SCIENTIFIC OPINION

Scientific Opinion on application (EFSA-GMO-NL-2005-16) for the placing on the market of insect resistant genetically modified cotton (*Gossypium hirsutum* L.) 281-24-236 x 3006-210-23 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Dow AgroSciences[1]

EFSA Panel on Genetically Modified Organisms (GMO)[2, 3]

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ABSTRACT

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

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

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

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

KEY WORDS

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

SUMMARY

Following the submission of an application (EFSA-GMO-NL-2005-16) under Regulation (EC) No 1829/2003 from Dow AgroSciences, The EFSA Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel) was asked to deliver a scientific opinion on the safety of the insect resistant genetically modified (GM) cotton 281-24-236 x 3006-210-23 (Unique identifier DAS-24236-5 x DAS-21Ø23-5) for food and feed uses, import and processing.

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

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

The molecular characterisation data established that event 281-24-236 contained a single insertion with the *cry1F* and *pat* genes and an additional *pat* fragment. The event 3006-210-23 contained a single insertion with the *cry1Ac* and *pat* genes. Analyses of the integration sites including sequence determination of the inserted DNA and flanking regions and bioinformatic analyses have been performed. Bioinformatic analyses of the flanking regions indicated that the 281-23-236 insertion occurred into the 3' untranslated region of a gibberellin 20-oxidase gene. Updated bioinformatic analyses of event 3006-210-23 did not indicate the interruption of any known endogenous coding or regulatory sequences. Bioinformatic analyses of junction regions demonstrated the absence of any new open reading frames (ORFs) potentially coding for known toxins or allergens. The expression of the genes introduced by genetic modification has been sufficiently analysed and the stability of the genetic modification has been demonstrated over several generations for both 281-24-236 and 3006-210-23 events. Moreover, the insert structure of both single events was retained in the stack 281-24-236 x 3006-210-23. The EFSA GMO Panel is of the opinion that the molecular characterisation of the DNA inserts and flanking regions of cotton 281-24-236 x 3006-210-23 does not raise any safety concern, and that sufficient evidence for the stability of the genetic modification was provided.

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

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

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

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

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

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BACKGROUND

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

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

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

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

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

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

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

TERMS OF REFERENCE

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

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

ASSESSMENT

1. Introduction

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

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

2. Issues raised by Member States

The scientific comments raised by Member States are addressed in details in Annex G of the EFSA overall opinion and have been considered in this EFSA GMO Panel scientific opinion[4].

3. Molecular characterisation

3.1. Evaluation of relevant scientific data

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

The GM cotton 281-24-236 x 3006-210-23 has been produced by conventional crossing between lines of the GM cotton events 281-24-236 and 3006-210-23 to combine resistance to certain lepidopteran insect pests. Cotton 281-24-236 x 3006-210-23 contains the *cry1F* from event 281-24-236, the *cry1Ac* from event 3006-210-23 and the *pat* genes from both events.

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

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

3.1.2. **Evaluation of the single cotton events**

3.1.2.1. Cotton 281-24-236

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

The vector pAGM281 used to generate cotton 281-24-236 contained two expression cassettes on the T-DNA, one for the *cry1F* and one for the *pat.* This *cry1F* is a synthetic chimeric gene with the N-terminal core toxin *cry1Fa* and the C-terminal part from *cry1Ca* and *cry1Ab* (Gao *et al*., 2006). The coding sequence was modified in order to introduce two restriction sites and this resulted in two amino acid substitutions (F604L and Q640R) (Gao *et al*., 2006). The *cry1F* coding sequence is driven by the constitutive mannopine synthase promoter from *A. tumefaciens* pTi15955 (Barker *et al*., 1983) fused with four copies of the octopine synthase (OCS) enhancer from *A. tumefaciens* pTiAch5 (Ellis *et al*., 1987) ((4OCS)ΔMas2' promoter). The *pat* expression cassette contained the synthetic plant-optimised *pat* gene based on the sequence from *Streptomyces viridochromogenes* Tu494 which results in tolerance to glufosinate-ammonium herbicides. The *pat* coding sequence is driven by the constitutive *Zea mays* ubiquitin 1 (ZmUbi1) promoter (Christensen *et al*., 1992) enhanced by its exon1 and intron1 (Gao *et al*., 2006). Termination of transcription of both transcripts is mediated by the bidirectional open reading frame 25 (orf25) terminator from *A. tumefaciens* pTi15955 (Barker *et al*., 1983).

