

3.2.1 Choice of comparator and production of material for the compositional assessment

For compositional studies, LLCotton25 was compared to its parent variety, Coker312 which is a commercial cotton variety grown in the Southern US since 1990. The comparison also included data from the scientific literature regarding the natural ranges of key compounds in conventional cotton. Field trials were performed in year 2000 and 2001 in Arkansas, Georgia, Mississippi, Missouri, North-Carolina and Texas, all belonging to the cotton growing regions of Southern United States. Each year trials were performed at 15 locations, three treatments at each location and three replications per treatment (except in one site where the sample plot was harvested three times per treatment). One site was excluded for the analysis of fatty acids as different methods had been used for the different treatment samples within the site. The three treatments consisted of: (a) non-GM cotton grown using conventional herbicide weed control, (b) GM cotton grown using conventional herbicide weed control, and (c) GM cotton grown with glufosinate-ammonium (Liberty®) herbicide weed control. Isolation distances of 12 m were maintained in order to avoid cross-pollination and herbicide treatment drift.

3.2.2. Compositional analysis

Materials were collected from the field trials for a compositional analysis of seeds and lint. The seeds were analysed for key nutrients, anti-nutrients, and toxicants as defined by the OECD consensus document for cotton (OECD, 2004). Thus besides proximates (moisture, total fat, total protein, ash, total carbohydrates, crude fibre, acid detergent fibre (ADF), and neutral detergent fibre (NDF), the samples were analysed for 18 amino acids, 10 fatty acids (C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C22:0, and C24:0), minerals (calcium, phosphorus, magnesium, potassium, iron, zinc), vitamin E, anti-nutrients (cyclopropenoid fatty acids and phytic acid) and the toxicant gossypol (free and total gossypol). Lint samples were only analysed for proximates.

The statistical analysis of compositional data collected each year was carried out on a per location basis, using data from 3 replicates per location, and on the combined data from all sites each year. In addition to comparing the composition of LLCotton25 with that of the non-GM parent variety, Coker312, the composition of the GM cotton was also compared to data from commercial cotton lines available in the literature (see Section 3.2.1.). The GMO Panel found the presentation of data adequate.

Although the PAT protein was detected at low amounts varying from 0.13 to 0.44 µg/g fresh weight (FW) in some non-GM seed samples (1 sample out of 4 in year 2000 and 5 out of 27 kernel samples in year 2001 with levels ranging from 0,132 µg/g to 0,365 µg/g FW), data from all control samples were used in the statistical evaluation of the composition of LLCotton25 as compared to Coker312. For comparison, the level of PAT protein in LLCotton25 seeds is 61.3–74.1 µg/g FW. The low level of the PAT protein in the control material is unlikely to have an impact on the outcome of the comparative compositional analysis and the GMO Panel therefore accepts the use of this control material.

The compositional comparisons occasionally revealed statistically significant differences of some compounds. In the analysis per site statistically significant differences were observed for a number of fatty acids i.e. myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid analysed in seeds. However, the reported levels all fell well within the natural ranges reported in the literature (OECD, 2004). In the analysis per year statistically significant differences were also observed in compounds i.e. calcium, total gossypol and aspartic acid analysed in seeds. For most of these compounds the differences were small and within the natural ranges reported in literature. Only the free gossypol levels (a toxicant) in both the GM LLCotton25 and the non-GM comparator fell outside the natural ranges reported in the literature. The applicant was approached for an explanation. In reply, the applicant presented an additional statistical analysis that showed that there were no significant differences in free gossypol levels between LLCotton25 and the non-GM comparator when analysed over the 15 sites tested, and that the levels fell within ranges reported in the ILSI (International Life Science Institute) crop composition database (http://www.cropcomposition.org/).

The GMO Panel considered the observed compositional differences between LLCotton25 and its comparator in the light of the field trial design, measured biological variation and the level of the studied compounds in conventional cotton varieties, and concluded that LLCotton25 can be considered to have a composition equivalent to the non-GM counterpart and other conventional cotton lines, except for the introduced trait.

3.2.3. Agronomic traits and GM phenotype

The applicant provided information on agronomic performance and phenotypic characteristics derived from several field trials in the USA, Australia and Brazil during multiple seasons. The characteristics that were analyzed in these studies included parameters related to plant morphology, seeds and plant development, reproductive traits, disease and pest susceptibility, weediness, weed control, volunteers, yields, cotton seed and fibre quality.

