SCIENTIFIC OPINION

Scientific Opinion on application EFSA-GMO-RX-MON531 for renewal of the authorisation for continued marketing of existing cottonseed oil, food additives, feed materials and feed additives produced from MON 531 cotton that were notified under Articles 8(1)(a), 8(1)(b) and 20(1)(b) of Regulation (EC) No 1829/2003 from Monsanto[1]

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

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ABSTRACT

This scientific opinion evaluates the risk assessment for the authorisation for continued marketing of genetically modified insect resistant cotton MON531 for food and feed produced from it. MON531 was transformed via *Agrobacterium*. It contains single copies of functional Cry1Ac and NPTII expression cassettes; two fragments of the Cry1Ac cassette and *aad*A as non-functional elements. Stability of the inserted DNA was confirmed over several generations. Bioinformatic analyses and the levels of recombinant proteins did not reveal safety concerns. Analysis of compositional, phenotypic and agronomic characteristics indicated that MON531 is not different from its conventional counterpart and is compositionally within the range observed among conventional cotton varieties, except for Cry1Ac and NPTII. Safety assessment of Cry1Ac and NPTII proteins and cotton MON531 identified no concerns regarding potential toxicity and allergenicity. Products from MON531 do not contain viable plant parts. The *aad*A and *ori*V sequences in MON531 may facilitate the stabilisation of *npt*II through double homologous recombination in plasmid sequences in the environment. However, considering the expected low frequency of gene transfer from MON531 to bacteria compared to that between bacteria, and the very low exposure to MON531 DNA, the GMO Panel concludes that gene transfer from MON531 to bacteria does not indicate a risk to human or animal health or to the environment in the context of its intended uses. The exposure of potentially sensitive non-target organisms to Cry1Ac protein is likely to be low and of no biological relevance. A PMEM plan is not required. The EFSA GMO Panel considers that information available for cotton MON531 addresses the questions raised by the Member States and that cotton MON531, as described in this application, is as safe as its conventional counterpart and is unlikely to have adverse effects on human and animal health and the environment in the context of its intended uses.

[1] On request from the European Commission on application (EFSA-GMO-RX-MON531) submitted by Monsanto, Question No EFSA-Q-2007-151, adopted on 7 September 2011.

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Groups on Molecular Characterisation, Food and Feed Safety and Environment for the preparatory work on this scientific opinion, Willem Seinen as external expert and EFSA's staff members Yann Devos (ENV), Zoltán Divéki (MC), Andrea Germini (FF), Ana Gomes (MC), Karine Lheureux (ENV) and Nancy Podevin (MC) for the support provided to this EFSA scientific opinion.

Suggested citation: EFSA Panel on Genetically Modified Organisms (GMO); Scientific Opinion on application EFSA-GMO-RX-MON531 for renewal of the authorisation for continued marketing of existing cottonseed oil, food additives, feed materials and feed additives produced from MON 531 cotton that were notified under Articles 8(1)(a), 8(1)(b) and 20(1)(b) of Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2011;9(9):2373. [1-30] doi:10.2903/j.efsa.2011.2373. Available online: www.efsa.europa.eu/efsajournal

KEY WORDS

GMO, cotton, MON 531, insect resistance, risk assessment, food and feed safety, environment, food and feed produced from, Regulation (EC) No 1829/2003, renewal.

SUMMARY

This document provides a scientific opinion of the Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) on application EFSA-GMO-RX-MON531 submitted by Monsanto under Regulation (EC) No 1829/2003[4] for renewal of the authorisation for continued marketing of (1) foods produced from cotton MON 531 (cottonseed oil); (2) foods produced from cotton MON 531 (food additives); (3) feed produced from cotton MON 531 (feed materials and feed additives).

The scope of the renewal application covers the continued marketing of:

- cottonseed oil notified as existing food falling within the scope of Article 8(1)(a) of Regulation (EC) 1829/2003, which is produced from a genetically modified organism (GMO) and which has been placed on the market in accordance with Article 5 of Regulation (EC) 258/97[5], notification forwarded to Member States on 19/12/2002, opinion on substantial equivalence by the UK Advisory Committee on Novel Foods and Processes;

- existing foods produced from cotton MON 531 (food additives) notified as existing food additives within the meaning of Article 8(1)(b) of Regulation (EC) 1829/2003, authorised under Directive 89/107/EEC[6] and complying with the relevant specifications laid down under this legislation;

- existing feed produced from cotton MON 531 (feed materials and feed additives) notified as existing feed falling within the scope of Article 20(1)(b) of Regulation (EC) 1829/2003, namely as feed materials and feed additives (subject to Directive 70/524/EEC[7]) which are produced from genetically modified organism (GMO).

After the date of entry into force of the Regulation (EC) 1829/2003, the products mentioned above were notified to the European Commission according to Articles 8(1)(a), 8(1)(b) or 20(1)(b) of this Regulation and subsequently included in the Community Register of GM food and feed.

Cotton MON 531 has been developed for resistance to specific lepidopteran cotton pests by the introduction, via *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation, of a gene coding for a synthetic variant of Cry1Ac insecticidal protein from *Bacillus thuringiensis*. MON 531 also carries genes coding for neomycin phosphotransferase type II (NPTII) and 3′(9)-O-nucleotidyltransferase (AAD, not expressed in MON 531), which were used as antibiotic-resistance marker genes during product development. In delivering its scientific opinion, the EFSA GMO Panel considered the renewal application EFSA-GMO-RX-MON531; additional information submitted by the applicant on request of the EFSA GMO Panel; the scientific comments submitted by Member States; and relevant scientific publications. In accordance with the Guidance Document for renewal of authorisations of existing GMO products (EFSA, 2006b), the EFSA GMO Panel has taken into account the new information, experience and data on cotton MON 531, which have become available during the authorisation period.

The EFSA GMO Panel assessed cotton MON 531 with reference to the intended uses and appropriate principles described in the Guidance Document of the EFSA GMO Panel for the Risk Assessment of Genetically Modified Organisms and Derived Food and Feed (EFSA, 2006a) and the Guidance Document of the Scientific Panel on Genetically Modified Organisms for renewal of authorisations of existing GMO products lawfully placed on the market (EFSA, 2006b). The scientific evaluation of the risk assessment included molecular characterisation of the inserted DNA and expression of the target

[4] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 1-23.
[5] Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. OJ L 43, 1-6
[6] Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption. OJ L 40, 27-33
[7] Council Directive 70/524/EEC of 23 November 1970 concerning additives in feeding-stuffs. OJ L 270, 1-17

proteins. A comparative analysis of agronomic traits and composition was undertaken and the safety of the new protein and the whole food/feed were evaluated with respect to potential toxicity, allergenicity and nutritional quality. An assessment of environmental impacts and the post-market environmental monitoring plan were undertaken.

The molecular characterisation data establish that the genetically modified cotton MON 531 contains two separate T-DNA elements. The functional insert contains the Cry1Ac cassette, the *aad*A gene, and the NPTII cassette and the *ori*V origin of replication. Adjacent to this insert, a fragment containing the 3' portion of the Cry1Ac cassette was inserted. Additionally, a non-functional fragment containing a portion of the 7S 3' transcriptional termination sequence was separately inserted into cotton MON 531. There is sequence similarity between parts of the functional insert flanking the *npt*II gene and naturally occurring bacterial plasmid sequences. Appropriate bioinformatic analyses of the integration site, including flanking sequences have been performed to characterise the MON 531 event. The stability of the genetic modification has been demonstrated over several generations. The expression of the Cry1Ac and NPTII proteins has been analysed sufficiently and data from various plant tissues were provided from field trials performed between 1992 and 2001 in the USA. The MON 531 event includes two bacterial antibiotic resistance genes and other sequences of bacterial origin, which may allow double homologous recombination to plasmid sequences present in the environment.