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

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

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

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

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

3.1.2.2. Cotton 281-24-236: conclusion

Cotton 281-24-236 contained one intact copy of the pAGM281 T-DNA, with the *cry1F* and *pat* genes, and in addition one partial *pat* gene. The expression of the partial *pat* was at least 16 times lower than the full-length *pat* gene at the level of RNA and was undetectable at the protein level. The absence of vector backbone sequences in event 281-24-236 was confirmed. Bioinformatic analyses of the regions flanking the insert indicated that the 281-23-236 insertion occurred into the 3'untranslated region of the GA 20-oxidase gene. However, compositional and agronomic analyses showed that event 281-24-236 is equivalent to its conventional counterpart except for the newly introduced traits (see section 4.2). No biologically relevant similarity to allergens, toxins, or bioactive proteins was observed for any of the ORFs spanning the junctions.

3.1.2.3. Cotton 3006-210-23

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

The vector pMYC3006 used to generate cotton 3006-210-23 contained the *cry1Ac* gene on the T-DNA driven by the ZmUbi1 promoter and the *pat* coding sequence driven by the (4OCS)ΔMas2' promoter. Termination of transcription of both genes is mediated by the bidirectional orf25 terminator. This *cry1Ac* is a synthetic chimeric gene with the N-terminal core toxin from *cry1Ac* and the C-terminal part from *cry1Ca* and *cry1Ab* and codon optimised for plants (Gao *et al*., 2006; Shan *et al*., 2007). The *pat* coding sequence is identical to the one present in the pAGM281 vector.

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

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

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

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

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

3.1.2.4. Cotton 3006-210-23: conclusion

The cotton 3006-210-23 contains one intact copy of the pMYC3006 T-DNA, with the *cry1Ac* and *pat* genes. The absence of vector backbone sequences in event 3006-210-23 was confirmed. Updated bioinformatic analyses on sequences flanking the insertion site of event 3006-210-23 did not indicate that any endogenous coding or regulatory sequences had been interrupted by the insertion. No biologically relevant similarity to allergens, toxins, or bioactive proteins was observed for any of the putative peptides spanning the junctions.

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

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

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

3.1.3.2. Information on the expression of the inserts

Analyses of Cry1F, Cry1Ac and PAT protein levels was carried out by enzyme-linked immunosorbent assays (ELISA) using plants grown at six different sites in the major cotton growing regions of the USA during the 2001, 2003 and 2007 growing seasons. The trial locations provide a range of environmental conditions that would be encountered in commercial production of cotton. Protein expression levels were determined in young and terminal leaves, squares, bolls, whole plant, root, pollen, nectar, cottonseed and cottonseed processed fractions in 2001 and in cottonseed and cottonseed processed fractions in 2003 and 2007. Since the *pat* gene was used only as a selectable marker during transformation process, the EFSA GMO Panel does not require protein expression data on cotton 281-24-236 x 3006-210-23 treated with glufosinate-ammonium herbicides.
The scope of the application covers food and feed uses, import and processing, therefore protein expression data related to the cottonseed is considered most relevant, which are summarised in Table 1. Levels of proteins in the stacked cotton are comparable to levels in the single events.

Table 1. Summary of protein expression levels in cottonseed of 281-24-236 x 3006-210-23, 281-24-236 and 3006-210-23 (µg/g dry weight)

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

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

3.1.3.3. Inheritance and stability of inserted DNA

Stability of the inserts in cotton events 281-24-236 and 3006-210-23 was established by segregation analysis. The segregation ratios of the 281-24-236 and 3006-210-23 plants sprayed with glufosinate-ammonium herbicide showed the expected pattern for the dominant herbicide tolerant marker. In addition, segregation analysis was performed by crossing the hemizygous lines 281-24-236 and 3006-210-23 to produce the segregating F_1 generation. Both the F1 and the F_2 plants, resulting from self-pollination of the $F_{1,}$ showed the expected segregation pattern using ELISA for the Cry1Ac and Cry1F proteins. In both generations the Chi square values indicated no significant difference from the expected ratios. Southern analyses were conducted on two different generations for the single and stacked events, confirming the stability of the inserts. Moreover, Southern analyses showed that the integrity of the inserts present in the single events was retained in 281-24-236 x 3006-210-23.