The GMO Panel noted that differences were observed in some instances with regard to several characteristics related to plant density, fibre quality, and phenotype (plant, seed, and flower). However these differences did not occur consistently in the various studies and, therefore, were not considered to be related to the genetic modification. The GMO Panel concludes that LLCotton25 is not agronomically different from other currently grown non-GM cotton varieties, with the exception of the newly introduced trait.

3.3 Conclusion

Compositional and agronomic analyses carried out on glufosinate-treated and conventionally treated LLCotton25, its non-GM counterpart Coker312 and other conventional cotton lines indicated that the LLCotton25 was compositionally and also agronomically equivalent to conventional cotton lines, except for the introduced transgenic trait. The comparative analysis of LLCotton25 therefore provided no indication for unintended effects resulting from the genetic modification.

4. Food/Feed safety assessment

4.1. Issues raised by Member States

Questions were raised regarding the need for further animal feeding studies, such as a 90-day subchronic toxicity study in rats, nutritional studies in ruminants, as well as allergenicity studies.

4.2. Evaluation of relevant scientific data

4.2.1. Product description and intended use

The scope of application EFSA-GMO-NL-2005-13 includes the import and processing of LLCotton25 and its derived products for use as food/feed. Thus the possible uses of LLCotton25 includes the production of refined oil from seeds and cellulose from linters for use as human food, and use of cottonseed meal (or cake), hulls and linters in animal feed.

4.2.2. Stability during processing

Since LLCotton25 has been found to be compositionally equivalent to conventional cotton, except for the newly expressed trait (see Section 3.2.2), the stability during processing is not expected to be different from conventional cotton varieties.

4.2.3. Toxicology

4.2.3.1. PAT protein used for safety assessment

Due to the low expression level of the PAT protein in LLCotton25 most of the safety studies were conducted with a PAT protein encoded in *E. coli* by the *bar* gene (PAT/*bar* protein). Examination of the structure and function of these plant and bacterial PAT proteins have shown a high degree of similarity, based on their size and sequence homology, enzymatic activity, immunoreactivity and absence of glycosylation. The PAT/*pat* and PAT/*bar* proteins have been shown to be structurally and functionally equivalent (Wehrmann *et al.*, 1996; Herouet *et al.*, 2005). Therefore the GMO Panel accepts the PAT/*bar* as well as the PAT/*pat* test material derived from *E. coli* for the safety assessment of PAT protein present in LLCotton25.

4.2.3.2. Toxicological assessment of expressed novel protein in LLCotton25

(a) Acute and repeated short term toxicity testing

The applicant provided data on an acute toxicity study in mice with a PAT protein encoded by the *bar* gene generated in *E. coli*. Because of the expected fast proteolytic degradation in digestive environments, the potential toxicity of the protein was studied after intravenous injection at the dose levels of 1 and 10 mg/kg body weight. Even at the relatively high dose of 10 mg/kg body weight, no signs of systemic toxicity were observed.

No oral toxicity studies with the *bar* encoded PAT protein are available in this application. However, a 14-day repeated dose feeding study conducted in rats with the PAT protein encoded by the *pat* gene was provided. Groups of five male and female Wistar rats (HanIbm:WIST) received diets containing the PAT protein (lyophilized powder) at levels of 0 (group 4), 5 (group 2) and 50 (group 3) g/kg diet. The high level corresponded to a dose of 7.6 and 7.9 mg/kg BW/day for males and females, respectively. A second control group (group 1) was fed a standard rodent diet. In the study there were no remarkable findings apart from statistically significant increases in blood cholesterol levels (males of groups 2 and 3) and phospholipid levels (females of group 3 and males of groups 2 and 3). These effects, which also occurred in one of the control groups (group 4), are not regarded as toxicologically relevant. In conclusion, feeding the PAT protein to rats for 14 days revealed no indications for adverse effects up to the highest dose tested.