Based on the results of a comparative analysis of data and in the light of the field trial design and the ranges of constituents reported for conventional cotton varieties, the EFSA GMO Panel concludes that seeds from cotton MON 531 are compositionally not different from seeds obtained from the conventional counterpart and compositionally within the range observed among conventional cotton varieties, except for expression the Cry1Ac and NPTII proteins. Based on the analysis of the data obtained from the field trials with regard to seed and plant development, disease and pest susceptibility, reproduction and yield, the EFSA GMO Panel concludes that cotton MON 531 is agronomically not different from its conventional counterpart and within the range observed among conventional cotton varieties, with the exception of the newly introduced trait. The safety of the Cry1Ac and NPTII proteins expressed in cotton MON 531 is supported by bioinformatics analysis and specific studies on stability during processing, digestibility in simulated gastric and intestinal fluids and toxicity in mice models. The potential allergenicity of the Cry1Ac and NPTII proteins has been assessed, and it was found unlikely that they are allergenic. The EFSA GMO Panel concludes that cotton MON 531 and its derived products obtained through seed processing are unlikely to have any altered potential to induce adverse effects on human and animal health as compared to conventional cotton in the context of their intended use.

According to the information provided by the applicant, food and feed products produced from cotton MON 531 have been consumed without reports of adverse effects since they have been placed on the market in the EU. Scientific publications which have become available since the previous evaluation of cotton MON 531 by the Scientific Committee on Plants (SCP, 1998) and the Advisory Committee of the Competent Authority of the United Kingdom (ACNFP, 2002) did not raise safety issues.

Cotton MON 531 is being assessed for food and feed produced from cotton MON 531, thus the scope only includes products produced from cotton MON 531 which contain no viable plant parts. Therefore, there are no requirements for scientific information on environmental risks associated with the accidental release or cultivation of cotton MON 531. No risk arising from a HGT of the *cry1Ac* gene from cotton MON 531 to bacteria has been identified. A hazard of an increased likelihood of stabilisation of the *npt*II gene from cotton MON 531 DNA in bacteria was postulated. However, considering the expected low frequency of gene transfer from MON 531 to bacteria compared to that between bacteria, and the very low exposure to MON 531 DNA, the GMO Panel concludes that the contribution of HGT to the environmental prevalence of *npt*II genes is negligible. The analysis of HGT from cotton MON 531 to bacteria does not indicate a risk to human or animal health or to the environment in the context of its intended uses. Considering the scope of the application, it can be concluded that the exposure of potentially sensitive non-target organisms to the Cry1Ac protein is

likely to be very low and of no biological relevance. A post-market environmental monitoring plan for cotton MON 531 is not required.

In conclusion, the EFSA GMO Panel considers that information available for cotton MON 531 addresses the questions raised by the Member States and that cotton MON 531, as described in this application, is as safe as its conventional counterpart and is unlikely to have adverse effects on human and animal health and the environment in the context of its intended uses.

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BACKGROUND

On 29 June 2007, the European Food Safety Authority (EFSA) received from the European Commission an application submitted under Regulation (EC) No 1829/2003 for renewal of the authorisation of (1) foods produced from cotton MON 531 (food additives); (2) feed produced from cotton MON 531 (feed materials and feed additives), developed by Monsanto to provide resistance to specific lepidopteran cotton pests. On 30 June 2011, the European Commission acknowledged the applicant's request to expand the scope also to foods produced from cotton MON 531 (cottonseed oil).

The scopes of the renewal application cover the continued marketing of:

- cottonseed oil notified as existing food falling within the scope of Article 8(1)(a) of Regulation (EC) 1829/2003, which is produced from a genetically modified organism (GMO) and which has been placed on the market in accordance with Article 5 of Regulation (EC) 258/97, notification forwarded to Member States on 19/12/2002, opinion on substantial equivalence by the UK Advisory Committee on Novel Foods and Processes (ACNFP, 2002);

- existing foods produced from cotton MON 531 (food additives) notified as existing food additives within the meaning of Article 8(1)(b) of Regulation (EC) 1829/2003, authorised under Directive 89/107/EEC and complying with the relevant specifications laid down under this legislation;

- existing feed produced from cotton MON 531 (feed materials and feed additives) notified as existing feed falling within the scope of Article 20(1)(b) of Regulation (EC) 1829/2003, namely as feed materials and feed additives (subject to Directive 70/524/EEC) which are produced from genetically modified organism (GMO).

The scope of the present renewal application covers both *Gossypium hirsutum* and *Gossypium barbadense* cotton species.

After the date of entry into force of the Regulation (EC) No 1829/2003, the products mentioned above were notified to the European Commission according to Articles 8(1)(a), 8(1)(b) or 20(1)(b) of this Regulation and subsequently included in the Community Register of GM food and feed.

Cotton MON 531 was the subject of earlier safety assessments (ACNFP, 2002; SCP, 1998) and has been placed on the market on 1 January 1996 (as food additives, feed additives and feed materials) and on 19 December 2002 (as food).

After receiving the renewal application EFSA-GMO-RX-MON531 and in accordance with Articles 5(2)(b) and 17(2)b of Regulation (EC) No 1829/2003, EFSA informed Member States as well as the European Commission and made the summary of this application publicly available on the EFSA website.[8] EFSA initiated a formal review of the renewal application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of Regulation (EC) No 1829/2003. On 26 May 2008, EFSA received additional information requested under completeness check (requested on 19 December 2007) and on 11 June 2008, 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[9], to request their scientific opinion. The Member State bodies had 3 months after the date of receipt of the valid application (until 11 September 2008) within which to make their opinion known.

[8] http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2007-151

[9] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L106, 1-39.

The EFSA GMO Panel carried out the safety evaluation of the renewal application of the cotton MON 531 in accordance with the appropriate principles described in the EFSA GMO Panel Guidance Document for the risk assessment of GM plants and derived food and fed (EFSA, 2006a) and the Guidance Document of the Scientific Panel on Genetically Modified Organisms for renewal of authorisations of existing GMO products lawfully placed on the market (EFSA, 2006b). In addition, the scientific comments of Member States, the additional information provided by the applicant and relevant scientific publications were taken into consideration.

The EFSA GMO Panel requested additional information from the applicant on 24 June 2008, 09 September 2008, 23 October 2008, 07 April 2009, 24 July 2009, 16 September 2009, 25 May 2010, 02 August 2010, 04 October 2010 and on 31 January 2011. The applicant provided the requested information on 30 September 2008, 02 March 2009, 23 April 2009, 03 February 2010, 08 June 2010, 14 September 2010, 15 September 2010, 02 December 2010 and on 11 April 2011.

In giving its scientific opinion on cotton MON 531 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 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 assessment of cotton MON 531 for the renewal of authorisation of (1) cottonseed oil notified as existing food falling within the scope of Article 8(1)(a) of Regulation (EC) 1829/2003, which is produced from a genetically modified organism (GMO) and which has been placed on the market in accordance with Article 5 of Regulation (EC) 258/97, notification forwarded to Member States on 19/12/2002, opinion on substantial equivalence by the UK Advisory Committee on Novel Foods and Processes; (2) existing foods produced from cotton MON 531 (food additives) notified as existing food additives within the meaning of Article 8(1)(b) of Regulation (EC) 1829/2003, authorised under Directive 89/107/EEC and complying with the relevant specifications laid down under this legislation; (3) existing feed produced from cotton MON 531 (feed materials and feed additives) notified as existing feed falling within the scope of Article 20(1)(b) of Regulation (EC) 1829/2003, namely as feed materials and feed additives (subject to Directive 70/524/EEC) which are produced from genetically modified organism (GMO). 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/environments 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 of the Cartagena Protocol. Furthermore, the EFSA GMO Panel did 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 assessment presented here is based on the information provided by the applicant in the renewal application EFSA-GMO-RX-MON531 for continued marketing of (1) foods produced from cotton MON 531 (cottonseed oil); (2) foods produced from cotton MON 531 (food additives); (3) feed produced from cotton MON 531 (feed materials and feed additives), additional information submitted by the applicant in response to questions asked by the EFSA GMO Panel, as well as scientific comments from Member States and relevant scientific publications. The assessment has taken into account the appropriate principles described in the Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a), and the Guidance Document of the Scientific Panel on Genetically Modified Organisms for renewal of authorisations of existing GMO products lawfully placed on the market, notified according to Articles 8 and 20 of Regulation (EC) No 1829/2003 (EFSA, 2006b).

Information in the application include 1) updated information on the comparative compositional analysis; 2) an estimation of the human and live-stock exposure in Europe to cotton MON 531; 3) an update on peer-reviewed scientific data on cotton MON 531, and 4) updated information on potential for allergenicity and toxicity, including updated homology searches between the newly expressed proteins and known toxic and allergenic proteins.