3.2. Conclusion

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

4. Comparative analysis

4.1. Evaluation of relevant scientific data

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

In field trials, a comparison was made between the single events 281-24-236 and 3006-210-23 the stacked cotton 281-24-236 x 3006-210-23, and controls. In the first year (2001), the control was a null-segregant that was selected in the F1 generation after stacking and further bred by four rounds of self-pollination. In the second and third years (2003, 2007), the control was cotton variety PSC355, which has a comparable genetic background to the test lines, but lacks all newly introduced genes. It is realized by the EFSA GMO Panel that because *G. hirsutum* L. is a tetraploid species, conventional counterparts derived from the same progenitor do not have a completely identical genetic background. In the opinion of the EFSA GMO Panel, variety PSC355 constitutes an appropriate conventional

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

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

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

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

4.1.2. Compositional analysis

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

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

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

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

4.1.2.1. Cotton 281-24-236

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

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

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

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

4.1.2.2. Cotton 3006-210-23

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

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

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

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

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

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

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

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

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

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

4.1.3. Agronomic traits and GM phenotype

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

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

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

4.2. Conclusion

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

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

5. Food/Feed safety assessment

5.1. Evaluation of relevant scientific data

5.1.1. Product description and intended use

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

5.1.2. Effect of processing

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

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

5.1.3. Toxicology

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

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

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

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

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

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

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

(a) Acute toxicity testing

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

(b) Degradation in simulated digestive fluids

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

(c) Susceptibility to processing conditions

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

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

(d) Bioinformatic studies

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

5.1.3.3. Toxicological assessment of new constituents other than proteins

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

5.1.3.4. Toxicological assessment of the whole GM food/feed

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

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

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

5.1.4. **Allergenicity**

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

5.1.4.1. Assessment of allergenicity of the newly expressed proteins

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

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

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

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

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

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

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

5.1.4.2. Assessment of allergenicity of the whole GM plant

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

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

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

5.1.5. **Nutritional assessment of GM food/feed**

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

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

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

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

5.1.6. Post-market monitoring of GM food/feed

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

5.2. Conclusion

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

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

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

6. Environmental risk assessment and monitoring plan

6.1. Evaluation of relevant scientific data

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

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

6.1.1. Environmental risk assessment

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

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

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

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

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

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

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

6.1.1.2. Potential for gene transfer

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

(a) Plant to bacteria gene transfer

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

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

Cotton 281-24-236 x 3006-210-23 contains the *cry1Ac*, *cry1F* and *pat* genes originating from bacteria. Thus, in theory, the *cry1Ac*, *cry1F* and *pat* genes of the recombinant DNA insert could provide sufficient DNA similarity for homologous recombination to take place in environmental bacteria. However, as discussed further below, such hypothesized horizontal gene transfer event is not likely to be maintained in bacterial population due to a predicted lack of efficient expression and no identified selective advantage of gene transfer recipients.

In case of illegitimate recombination into genomes of environmental bacteria, it is unlikely that the *cry1Ac* gene regulated by eukaryotic plant promoter in cotton 281-24-236 x 3006-210-23 would be expressed. The *cry1F* and *pat* genes are regulated by *Agrobacterium* promoters. The activity of these promoters in bacteria may be possible. However, no selective advantage of a hypothesized bacterial uptake all of the above mentioned genes is anticipated, because *cry* and *pat* genes are distributed in various bacterial species in the natural environment. Thus, the hypothesized low level exposure of bacterial communities to the cotton 281-24-236 x 3006-210-23 *cry1Ac*, *cry1F* and *pat* genes must be

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

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

(b) Plant to plant gene transfer

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

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

6.1.1.3. Interactions of the GM plant with target organisms

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

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

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

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

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

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

6.1.1.5. Interactions with the abiotic environment and biogeochemical cycles

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

6.1.2. **Post-market environmental monitoring**

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

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

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

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

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

6.2. Conclusion

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

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

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

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

OVERALL CONCLUSIONS AND RECOMMENDATIONS

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

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

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

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

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

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

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

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

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Competent Authority of The Netherlands, dated 28 June 2005, concerning a request for placing on the market of cotton 281-24-236 x 3006-210-23 in accordance with Regulation (EC) No 1829/2003.