(b) Degradation in simulated digestive fluids

The PAT/bar protein expressed in *E. coli* was used in the degradation studies. The PAT protein was tested for *in vitro* digestibility in simulated gastric fluid containing pepsin. Degradation occurred rapidly, as shown by polyacrylamide gel electrophoresis (within 30 seconds at pH 2). Rapid degradation was also demonstrated by western blots in simulated intestinal fluid (pH 7.5) in presence of pancreatin. During degradation fragments of 7 kD appeared transiently. These fragments disappeared after 5 minutes of incubation. These *in vitro* digestion experiments demonstrate that the PAT protein encoded by the *bar* gene is rapidly degraded in simulated gastric and intestinal conditions.

(c) Bioinformatic studies

Searches for sequence homology between the *bar* gene encoded PAT protein in LLCotton25 and other proteins indicated significant homology only with other acetyltransferases. No sequence homology with known toxins was shown.

4.2.3.3. Toxicological assessment of new constituents other than proteins

No new constituent other than the PAT protein is expressed in LLCotton25 and no relevant changes in composition were detected by the compositional analysis.

4.2.4. Toxicological assessment of the whole GM food/feed

The comparative compositional analysis and agronomic analyses showed that LLCotton25 is substantially equivalent to its non-GM counterpart Coker312 and other commercially grown cotton varieties except for the introduced trait. In addition, the analyses provided no indication for unintended effects of the genetic modification and therefore the GMO Panel concluded that no additional safety studies with laboratory animals are needed.

4.2.5. 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).

4.2.5.1. Assessment of allergenicity of the newly expressed proteins

Potential expression products were analysed for possible homology to known allergens. The total amino acid sequence of the PAT/*bar* protein was compared to that of known allergens. The results of a linear epitope homology search over 8 contiguous amino acids showed no similarities between epitopes of known allergens and the PAT protein expressed by LLCotton25. Further, bioinformatic search with 80 amino acids window indicated no similarity with potential allergenic proteins applying a 35 % identity criterion. Based on these results PAT protein presented a high structural similarity only with non-allergenic PAT proteins, while no evidence for any homology to known toxic or allergenic proteins was found. Searches for potential N-glycosylation sites, which are often found on allergens, were negative. PAT is not stable in an acidic environment and is rapidly degraded under simulated gastric and intestinal conditions. It is also rapidly degraded and inactivated in stomach fluids of cattle and pig. Based on these results the GMO Panel considers that the newly expressed PAT protein is not likely to be allergenic.

4.2.5.2. 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 transgene 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 GMO Panel since cotton (*Gossypium hirsutum* L.) is not considered to be an allergenic food. Furthermore, the main cotton seed product in human food, cotton seed 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. The applicant states that no toxic or allergic effects in workers handling LLCotton25 in the field since its first field release in 1999 have been reported.

The GMO Panel concludes that the information presented confirms that the overall allergenicity of the whole plant is not changed.

4.2.6. Nutritional assessment of GM food/feed

A total of 560 Ross commercial one-day old broiler chicks were used in a 33-day study to evaluate the nutritional characteristics of cotton seed meal derived from LLCotton25. The study consisted of four treatments in which 10% of the diet consisted of cotton seed meal derived from either LLCotton25 not treated with glufosinate-containing herbicide, LLCotton25 treated with glufosinate-containing herbicide, the near isogenic counterpart (Coker312), or a commercial variety. There were 10 birds and 14 replicates in each treatment group. There were no statistically significant differences between treatments in total feed consumption, total live-weight gain, and feed conversion efficiency. Although the thigh and breast weight from broilers fed the diet containing cotton seed meal from LLCotton25 not treated with glufosinate-containing herbicide was significantly lower when compared with the values for broilers receiving cotton seed meal from the commercial variety, there were no statistically significant differences in any of the weight variables between chickens fed the diet containing cotton seed meal from LLCotton25 treated with herbicide and the other three dietary treatments. These results indicate that the cotton seed meal derived from LLCotton25 treated with glufosinate-containing herbicide is nutritionally comparable with its near isogenic non-GM counterpart and the commercial varieties included in the study.

As the extensive comparative compositional analysis of LLCotton25 provided no indication for unintended effects of the genetic modification under consideration in this opinion, the GMO Panel concluded that no additional safety or nutrition study with laboratory animals is needed.