The original transformed MON 531 cotton plant used in the application was from the species *Gossypium hirsutum.* Since there are no known genetic barriers to interspecies hybridization between the tetraploid *Gossypium* (Percival et al., 1999), the MON 531 event could be introgressed in *G. barbadense* through conventional breeding using the original transformed variety of *G. hirsutum*. On request of the EFSA GMO Panel the applicant provided information that the composition of cottonseed from *G. barbadense* does not differ from that of *G. hirsutum* regarding nutrients, anti-nutrients and toxicants, to such an extent that a food and feed risk assessment of one of these species would not be applicable also for the other species.[10] Therefore the food and feed and environmental risk assessment of the MON 531 event in cotton in this opinion is applicable to both *G. barbadense* and *G. hirsutum*.

2. Issues raised by the Member States

The comments raised by the Member States are addressed in Annex G of the EFSA overall opinion and have been considered in this scientific opinion.[11]

3. Molecular characterisation

3.1. Evaluation of relevant scientific data

3.1.1. Transformation process and vector constructs[12]

The cotton event MON 531 was developed through *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation of hypocotyl cells. The plasmid vector PV-GHBK04 contained two plant expression cassettes.

[10] Additional information, April 2011
[11] http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2007-151
[12] Technical dossier / Section C1

One of the cassettes consisted of a synthetic *cry1A* gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and the 3' untranslated region of the soybean 7S seed protein gene. The synthetic gene codes for a Cry1Ac variant protein with 99.4 % amino acid sequence identity to the Cry1Ac protein of *Bacillus thuringiensis*. The nucleotides 1 to 1398 were derived from the *cry1Ab* gene and nucleotides 1399 to 3534 from the *cry1Ac* gene, after optimising their codon usage for expression in the plant. The Cry1Ab-derived part differs by six amino acids from the corresponding region of the Cry1Ac protein. The Cry1Ac-derived part contains one unintended amino acid change (L766S). Because this amino acid change is located outside the insecticidally-active, trypsin-resistant core protein, it is not expected to affect the host range of the active protein.

The other cassette contained a DNA fragment derived from transposon Tn*5* consisting of the coding sequence of the neomycin phosphotransferase II gene (*npt*II) and part (153 bp) of the 378 bp bleomycin binding protein (*ble*)-encoding gene. This fragment was combined with the CaMV 35S promoter and the 3' untranslated region of the nopaline synthase (*nos*) gene from *A. tumefaciens*. The expression of the *npt*II gene allowed selection of the transformed plant cells with kanamycin.

The backbone of vector PV-GHBK04 contained the origins of replication from the RK2 plasmid (*ori*V) and from pBR322 allowing replication of the plasmid in *A. tumefaciens* and *E. coli,* respectively. It also contained the *rop* gene, which is involved in controlling the plasmid copy number, the *bom* site for the conjugational transfer into *A. tumefaciens,* the right T-DNA border from the pTiT37 plasmid, and the prokaryotic gene *aad*A from transposon Tn7, conferring bacterial resistance to the antibiotics spectinomycin and streptomycin.

3.1.2. Transgene constructs in the genetically modified plant[13]

In the MON 531 cotton, two separate T-DNA elements are inserted. The functional insert contains 7916 bp from the 11407 bp PV-GHBK04 plasmid, ranging from the right T-DNA border through the Cry1Ac cassette, the *aad*A gene, and the NPTII cassette up to the *ori*V; the *ori*-pBR322 and the *rop* gene are not inserted, which was confirmed by Southern analysis using the vector fragment as a probe. Adjacent to this insert, a fragment containing the 3' portion of the Cry1Ac cassette up to the right T-DNA border was inserted in the opposite orientation, arranged as an inverted repeat. Additionally, a non-functional fragment of 242 bp containing the right border T-DNA sequence and a portion of the 7S 3' transcriptional termination sequence was separately inserted.

The nucleotide sequences of both inserts in MON 531 have been determined. Flanking sequences extending 307 bp at the 5' end and 211 bp at the 3' end of the functional insert and 499 bp at the 5' end and 380 bp at the 3' end of the non-functional insert were determined. The sequences of both pre-insertion loci (349 bp for the functional insert and 185 bp for the non-functional insert) were also determined. In the functional insert locus, 85 bp of the endogenous cotton sequence are missing, whereas no modifications occurred in the non-functional insert locus with the exception of the insertion.

The sequence of the region between the two repeats of the functional insert (c*ry1Ac* – 7S 3' junction – 7S 3' *cry1Ac*) was not determined because of technical difficulties. The applicant indicated that the secondary structure of the inverted repeat prevented the isolation of the region (the RB/RB junction). Therefore, no bioinformatic analysis of the putative open reading frames (ORFs) could be performed. Southern analysis indicated that this region is less than 100 bp. Furthermore, the RB/RB junction is flanked on both sides by 7S 3' ends that terminate transcription and should, therefore, prevent transcription of the RB/RB junction from any promoter that is in or near the insert. Moreover, the inverted repeat sequences contain stop codons in all three reading frames leading into the RB/RB junction. Therefore, it is unlikely that the RB/RB junction would be included in a transcript, and if it were, translation would be terminated prior to reaching the sequences of the RB/RB junction.

[13] Technical dossier / Section D2

The newly created ORFs present at the junctions of the inserts (5 ORFs at each junction of the functional insert; 4 and 5 ORFs at the 5' and 3' junctions of the non-functional insert, respectively) were analysed for homology with known allergens, toxins or other proteins. Bioinformatic analysis indicated no significant homology with known allergens or toxins and that no known endogenous genes or regulatory regions were interrupted by either insert.

Two antibiotic resistance genes are present in MON 531 cotton as a consequence of the genetic modification process. The transfer of antibiotic resistance marker genes from GM plants to bacteria has not been shown to occur either in natural conditions or in the laboratory in the absence of sequence identity in the recipient bacterial cell (EFSA, 2009). One of the key factors determining the stabilisation rates for foreign DNA in bacteria is the presence of DNA sequence similarity, which influences the frequency of homologous recombination. In MON 531, the *npt*II gene under the control of the CaMV 35S promoter is flanked upstream by the *aad*A gene and downstream by the broad host-range origin of replication from the RK2 plasmid (*ori*V). To analyse the possibility of homologous recombination, a bioinformatic analysis was performed with the insert against all bacterial, plasmid and viral sequences (Genbank, 2010). All hits with sufficient homology to allow homologous recombination were bacterial gene sequences with the same function as in the transgene. No cryptic targets of homologous recombination were identified. The possibility of double homologous recombination was investigated. Two alignment pairs were found showing in the same plasmid the presence of an *aad*A gene and *ori*V sequence with sufficient homology (*aad*A: 92-100 % identity in a stretch of 800-854 bp; *ori*V: 100 % identity in a stretch of 170-639 bp) to allow homologous recombination. The hits were found in plasmids from two bacteria, one isolated from soil and one from activated sludge. Homologous recombination involving the *aad*A gene and the *ori*V would lead to the insertion of the *npt*II gene cassette in the plasmid sequence.

3.1.3. Information on the expression of the insert[14]

The expression levels of the Cry1Ac and the NPTII proteins in MON 531 were measured by ELISA in four field trials in the USA (1992 [six locations]; 1998 [four locations]; 1999 [four locations] and 2001 [five locations]). The expression levels of Cry1Ac and NPTII proteins in seeds varied between 0.40 – 5.99 µg/g fw and 1.97 – 15.9 µg/g fw, respectively (Table 1)..As expected, AAD protein was not detected in any of the samples analysed since the *aad*A gene is under the control of a prokaryotic promoter.

Table 1. Ranges of Cry1Ac and NPTII expression levels in MON 531 seed (µg / g fresh weight)

For the 1992 trial, the confidence interval is indicated. All other trials are represented with minimum-maximum values.

	1992	1998 line DP50BG	1998 line DP5415BG	1998 line DP5690BG	1999 line DP5415BG	2001 line SureGrow 125
Cry1Ac	0.40 – 1.32	1.94 – 3.27	2.77 – 4.27	2.77 – 4.66	2.63 – 5.99	1.6 – 1.8
NPTII	1.97 – 2.93	11.4 – 12.1	10.0 – 15.9	10.1 – 11.6	4.21 – 7.01	4.7 – 6.0

3.1.4. Inheritance and stability of inserted DNA[15]

The stability of the insert was investigated by Southern analysis in plants derived from two generations of backcrossed MON 531 and in two commercial lines containing the MON 531 event. The integrity of the functional insert was confirmed in all materials tested, whereas the non-functional insert was absent from the commercial lines, because of segregation of the inserts.