2. Acknowledgement letter, dated 30 June 2005, from EFSA to the Competent Authority of The Netherlands (Ref. SR/SM/jq (2005)804).

3. Letter from EFSA to applicant, dated 8 August 2005, delivering the 'Statement of Validity' for application NL-2005-16, cotton 281-24-236 x 3006-210-23 submitted by Dow AgroSciences under Regulation (EC) No 1829/2003 (Ref. SR/SM/jq (2005)1002).

4. Letter from EFSA to applicant, dated 21 October 2005, requesting additional information and stopping the clock (1) (Ref. SR/SM/cz (2005)).

5. Letter from applicant to EFSA, dated 2 November 2005, providing the timeline for submission of response.

6. Letter from EFSA to applicant, dated 15 December 2005, requesting additional information and maintaining the clock stopped (2) (Ref. SR/SM/cz (2005)1447).

7. Letter from applicant to EFSA, dated 6 March 2006, providing additional information (requested by EFSA on 21 October 2005).

8. Letter from DG SANCO to applicant, dated 13 December 2005, requesting clarification of scope of the application EFSA-GMO-NL-2005-16 submitted under Regulation (EC) No 1829/2003 (Ref. SANCO D4/SG/cc-D(05)441004).

9. Letter from applicant to EFSA, dated 8 March 2006, providing clarification of scope and proposal to update the application package.

10. Letter from EFSA to applicant, dated 24 March 2006, accepting the proposal to update the application EFSA-GMO-NL-2005-16, based on the updated scope launching a second consultation with Member States for a period of 6 weeks (Ref. SR/KL/jp (2006) GMO/262).

11. Letter from EFSA to applicant, dated 7 July 2006, requesting additional information and clarifications on the additional information already provided, maintaining the clock stopped (3) (Ref. SR/SM/jq (2006) 1623208).

12. Letter from applicant to EFSA, dated 23 August 2006, providing clarifications requested on additional information (requested by EFSA on 7 July 2006).

13. Letter from EFSA to applicant, dated 14 November 2006, request for additional information and maintaining the clock stopped (4) (Ref. SR/CP/jq (2006)1832026).

14. Letter from applicant to EFSA, dated 27 November 2006, providing the timeline for submission of response.

15. Letter from EFSA to applicant, dated 8 November 2007, reminding the timeline for submission of response (Ref. SR/SM/shv (2007)2493539).

16. Letter from applicant to EFSA, dated 18 December 2007, providing new timeline for submission of response.

17. Letter from EFSA to applicant, dated 31 July 2008, reminding that the new timeline for submission of response was not met (Ref. SR/SM/Shv (2008)3205270).

18. Letter from applicant to EFSA, dated 29 August 2008, providing another new timeline for submission of response.

19. Letter from applicant to EFSA, dated 9 December 2008, providing additional information (requested by EFSA on 14 November 2006).

20. Letter from EFSA to applicant, dated 17 March 2009, request for additional information and maintaining the clock stopped (5) (Ref. PB/SM/md (2009)3810863).

21. Letter from applicant to EFSA, dated 29 April 2009, requesting clarifications on proposed approach for answering some questions, and providing the timeline for submission of response.

22. Letter from EFSA to applicant, dated 20 May 2009, providing clarifications to the proposed approach for answering some questions (Ref. PB/SM/md (2009)3978423).

23. Letter from applicant to EFSA, dated 29 May 2009, providing renamed reference files.

24. Letter from applicant to EFSA, dated 8 July 2009, providing two references with adjusted names.

25. Letter from applicant to EFSA, dated 29 September 2009, providing additional information (requested by EFSA on 17 March 2009).

26. Letter from EFSA to applicant, dated 21 October 2009, request for clarification concerning additional information and maintaining the clock stopped (6) (Ref. PB/KL/YL/ls (2009) 4371610).

27. Letter from applicant to EFSA, dated 25 January 2010, providing timeline for submission of response.

28. Letter from applicant to EFSA, dated 25 January 2010, providing new timeline for submission of response.

29. Letter from applicant to EFSA, dated 25 January 2010, providing another new timeline for submission of response.

30. Letter from applicant to EFSA, dated 12 March 2010, providing additional information (requested by EFSA on 21 October 2009).

31. Letter from EFSA to applicant, dated 30 March 2010, restarting the clock (Ref. PB/KL/YL/shv (2010)4767809).

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