4.2.7. Post-market monitoring of GM food/feed

The risk assessment concluded that no data have emerged to indicate that LLCotton25 is any less safe than its non-GM comparator. In addition, LLCotton25 is, from a nutritional point of view, substantially equivalent to conventional cotton. Therefore, and in line with the Guidance document (EFSA, 2006a), the GMO Panel is of the opinion that post-market monitoring of the GM food/feed is not necessary.

4.3. Conclusion

No toxicity of the PAT protein was observed in the 14-day repeated dose feeding study conducted in rats and in the acute toxicity study in mice after intravenous injection. The PAT protein is rapidly degraded in simulated gastric and intestinal conditions. The PAT protein shows no homology with known toxins and/or allergens. An extensive compositional analysis showed no consistent compositional differences to conventional cotton with relation to key nutrients and anti-nutrients. A 33-day feeding study with broiler chickens did not provide any indications that the cotton seed meal derived from LLCotton25 treated with glufosinate-containing herbicides is nutritionally different from meal produced from its near isogenic non-GM counterpart or commercial varieties included in the study. The GMO Panel considers that no additional animal safety or nutritional study is needed. The GMO Panel is therefore of the opinion that the LLCotton25 is as safe as its non GM counterparts and that the overall allergenicity of the whole plant is not changed.

5. Environmental risk assessment and monitoring plan

5.1 Issues raised by Member States

Questions were raised regarding (1) the interactions of LLCotton25 with the biotic environment, (2) the need for more information on the application of herbicides, from season to season and in all the intended LLCotton25 growing countries, (3) the cotton-weeds for those member countries of the EC, where cotton is cultivated, (4) the gene-environment interactions, unintended or pleiotropic effects and (5) the need for data on the overwintering capacity of LLCotton25 and its parental variety Cocker 312 seeds.

Further comments were raised with respect to the environmental monitoring plan regarding (6) the need for an updated case-specific monitoring plan and (7) a more detailed general surveillance plan.

5.2. Evaluation of relevant scientific data

5.2.1. Environmental risk assessment

The scope of application EFSA-GMO-NL-2005-13 includes import, processing and food/feed uses of LLCotton25. Considering the proposed uses of LLCotton25, excluding cultivation purposes, the environmental risk assessment is limited to unintentional release into the environment of GM seeds during transportation and processing or when cotton seeds are used as food or feed.

As this application is not for cultivation, concerns regarding the use of glufosinate-containing herbicides on LLCotton25 apply only to imported and processed cotton products that may have been treated with those herbicides in the countries of origin. However the GMO Panel is aware that glufosinate-containing herbicides are used in Europe on other crops and that the risk assessment of such compounds is within the scope of Directive 91/414/EEC concerning the placing of plant protection products on the market.

5.2.1.1. Potential unintended effects on plant fitness due to the genetic modification

Gossypium herbaceum and *Gossypium hirsutum* are highly domesticated crops which have been grown in Southern Europe since the 19th century, giving rise to feral plants which can occasionally be found in the same area (Davis, 1967; Todaro, 1917). There are no wild relatives in Europe. The main cultivated cotton (*Gossypium hirsutum* L.) is an annual self-pollinating crop which has a relatively low percentage of cross-pollination (Xanthopoulos & Kechagia, 2000; Turley & Kloth, 2002). Seed and pollen dispersal are potential sources of gene flow to conventional varieties and to occasional feral cotton plants. Cotton pollen is heavy and sticky so that the natural crossing is made mostly by insect pollinators (wild bees, honeybees, etc). Seeds are the only survival structures.

However, if accidental release into the environment occurs, these GM cotton plants will only be fitter in the presence of glufosinate-containing herbicides which are not currently used on cultivated cotton or in most areas where the GM cotton might be spilled.

In addition to the data presented by the applicant, the GMO Panel is not aware of any scientific report of increased fecundity or ferality of herbicide tolerant cotton in regions where GM cotton is cultivated. There is no information to indicate change in survival capacity (including

overwintering). Furthermore there is no evidence that the herbicide tolerance trait introduced by genetic modification result in increased invasiveness of any crop species, except in the presence of the herbicide. 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 GMO Panel is thus of the opinion that, even in case of accidental release into the environment, LLCotton25 is very unlikely to show any enhanced fitness and would behave as conventional cotton.