[14] Technical dossier / Section D3
[15] Technical dossier / Section D5

The expected inheritance ratio was observed for the Cry1Ac protein over several generations, indicating the presence of a stable single Mendelian locus.

The insect resistance phenotype of MON 531 has been demonstrated under field conditions on a commercial scale since 1996 e.g. in the United States and Australia.

3.2. Conclusion

Appropriate bioinformatic analyses of the integration site, including flanking sequences has been performed to characterise the MON 531 event. The stability of the genetic modification has been demonstrated over several generations. The expression of the Cry1Ac and NPTII proteins has been analysed sufficiently. The MON 531 event includes two bacterial antibiotic resistance genes and other sequences of bacterial origin, which may allow double homologous recombination to plasmid sequences present in the environment (further discussed in Section 5.1.1 of this opinion).

4. Comparative analysis

4.1. Evaluation of relevant scientific data

4.1.1. Choice of comparator and production of material for the compositional assessment[16]

Seed from cotton MON 531 and the conventional counterpart Coker 312 were harvested from field trials performed at six locations in the major cotton growing regions of the USA in 1992 as well as in 1993. The seed were used for a comparative compositional analysis. In 1993 cottonseed material from the same field trial were pooled per line and processed into toasted meal and refined cottonseed oil fractions for compositional analysis.

Additional compositional data were obtained from cottonseed harvested at four field trial sites in the USA in 1999, each site having four replicates of cotton MON 531 and its conventional counterpart. As the cotton MON 531 event studied in these field trials had been bred into the DP5415 genetic background, the conventional counterpart in the field trials in 1999 was DP5415 instead of Coker 312. In addition, these field trials included 11 non-GM reference varieties to determine the range in cotton constituent levels in these field trails. The 99 % tolerance interval of this range was calculated to establish the range naturally occurring for the various compositional parameters studied. Historical and literature values were also provided for these parameters in cottonseed.

The harvested material and produced products were analysed for key nutrients, anti-nutrients and toxicants as defined by (OECD, 2009). Vitamin E (alpha-tocopherol) was only reported for refined cottonseed oil produced in 1993 and crude fibre only in the cottonseed harvested in 1999. Cotton fibres and linters obtained from the 1993 field trial were analyzed for the presence of Cry1Ac and NPTII proteins.

4.1.2. Compositional analysis[17]

When the compositional data for cottonseed produced in 1992 were analysed across the six sites of field trials, statistically significant differences in the levels of fatty acids myristic acid, stearic acid and oleic acid were observed between MON 531 and its conventional counterpart. The mean value for myristic acid was slightly lower in MON 531 compared with Coker 312 (0.88 and 0.94 % of total lipid), whereas the stearic acid and oleic acid levels were slightly higher in MON 531 (10 % and 1 % respectively). Although the levels of these fatty acids differed between MON 531 and its conventional counterpart at all field trial sites, the mean values were within the range reported for each of these fatty

[16] Technical dossier / Section D7.2
[17] Technical dossier / Section D7.1 and Annex 3.4.c

acids in the scientific literature. Notably, the level of the toxicant gossypol (total and free) and the cyclopropenoid fatty acids did not differ between cotton MON 531 and Coker 312.

In contrast to the cottonseed produced in 1992, cottonseed produced in 1993 showed no alteration in the level of fatty acids as compared to cotton Coker 312. In 1993, statistically significant differences were found in the level of the amino acids glutamic acid, valine, methionine, isoleucine, tyrosine, lysine, and histidine. However, differences were small and did not occur consistently across all locations. Total protein and total and free gossypol levels were determined in toasted meal obtained from MON 531 and Coker 312 and no significant differences were observed. Also no biologically relevant differences were observed in the fatty acid profile and alpha-tocopherol content of refined oil obtained from MON 531 and Coker 312. No gossypol and no proteins were detected in refined oil from cotton MON 531 and Coker 312.

In the 1999 field trial performed with cotton MON 531 in the genetic background DP5415 statistically significant differences between cotton MON 531 and DP5415 were observed for the level of total fat, carbohydrates, palmitic acid, linoleic acid, calcium and iron in the overall analysis. Although these differences were found in cottonseeds from each individual test site, the differences were small and, with the exception of a single iron value in cotton MON 531, fell within the 99 % tolerance interval for the 11 non-GM reference varieties. With regard to the statistically significantly altered levels of myristic acid, stearic acid and oleic acid found in 1992, in the 1999 field trial no significant differences were noted in the levels of these fatty acids in the seeds from MON 531 and its conventional counterpart DP5415.

The EFSA GMO Panel considered the compositional data on cotton MON 531 and its conventional counterpart in the light of the field trial design and the ranges in constituents reported for conventional cotton varieties. The EFSA GMO Panel concluded that cotton MON 531 is compositionally not different from its conventional counterpart and that its composition falls within the range observed among conventional cotton varieties, except for expressing the Cry1Ac and NPTII proteins.

4.1.3. Agronomic traits and GM phenotype[18]

Agronomic and phenotypic characteristics of cotton MON 531 together with its conventional counterpart were studied in field trials conducted at four sites in the USA in 1998 and 1999.[19] In 1998 the MON 531 event was tested in three genetic backgrounds (DP50, DP5690 and DP5415), each having its appropriate conventional counterpart whereas in 1999 only cotton MON 531 in the DP5415 background was tested. The agronomic and phenotypic parameters studied were related to seed and plant development, disease and pest susceptibility, reproduction and yield. The studies showed significantly more cracked bolls in cotton MON 531 than in the conventional counterpart (mean number of cracked bolls per plot for MON 531 in the 1998 field trials was 427 versus 285 for the genetic background line DP5415 whereas the numbers were 464 versus 355 in the 1999 trial). According to the applicant, this difference might indicate a later maturity of cotton MON 531, possibly related to minor differences in insect damage. No other alteration related to cotton development and maturity was observed. Other agronomic or phenotypic characteristics of the tested cotton materials did not differ between cotton MON 531 and its conventional counterpart. The EFSA GMO Panel concludes that cotton MON 531 is agronomically not different from its conventional counterpart, with the exception of differences related to the newly introduced trait.

4.2. Conclusion

Analyses carried out on cotton MON 531, its conventional counterpart and other non-GM cotton varieties indicate that cotton MON 531 is compositionally, phenotypically and agronomically not different from its conventional counterpart and compositionally within the range observed among conventional cotton varieties, except for expressing the introduced trait. The comparative analysis of

[18] Technical dossier / Annex 3.4b
[19] Additional information, February 2010

cotton MON 531 therefore provided no indication for unintended effects resulting from the genetic modification that would raise a safety concern.

5. Food and feed safety assessment

5.1. Evaluation of relevant scientific data

5.1.1. Product description and intended uses[20]

The scope of the present application is renewal of the authorisation for continued marketing of (1) foods produced from cotton MON 531 (cottonseed oil); (2) foods produced from cotton MON 531 (food additives); (3) feed produced from cotton MON 531 (feed materials and feed additives). The possible uses of cotton MON 531 will include the production of refined oil from seeds and cellulose from linters for use as food or food ingredients, and the production of cottonseed meal, hulls and linters for use as animal feed.

The genetic modification of cotton MON 531 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.

Cotton MON 531 was first cultivated in the USA in 1996. By 2006 cotton MON 531 was also cultivated in Argentina, Australia, Brazil, China, Colombia, India, Mexico, and South Africa. Cotton MON 531, as single event or stacked with other events, is not commercially produced in any of the 27 countries of the European Community. Globally, the production of cotton MON 531 has grown rapidly since its introduction and MON 531 containing cotton traits reached in 2006 adoption rates exceeding 50 % of the total cotton production area in Mexico, South Africa, the USA and Australia. In recent years, production of cotton MON 531 outside Europe has levelled off or declined, being replaced by stacked cotton events containing MON 531.