5.2.1.2. Potential for gene transfer

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

(a) Plant to bacteria gene transfer

Based on present scientific knowledge and elaborated in more detail elsewhere (EFSA, 2004), gene transfer from GM plants to microorganisms under natural conditions is extremely unlikely, and its establishment would occur primarily through homologous recombination in microorganisms.

Transgenic DNA is a component of some or most of the food and feed products derived from the GM cotton. Therefore microorganisms in the digestive tract of humans and animals (domesticated animals and other animals feeding on fresh and decaying GM plant material) may be exposed to transgenic DNA.

The *bar* gene is known to be ubiquitous in soil microbial populations. Taking into account the origin and nature of *bar* gene and the lack of selective pressure in the intestinal tract, the likelihood that horizontal gene transfer would confer selective advantages or increased fitness of microorganisms is very limited. For this reason it is very unlikely that *bar* gene from LLCotton25 would become established in the genome of microorganisms in the environment or human and animal digestive tract. In the very unlikely event that such a horizontal gene transfer would take place, no adverse effects on human and animal health and the environment are expected as no new traits would be introduced into microbial communities.

(b) Plant to plant gene transfer

Considering the intended uses of LLCotton25 and the physical characteristics of cotton seeds, a possible pathway of dispersal is from seed spillage and pollen of occasional feral GM cotton plants originating from accidental seed spillage during transportation and/or processing.

Gossypium herbaceum is reported (Zohary and Höpf, 2000) to be a traditional fiber crop in the Eastern Mediterranean area already in the pre-Columbus period (before 1500 AD). The genus *Gossypium* consists of at least four crop species: *G. arboreum*, *G. barbadense*, *G. herbaceum* and *G. hirsutum*. 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 (Davis, 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 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 if feral populations of

LLCotton25 were established or transgene flow occurred to cultivated and feral cotton, a selective advantage would only occur if the complementary glufosinate-containing herbicides were applied.

5.2.1.3. Potential interactions of the GM plant with non-target organisms

Because the level of exposure to PAT protein is so low, potential effects on non-target organisms are considered by the GMO Panel as very unlikely.

5.2.1.4. Potential interaction with the abiotic environment and biogeochemical cycles

Because the level of exposure to PAT protein is so low, potential effects on the abiotic environment and biogeochemical cycles are considered by the GMO Panel as very unlikely.

5.2.2. 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. 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.

General surveillance is related to risk management, and thus a final adoption of the general surveillance plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific quality of the general surveillance plan provided by the applicant (EFSA, 2006a). The only significant exposure of the environment to the genetically modified cotton would be related to accidental spillage. The GMO Panel is aware that, due to the physical characteristics of cotton seeds and methods of transportation, accidental spillage is unavoidable. Therefore the GMO Panel recommends that specific measures are introduced to actively monitor the occurrence of feral cotton plants in areas where seed spillage is likely to occur as proposed in the EFSA Guidance document (EFSA, 2006a) and the opinion of the GMO Panel on post-market environmental monitoring (EFSA, 2006b).

In other respects the GMO Panel is of the opinion that the general approaches and measures of the monitoring plan proposed by the applicant are in line with the EFSA opinion on post-market environmental monitoring (EFSA, 2006b) as well as with the intended uses of LLCotton25. Since the environmental risk assessment does not cover cultivation and identifies no potential adverse environmental effects, no case-specific monitoring is necessary.

The GMO Panel agrees with the proposal made by the applicant on the reporting intervals.

5.3. Conclusion

LLCotton25 is being assessed for import, processing and food/feed uses and thus there is no requirement for scientific information on environmental effects associated with cultivation. The GMO Panel considered the environmental issues raised by Member States in the above sections of Chapter 5 and concludes as follows: *Gossypium 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 LLCotton25 seeds occurs, LLCotton25 plants will only be fitter in the presence of glufosinate-containing herbicides which are not currently used on cultivated cotton or in most areas where the GM cotton might be spilled. Therefore the GMO Panel is of the opinion that the likelihood of the establishment and spread of LLCotton25 is very low and that unintended environmental effects due to this GM cotton will be no different from that of conventional cotton varieties. Furthermore the scope of the monitoring plan provided by the applicant is in line with the intended uses of LLCotton25 since this does not include cultivation.

The GMO Panel is aware that, due to the physical characteristics of cotton seeds and methods of transportation, accidental spillage is unavoidable. Therefore the GMO Panel recommends that, within general surveillance, specific measures are introduced to actively monitor the occurrence of feral cotton plants in areas where seed spillage is likely to occur.

CONCLUSIONS AND RECOMMENDATIONS

The GMO Panel was requested to carry out a scientific risk assessment of the LLCotton25 for food and feed uses, import and processing.

LLCotton25 has been modified to express the *bar* gene providing tolerance to glufosinate-containing herbicides. The GMO Panel has evaluated the molecular analysis of the GMO and recognised that only the intended DNA fragment has been integrated at a single locus. From the sequence data provided by the applicant there is no reason to assume that the DNA regions transferred code for toxic and/or allergenic products.

Comparative analysis has shown that the LLCotton25 is compositionally and agronomically equivalent to conventional cotton lines, except for the introduced transgenic trait. The risk assessment included an analysis of data from appropriate animal feeding studies. The GMO Panel concluded that the LLCotton25 is as safe as its non GM counterparts and that the overall allergenicity of the whole plant is not changed.

The application EFSA-GMO-NL-2005-13 concerns import, processing and food/feed uses. There is therefore no requirement for scientific information on possible environmental effects associated with the cultivation of LLCotton25. However the GMO Panel is aware that, due to the physical characteristics of cotton seeds and methods of transportation, accidental spillage is unavoidable. Therefore the GMO Panel recommends that, within general surveillance, specific measures are introduced to actively monitor the occurrence of feral cotton plants in areas where seed spillage is likely to occur.

In conclusion, the GMO Panel considers that information available for LLCotton25 addresses the outstanding questions raised by the Member States and considers it unlikely that LLCotton25 will have any adverse effect on human and animal health or on the environment in the context of its proposed uses.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Dutch Competent Authority (VROM), dated 3 March 2005, concerning a request for placing on the market of glufosinate-ammonium tolerant cotton LLCotton25 in accordance with Regulation (EC) 1829/2003, submitted by Bayer Crop Science (ref. 050303-BG01).

2. Letter from EFSA to applicant, dated 14 July 2005, with request for clarifications/additional information (ref. SR/SM/sp (2005) 933).

3. Letter from the applicant, dated 5 August 2005, providing EFSA with an updated version of the application EFSA-GMO-NL-2005-13 submitted by Bayer Crop Science under Regulation (EC) 1829/2003:

   Part I – Technical dossier
   Part II – Summary
   Part III – Cartagena Protocol
   Part IV – Labelling and Unique Identifier
   Part V – Samples and Detection
   Part VI – Additional information for GMOs

4. Letter from EFSA to applicant, dated 2nd September 2005, delivering the 'Statement of Validity' for application EFSA-GMO-NL-2005-13, LLCotton25 submitted by Bayer Crop Science under Regulation (EC) 1829/2003 (ref. SR/SM/sp (2005) 1110).

5. Letter from EFSA to applicant, dated 15 September 2005, with request for additional information on detection method/reference material (ref. SR/KL/jq (2005) 1154).

6. Letter from EFSA to applicant, dated 3 November 2005, regarding additional data received from the applicant and the time-schedule for application EFSA-GMO-NL-2005-13 (ref. SR/KL/cz (2005) 1326).

7. Letter from EFSA to applicant, dated 26 January 2006, with request for additional information (ref. SR/SM/cz (2006) 1336033).

8. Letter from applicant to EFSA, dated 17 May 2006, providing additional information upon EFSA request.

9. Letter from EFSA to applicant, dated 23 October 2006, with respect to the time-schedule for application EFSA-GMO-NL-2005-13 (ref. SR/SM/jq (2006) 1797821).

10. Letter from EFSA to applicant, dated 27 October 2006, with request for additional information on detection method/reference material (ref. SR/SM/jq (2006) 1806662).

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ACKNOWLEDGEMENT

The GMO Panel wishes to thank Thomas Frenzel, Boet Glandorf, Esther Kok, Gijs Kleter and Richard Phipps for their contribution to the opinion.

Appendix III

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 (–) entiomer 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 administrated 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, 2008).

Gossypol levels in the reported feeding trials

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

Total or free gossypol contents in cottonseed, toasted cottonseed meal or formulated experimental diets were not provided (Stafford, 2004).