Based on import data of cottonseed oil and cottonseed meal from cotton MON 531 producing countries into the 27 countries of the European Community over the years 2003-2005, the applicant has calculated that around 0.0026 % of cottonseed oil and 0.36 % of cottonseed meal used in the EU might be derived from cotton MON 531 and its combined trait products. It should be noted, however, that the calculations giving these figures are based on several assumptions. Because operators in the food and feed chain in some Member States of the European Community have made efforts to preferentially source non-GM products, the actual consumption of products derived from cotton MON 531 in food and feed may vary between Member States.

Based on FAO Statistics from 1997 to 2001, the human cottonseed oil consumption in Europe was calculated to be 0.0 and 4.9 g/person/day in central and southern Europe, respectively. Assuming 0.0026 % of cottonseed oil being derived from cotton MON 531, its estimated average dietary intake by the southern European consumer would be about 0.13 mg/person/day. For comparison the average dietary intake of cottonseed oil derived from MON 531 containing traits in the USA would be approximately 750 mg/person/day.

Animal feed is the major end use of cottonseed meal. The applicant calculated, based on data from 2005, that the maximum inclusion levels (% of the diet) of MON 531 derived cottonseed meal in the EU would be 0, 0.018 and 0.054 % in livestock diets for pigs, broiler chickens and dairy cattle respectively. For comparison, the maximum inclusion rates of MON 531-derived cottonseed meal in livestock diets in cotton producing countries such as the USA is up to 460-fold higher than in the European Union.

[20] Technical dossier / Annex 3.2

Although no post-market monitoring for food and feed safety of cotton MON 531 has formally been performed, there is no evidence of any adverse effects being associated with the consumption of MON 531 derived products as food or feed.

5.1.2. Effects of processing

Since cotton MON 531 is compositionally not different from its conventional counterpart and within the range observed among conventional cotton varieties, except for the newly expressed proteins (see Section 4.1.2), the effect of processing cotton MON 531 is not expected to result in any different products than obtained after processing conventional cotton varieties. The effect of processing on the levels of the newly expressed proteins (Cry1Ac and NPTII) in cottonseed derived products (processed cottonseed meals, oil and linters) was studied in cotton MON 531, using Western analysis. In processed cottonseed meal derived from cotton MON 531 the Cry1Ac protein was not detected. In raw linters trace amounts of Cry1Ac were detected, which was not the case in processed material. More than 96.7 % of the NTPII protein in seeds of cotton MON 531 could not be detected by Western analysis after processing to toasted cottonseed meal. No NPTII protein was detected in fibre fractions obtained from cotton MON 531. In refined cottonseed oil, whether produced from conventional cottonseed or from seeds of cotton MON 531, no protein or DNA was detected.

Upon request of the EFSA GMO Panel to provide data on the presence/absence of DNA fragments carrying the *npt*II and the *aad*A antibiotic resistance marker (ARM) genes in the various cottonseed products intended to be placed on the market within the European Union, the applicant submitted data on the presence of genetic material in cottonseed processed products.[21] Transgenic DNA fragments spanning a functional gene length could not be detected in refined, bleached and deodorized cotton oil and in methylcellulose preparations obtained by further processing of cotton linters. It can be assumed that no ARM functional genes are present in these products. In linters and cottonseed meal, DNA fragments spanning a functional gene length are still present. In cottonseed meal their concentration is reduced to a level of about 3 % compared to cottonseed due to degradation of the DNA.

5.1.3. Toxicology[22]

5.1.3.1. Proteins used for safety assessment

The Cry1Ac and NPTII proteins used in safety testing were produced in *E. coli* due to the low levels expression of these proteins in the genetically modified cotton and difficulties in purifying adequate quantities of protein from plant tissues.

The Cry1Ac protein produced in *E. coli* was found to be identical to the protein expressed in cotton MON 531, with the exception of leucine at position 766 in the plant protein being exchanged for serine in the *E. coli* protein, this change being located outside the trypsin resistant core of the protein. Both proteins showed similar properties in analyses by SDS-PAGE, Western analysis, protein glycosylation and insect bioassays.

The NPTII protein produced in *E. coli* was characterized by means of mobility in SDS-PAGE, Western analysis, amino acid sequencing, protein glycosylation and enzymatic activity. The amino acid sequence of the NPTII protein expressed in *E. coli* was found to be equivalent to the NPTII protein expressed in cotton MON 531.

E. coli produced Cry1Ac and NPTII proteins were shown to be structurally and functionally equivalent to the plant produced proteins. Based on the identified similarity in structure and function, the GMO Panel accepts the use of the Cry1Ac and NPTII proteins expressed in *E. coli* for the safety testing of the Cry1Ac and NPTII proteins present in cotton MON 531.

[21] Additional information, December 2010
[22] Technical dossier / Section D7.8

5.1.3.2. Toxicological assessment of expressed novel proteins in cotton MON 531

Cry1Ac

Cry1Ac belongs to the class of delta-endotoxins, which are found in *B. thuringiensis* and are known to have highly specific insecticidal properties. Humans and other mammalian species are not susceptible to toxic activity from Cry1Ac because of the absence of δ-endotoxin receptors in mammalian species. No adverse effects associated with the intake of this protein in mammalian species have been identified. Furthermore, the Cry1Ac protein has been previously assessed by the EFSA GMO Panel in the context of the evaluation of applications concerning other GM events expressing the protein, which were found to be as safe as their conventional counterpart for human and/or animal consumption (EFSA, 2010a, 2011).

(a) Acute toxicity testing

In an acute oral toxicity study, the Cry1Ac protein expressed in *E. coli* did not induce adverse effects in mice after administration by gavage at dosages up to 4200 mg/kg bw.

(b) Degradation in simulated digestive fluids

The stability of the Cry1Ac protein purified from *E. coli* was tested in simulated gastric (SGF, pH 1.2) and intestinal fluids (SIF, pH 7.5). Western analysis of Cry1Ac digests separated on SDS-gel showed that in SGF the protein is readily degraded to its tryptic core in 30 seconds and completely degraded after 7 minutes; in SIF the protein degrades to its tryptic core in 30 minutes, which remains stable up to 20.8 hours. The biological activity of the Cry1Ac protein was reduced by more than 91 % within 5 min incubation in SGF, while it remained essentially unaltered after 20.8 hours incubation in SIF.

(c) Effect of processing

The level of active Cry1Ac protein in cotton MON 531 was assessed in unprocessed and processed cottonseed using a biological test and Western analysis.[23] No detectable level of the Cry1Ac protein was found in processed cottonseed meal.

(d) Bioinformatics studies

Bioinformatics-supported comparison of the amino acid sequence of the Cry1Ac protein expressed in cotton MON 531 with amino acid sequences contained in protein databases were performed in 2009[23]. Apart from expected similarities to other insecticidal Cry family proteins in *B. thuringiensis* and related species, no relevant similarities between the sequence of the Cry1Ac protein and sequences of toxic proteins were found.

NPTII

The NPTII protein has been the subject of previous safety assessments in connection with the evaluation of applications of other NPTII-expressing genetically modified crops, including the maizes MON 863, MON 863 x MON 810 and MON 863 x MON 810 x NK603 and the potato EH92-527-1. In none of these cases safety concerns were identified. The issue of potential horizontal gene transfer was recently addressed in an opinion by the GMO Panel (EFSA, 2009) and assessed in more details in Section 6.1.1 of this opinion.

(a) Acute toxicity testing

In an acute oral toxicity study, the NPTII protein expressed in *E. coli* did not induce adverse effects in mice after administration by gavage at dosages up to 5000 mg/kg bw.

[23] Additional information, February 2010

(b) Degradation in simulated digestive fluids

The *E. coli* produced NPTII protein is readily degraded in gastric and intestinal fluids, as demonstrated by Western analysis of SDS-PAGE gels. In 10 seconds the NPTII protein is degraded in simulated gastric (SGF, pH 1.2) and in 5 minutes in intestinal fluids (SIF, pH 7.5). Using an enzymatic assay, the activity of the NPTII protein was completely lost after 2 minutes in SGF, the shortest incubation time, and after 15 minutes incubation in SIF.

(c) Effect of processing

The levels of active NPTII protein were assessed in processed cottonseed meal prepared from cotton MON 531 using enzymatic activity assay and Western blotting. It was estimated that most (> 96.7 %) of the NPTII protein in cottonseed meal was eliminated during processing.

(d) Bioinformatic studies

The amino acid sequence similarity of the NPTII protein to amino acid sequences of proteins in publicly available databases were evaluated using bioinformatics tools in 2009.[24] No relevant similarities between the sequence of the NPTII protein and sequences of toxic proteins were found.

5.1.3.3. Toxicological assessment of new constituents other than proteins

No new constituents other than the Cry1Ac and the NPTII protein are expressed in cotton MON 531 and no relevant changes in the composition of cotton MON 531 were detected in the comparative compositional analysis (see Section 4.1.2).

5.1.3.4. Toxicological assessment of the whole GM food/feed

The comparative analysis concluded that cotton MON 531 is compositionally and agronomically not different from its conventional counterpart and compositionally within the range observed among conventional cotton varieties, except for the introduced trait. Also the molecular characterisation provided no indication of unintended effects of the genetic modification. According to the EFSA GMO Panel guidance document, no animal safety studies with the whole food/feed are required under these conditions (EFSA, 2006a).

5.1.4. Allergenicity

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 (Codex Alimentarius, 2009; EFSA, 2006a, 2010b).

5.1.4.1. Assessment of allergenicity of the newly expressed proteins

The *cry1Ac* gene originates from *B. thuringiensis* subsp. *kurstaki*, a soil-borne and plant-interacting micro-organism that is not known to be allergenic. The Cry1Ac protein is expressed in various tissues of cotton MON 531 and has been quantified at a number of growth stages during the growing season (see Section 3.1.3). The most relevant tissue for the assessment of food allergenicity is the seeds, which contains less than 2 µg Cry1Ac/g fresh weight.

A bioinformatics-supported comparison of the amino acid sequence of the Cry1Ac and NPTII proteins with the sequences of known allergens, gliadins and glutenins collected in an updated proprietary database based on the FARRP database, has been performed.[25] This analysis included both overall

[24] Additional information, February 2010
[25] Additional information, February 2010

sequence alignments using the FASTA algorithm and searches for short identical stretches of at least eight contiguous amino acids. In the overall sequence alignment no identity higher than 35 % in polypeptides of 80 or more amino acids was found between the Cry1Ac or the NPTII protein and known allergens. Similarly, no identical sequence of eight contiguous amino acids was detected. These results indicate lack of relevant amino acid sequence similarities between the Cry1Ac and the NPTII protein and known allergens.

Furthermore, the potential allergenicity of the Cry1Ac and NPTII proteins have been assessed previously and it was found unlikely that they are allergenic, among others based on their fast degradability and the absence of any significant similarity with known protein allergens. The EFSA GMO Panel considers that the newly expressed proteins are unlikely to be allergenic under the intended conditions of exposure.

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 (Atkins et al., 1988; Malanin et al., 1988). Furthermore, the main cottonseed product in human food, cottonseed oil, is highly purified and contains negligible levels of proteins, if any. In general, edible oils that are refined, bleached and deodorised do not appear to pose a risk to allergic individuals, as they contain virtually no proteins. In the context of the present application the EFSA GMO Panel considers it unlikely that any interactions between the newly expressed proteins and metabolic pathways of cotton would alter the pattern of expression of endogenous proteins/potential allergens and thereby significantly change the overall allergenicity of the whole plant.

5.1.5. Nutritional assessment of GM food and feed

As the comparative compositional analysis of cotton MON 531 provided no indication for unintended effects of the genetic modification under consideration, the GMO Panel concludes that according to the Guidance document (EFSA, 2006a) no nutritional animal feeding study is required.

5.1.6. Post-market monitoring of GM food and feed

The risk assessment concluded that no data have emerged to indicate that cotton MON 531 is any less safe than its conventional counterpart. In addition, cotton MON 531 is, from a nutritional point of view, substantially equivalent to conventional cotton. Therefore, and in line with its Guidance document (EFSA, 2006a), the EFSA GMO Panel is of the opinion that post-market monitoring of the GM food/feed is not necessary.

5.2. Conclusion

The safety of the Cry1Ac and NPTII proteins is supported by bioinformatics analysis and investigations on stability, digestibility and toxicity. The potential allergenicity of the Cry1Ac and NPTII 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 indicated any unintended effects, an alteration in allergenic and nutritional properties of the GM-cottonseed appears to be unlikely.

The EFSA GMO Panel concludes that cotton MON 531 and its derived products obtained through seed processing are unlikely to have any altered potential to induce adverse effects on human and animal health as compared to conventional cotton in the context of their intended use.

6. Environmental risk assessment and monitoring plan

6.1. Environmental risk assessment

The scope of application EFSA-GMO-RX-MON531 is for food and feed products produced from GM cotton MON 531. Thus the scope of the application only includes products produced from cotton MON 531 which contain no viable plant parts. Considering the intended uses of cotton MON 531, there are no requirements for scientific information on environmental safety assessment of accidental release or cultivation of cotton MON 531.

6.1.1. Potential for gene transfer[26]

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 seed dispersal and cross-pollination.

Plant to bacteria gene transfer

The functional recombinant DNA insert in event MON 531 could hypothetically be acquired through horizontal gene transfer (HGT) by bacteria. However, current scientific knowledge of recombination processes in bacteria indicates that horizontal transfer of non-mobile, chromosomally-located DNA fragments between unrelated organisms (such as plants to bacteria) does not occur at quantifiable levels (EFSA, 2009). The hypothetical HGT of recombinant plant DNA to bacteria requires a genetic recombination mechanism, which, in theory, might be homologous or illegitimate recombination. The exposure of bacteria to the recombinant DNA fraction of plants should also be assessed in the context of their continuously ongoing exposure to a wide variety of other naturally occurring sources of DNA.

The probability and frequency of HGT of plant DNA (including the recombinant DNA fraction) to exposed bacteria in the environment is determined by the following factors: (1) The amount and quality of plant DNA accessible to bacteria in relevant environments; (2) The presence of bacteria with a capacity to develop genetic competence for transformation (to take up extracellular DNA); (3) The mechanism of genetic recombination by which the plant DNA can be incorporated and thus stabilized in the bacterial genome (including chromosomes or plasmids); (4) The mobility of the plant DNA in bacterial recipients i.e. whether they are located on chromosomes or mobile genetic elements, e.g., plasmids.

Furthermore, the risk assessment of an impact of rare HGT events considers the potential expression of the recombinant plant DNA in the bacterial cells, and most importantly, the selective advantage conferred by acquisition of recombinant DNA. Finally, the source of the recombinant DNA inserted into the GM plant is considered because many plant transgenes have been derived from the genomes of various soil bacteria. Information on the prevalence of similar genes and their encoded phenotypes within natural microbial communities are taken into account to understand alternative and naturally occurring exposure sources to the same genetic traits.

Hazard identification and characterisation

Cotton MON 531 contains recombinant genes originating from bacteria, i.e., *aad*A, *npt*II, *ori*V, and the *nos* promoter (see Section 3.1.2 of the scientific opinion). It also contains a synthetic *cry1Ac* gene encoding for a Cry1Ac variant protein with 99.4 % amino acid sequence identity to a natural insecticidal Cry1Ac protein of a *B. thuringiensis* strain. The *cry1Ac* and the *npt*II genes are both under the control of 35S promoter originating from the *Cauliflower mosaic virus*. Bioinformatic analysis indicates the possibility of double homologous recombination between the *aad*A gene and the *ori*V present in MON 531 with the same sequences present in bacterial plasmids isolated from soil and activated sludge. This homologous recombination would lead to the replacement of the genes in such plasmids between the two recombination sites by the *npt*II gene cassette as present in the DNA of

[26] Technical dossier / Section D9.2

cotton MON 531 and thus, the acquisition of novel genetic information. The stabilisation rate of the *npt*II gene cassette in such bacteria is estimated in laboratory experiments to be increased about 10^9 - 10^{10} times compared to stabilisation by the process of illegitimate recombination encountered for constructs where no flanking homology to bacterial sequences has been introduced (De Vries and Wackernagel, 2002; Hülter and Wackernagel, 2008). In addition to the double homologous recombination involving flanking regions of transgenes, homologous recombination may theoretically also occur between single transgenes and their natural counterparts in bacteria, i.e., the *aad*A, *npt*II or *cry1Ac*. Such substitutive recombination, however, would not introduce any novel trait, since only existing genes would be replaced. Such replacements are unlikely to provide a selective advantage in comparison to the natural genes which have evolved in their particular habitats. Furthermore, theoretically illegitimate recombination events would also be possible, but they have not been detected even in studies that have exposed bacteria to high concentrations of GM plant DNA (reviewed by EFSA, 2009) and are therefore not considered to significantly contribute to the HGT process.

Expression of *npt*II under the control of CaMV 35S promoter has been demonstrated in bacteria (Assaad and Signer, 1990; Lewin et al., 1998). Therefore, oral treatment with kanamycin or neomycin may create a selective advantage for the transformed bacterial cells with the capability to express the *npt*II-encoded neomycin phosphotransferase II and could enhance the further spread of *npt*II between bacteria by transformation or conjugation. The indicated uses of kanamycin or neomycin or similar substances include gut irrigation and the treatment of encephalopathy in humans (neomycin) and treatment of diarrhoea in farm animals and aerosol administration for respiratory infections in humans and animals (EFSA, 2009).

Since it cannot be excluded that HGT of the *npt*II gene cassette of MON 531 could lead to kanamycin and neomycin resistant bacteria emerging in some environments (e.g., the gastrointestinal tract and faeces) under selective conditions (usage of the corresponding antibiotic), a risk characterisation is performed below.

Exposure characterisation

DNA is a common component of many food and feed products derived from plants. During processing, the DNA of the plant material for food and feed may substantially be degraded or removed. Considering the scope of this application (foods produced from cotton MON 531 (cottonseed oil), foods produced from cotton MON 531 (food additives), feed produced from cotton MON 531 (feed materials and feed additives)), products that are covered in this application include oil for food and feed; meals, cake and hulls for feed; linters and derived products (e.g. viscose, food casins, cellulose esters and ethers for food). The applicant indicates that, in cottonseed meal and linters but not in methylcellulose or oil, DNA is still present.[27] Experimental evidence was provided that processing reduced the content of transgenic DNA spanning the *npt*II gene cassette in the cottonseed meal to a level of 1.6 to 5.1 % of what is present in unprocessed cottonseed.[28]

In case of products containing DNA, the main route of exposure to potential bacterial gene transfer recipient is generally in the gastro-intestinal systems of humans or animals. DNA present in food and feed is substantially degraded through digestion in the human and animal gastrointestinal tracts (see Section 5.1 of the scientific opinion). The highest exposure is expected for products containing intact transgenic DNA (such as unprocessed linters). Exposure is also possible for products in which the transgenic DNA is much degraded but the full gene length transgenic DNA could still be present at a reduced concentration (such as for cottonseed meal). No exposure is expected from highly processed and refined products (such as cottonseed oil and methylcellulose) which have no detectable transgenic DNA; this applies to MON 531 products for human consumption. It should be noted that cotton products are only used in trace amounts in animal feeding in the EU (FEDIOL, online), mainly due the presence of gossypol, which is highly toxic for non-ruminants (e.g. allowable upper limit of 60 mg/kg

[27] Additional information, December 2010
[28] Additional information, December 2010

in feed for pigs[29], Verstraete 2011). Even with accepted upper limits of 500 mg/kg gossypol in feed for ruminants[30], the feed can only contain 5 % cottonseed meal.

The probability of gene transfer depends on the presence of micro-organisms with a capacity to develop genetic competence for transformation, *i.e.*, to take up and recombine extracellular DNA. Several bacterial species with the potential to develop competence belong to the common gut microbial community (EFSA, 2009; Rizzi et al., 2011). However, actual competence development and transformation of such bacteria or other micro-organisms by genomic DNA of plants has not yet been observed in the lower gastrointestinal tract even with optimized model systems providing a selective advantage (EFSA, 2009; Nordgård et al., 2007; Rizzi et al., 2011). In contrast, some studies have shown that introduced bacteria can be naturally transformed in the oral cavity of humans and animals (Duggan et al., 2000, 2003; Mercer et al., 1999a, 1999b, 2001).

Risk characterisation

No exposure of humans is expected to MON 531 DNA due to food consumption. Exposure of animals to the *aad*A–*npt*II–oriV DNA fragment from cotton MON 531 is limited to unprocessed linters and cottonseed meal. Moreover, cottonseed meal contains mainly fragmented DNA with a size smaller than that of the above mentioned fragment.[31] In addition, DNA is further degraded in the gastrointestinal tract of animals (Jonas et al., 2001; Van den Eede et al., 2004). Furthermore, these products are only fed to animals in trace amounts in the EU (FEDIOL, online).

The molecular characterization of the genetic composition of MON 531 revealed that sequences with similar gene order as the *aad*A and *ori*V sites flanking the *npt*II gene in MON 531 are present in naturally-occurring bacteria. The theoretical probability of horizontal transfer of the transgene sequences into bacteria is therefore higher compared to plant transgenes that do not have flanking DNA sites with continual sequence similarity to bacterial DNA. The genetic composition of the inserted DNA in MON 531 would thereby lead to HGT to bacteria harbouring *aad*A and *ori*V sites in their DNA. Since such recombination sites can be located on mobile genetic elements, rare transfer of *npt*II from plant material to bacteria could theoretically be followed by higher frequency conjugative gene transfer to other bacteria and, thus, contribute to establishment of the *npt*II-encoded resistance trait in environmental bacterial populations.

The frequency of HGT of the recombinant *npt*II gene to the development and proliferation of antibiotic resistant bacteria should be seen in the context of the naturally ongoing gene transfer between bacteria, which is several orders of magnitude more frequent (Brigulla and Wackernagel, 2010). The contribution of the frequency of HGT of the recombinant *npt*II gene must likewise be regarded relative to the natural distribution and prevalence of *npt*II genes on mobile genetic elements in bacteria. Bacteria carrying *npt*II on mobile genetic elements are found in various environments, although with large spatial and temporal fluctuations (EFSA, 2009). Moreover, other resistance genes also lead to the distribution and prevalence of kanamycin and neomycin resistant bacteria in various environments. The exposure of environmental bacteria to recombinant DNA from cotton MON 531 of sufficient length is highly limited.

There is limited knowledge about the spatial and temporal variability in the selective conditions, and in the transferability and distribution of *npt*II genes in different environments. Also, there is a lack of experimental data on HGT from cotton MON 531. Notwithstanding these uncertainties, considering the expected low frequency of gene transfer from MON 531 to bacteria compared to that between bacteria, and the very low exposure to MON 531 DNA, the GMO Panel concludes that the contribution of HGT to the environmental prevalence of *npt*II genes is negligible.

[29] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 10-22
[30] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 10-22
[31] Additional information, December 2010

Conclusion

The environmental risk assessment indicates no risk arising from a HGT of the *cry1Ac* gene from cotton MON 531 to bacteria. However, it reveals that for products from cotton MON 531 containing transgenic DNA, there is an increased likelihood of stabilisation of the *npt*II gene from plant DNA in bacteria compared to plants not including sites for double homologous recombination. Nevertheless, this increased likelihood must be seen in the context of the naturally ongoing gene transfer between bacteria, which is several orders of magnitude more frequent.

Considering the expected low frequency of gene transfer from MON 531 to bacteria compared to that between bacteria, and the very low exposure to MON 531 DNA, the GMO Panel concludes that the contribution of HGT to the environmental prevalence of *npt*II genes is negligible.

In summary, the analysis of HGT from cotton MON 531 to bacteria does not indicate a risk to human or animal health or to the environment in the context of its intended uses.

6.1.2. Interactions of the GM plant with target organisms

Due to the intended uses of cotton MON 531, which exclude cultivation, this was not considered an issue by the EFSA GMO Panel.

6.1.3. Interactions of the GM plant with non-target organisms[32]

Due to the intended uses of cotton MON 531 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 the Cry1Ac protein might potentially affect non-target organisms by entering the environment through manure and faeces from animals fed this GM cotton. Cry proteins are degraded by enzymatic activity in the gastrointestinal tract, 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 the protein 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 micro-organisms 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 protein causing significant negative effects on soil micro-organisms.

Considering the scope of the application, it can be concluded that the exposure of potentially sensitive non-target organisms to the Cry1Ac protein is likely to be very low and of no biological relevance.

6.1.4. Monitoring[33]

Considering the scope of the application EFSA-GMO-RX-MON531 for food and feed materials produced from cotton MON 531, a post-market environmental monitoring plan for cotton MON 531 is not required.

[32] Technical dossier / Section D9.5
[33] Technical dossier / Section D11

6.2. Conlusion

Cotton MON 531 is being assessed for food and feed produced from cotton MON 531, thus the scope only includes products produced from cotton MON 531 which contain no viable plant parts. Therefore, there are no requirements for scientific information on environmental risks associated with the accidental release or cultivation of cotton MON 531. No risk arising from a HGT of the *cry1Ac* gene from cotton MON 531 to bacteria has been identified. A hazard of an increased likelihood of stabilisation of the *npt*II gene from cotton MON 531 DNA in bacteria was postulated. However, considering the expected low frequency of gene transfer from MON 531 to bacteria compared to that between bacteria, and the very low exposure to MON 531 DNA, the GMO Panel concludes that the contribution of HGT to the environmental prevalence of *npt*II genes is negligible. The analysis of HGT from cotton MON 531 to bacteria does not indicate a risk to human or animal health or to the environment in the context of its intended uses. Considering the scope of the application, it can be concluded that the exposure of potentially sensitive non-target organisms to the Cry1Ac protein is likely to be very low and of no biological relevance. A post-market environmental monitoring plan for cotton MON 531 is not required.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The EFSA GMO Panel was requested to deliver a scientific opinion for renewal of the authorisation for continued marketing of existing products from GM cotton MON 531 (references EFSA-GMO-RX-MON531) under Regulation (EC) No 1829/2003. The scope of this application cover the continued marketing of (1) foods produced from cotton MON 531 (cottonseed oil); (2) foods produced from cotton MON 531 (food additives); (3) feed produced from cotton MON 531 (feed materials and feed additives) which were lawfully placed on the market in the European Community before the date of entry into force of Regulation (EC) No 1829/2003 and included in the Community Register of genetically modified food and feed.

In delivering its scientific opinion, the EFSA GMO Panel considered the renewal application EFSA-GMO-RX-MON531; additional information submitted by the applicant on request of the EFSA GMO Panel; the scientific comments submitted by Member States; and relevant scientific publications. In accordance with the Guidance Document for renewal of authorisations of existing GMO products (EFSA, 2006b), the EFSA GMO Panel has taken into account the new information, experience and data on cotton MON 531, which have become available during the authorisation period.

The EFSA GMO Panel is of the opinion that the molecular characterisation data provided for cotton MON 531 are sufficient to conclude on this part of the risk assessment. The bioinformatic analyses of the inserted DNA and the flanking regions do not raise safety concerns. The levels of Cry1Ac and NPTII proteins in cotton MON 531 have been analysed and the stability of the genetic modification and the corresponding phenotype have been demonstrated. Molecular characterisation indicated that the two bacterial antibiotic resistance genes and other sequences of bacterial origin present in MON 531 may allow double homologous recombination to plasmid sequences present in the environment.

The results of the comparative analysis indicated that cotton MON 531 is compositionally, agronomically and phenotypically not different from its conventional counterpart and compositionally within the range observed among commercial varieties, except for the presence of newly expressed Cry1Ac and NPTII proteins. The safety of the Cry1Ac and NPTII proteins is supported by bioinformatics analysis and investigations on stability, digestibility and toxicity. The EFSA GMO Panel considers that cotton MON 531 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 MON 531 and its derived products obtained through seed processing are unlikely to have any adverse effects on human and animal health, as compared to conventional cotton, in the context of their intended uses.

The EFSA GMO Panel considers that there is no requirement for scientific information on environmental risk assessment associated with the accidental release or cultivation of cotton MON

531. No risk arising from a HGT of the *cry1Ac* gene from cotton MON 531 to bacteria has been identified. A hazard of an increased likelihood of stabilisation of the *npt*II gene from cotton MON 531 DNA in bacteria was postulated. However, considering the expected low frequency of gene transfer from MON 531 to bacteria compared to that between bacteria, and the very low exposure to MON 531 DNA, the GMO Panel concludes that the contribution of HGT to the environmental prevalence of *npt*II genes is negligible. The analysis of HGT from cotton MON 531 to bacteria does not indicate a risk to human or animal health or to the environment in the context of its intended uses. Considering the scope of the application, it can be concluded that the exposure of potentially sensitive non-target organisms to the Cry1Ac protein is likely to be very low and of no biological relevance. A post-market environmental monitoring plan for cotton MON 531 is not required.

In conclusion, the EFSA GMO Panel considers that information available for cotton MON 531 addresses the questions raised by the Member States and that cotton MON 531, as described in this application, is as safe as its conventional counterpart and is unlikely to have adverse effects on human and animal health and the environment in the context of its intended uses.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the European Commission, received 29 June 2007, concerning a request for renew the authorisation for placing on the market of GM cotton MON 531 in accordance with Articles 8(1)(b) and 20(1)(b) of Regulation (EC) No 1829/2003.

2. Acknowledgement letter, dated 20 July 2007, from EFSA to the European Commission.

3. Letter from EFSA to applicant, dated 19 December 2007, requesting additional information under completeness check.

4. Letter from applicant to EFSA, received 29 January 2008, providing the timeline for submission of responses.

5. Letter from applicant to EFSA, received 2 April 2008, changing the timeline for submission of responses.

6. Letter from applicant to EFSA, received 6 May 2008, providing the timeline for submission of responses.

7. Letter from applicant to EFSA, received 26 May 2008, providing additional information under completeness check.

8. Letter from EFSA to applicant, dated 11 June 2008, delivering the 'Statement of Validity' for application EFSA-GMO-RX-MON531, cotton MON 531 submitted by Monsanto under Regulation (EC) No 1829/2003.

9. Letter from EFSA to applicant, dated 24 June 2008, requesting additional information and stopping the clock.

10. Letter from applicant to EFSA, received 29 July 2008, providing the timeline for submission of responses.

11. Letter from EFSA to applicant, dated 9 September 2008, requesting additional information and maintaining the clock stopped.

12. Letter from applicant to EFSA, received 15 September, replying to letter dated 9 September 2008.

13. Letter from applicant to EFSA, received 30 September 2008, providing the additional information requested.

14. Letter from EFSA to applicant, dated 23 October 2008, requesting additional information and maintaining the clock stopped.

15. Letter from applicant to EFSA, received 2 December 2008, providing the timeline for submission of response.

16. Letter from applicant to EFSA, received 2 March 2009, providing additional information requested.

17. Letter from EFSA to applicant, dated 7 April 2009, requesting additional information and maintaining the clock stopped.

18. Letter from applicant to EFSA, received 23 April 2009, providing the additional information requested.

19. Letter from EFSA to applicant, dated 12 May 2009, restarting the clock.

20. Letter from EFSA to applicant, dated 24 July 2009, requesting additional information and stopping the clock.

21. Letter from applicant to EFSA, received 7 September 2009, providing the timeline for submission of responses.

22. Letter from EFSA to applicant, dated 16 September 2009, requesting additional information and maintaining the clock stopped.

23. Letter from applicant to EFSA, received 30 October 2009, providing the timeline for submission of responses.

24. Letter from applicant to EFSA, received 3 February 2010, providing additional information requested.

25. Letter from EFSA to applicant, dated 25 May 2010, requesting additional information and maintaining the clock stopped.

26. Letter from applicant to EFSA, received 8 June 2010, providing additional information requested.

27. Letter from applicant to EFSA, received 15 July 2010, providing the timeline for submission of responses.

28. Letter from EFSA to applicant, dated 2 August 2010, requesting clarifications.

29. Letter from applicant to EFSA, received 14 September 2010, providing additional information requested.

30. Letter from applicant to EFSA, received 15 September 2010, providing the clarifications requested.

31. Letter from EFSA to applicant, dated 4 October 2010, requesting additional information and maintaining the clock stopped.

32. Letter from applicant to EFSA, received 2 December 2010, providing additional information requested.

33. Letter from EFSA to applicant, dated 31 January 2011, requesting additional information and maintaining the clock stopped.

34. Letter from applicant to EFSA, received 21 March 2011, providing the timeline for submission of responses.

35. Letter from applicant to EFSA, received 11 April 2011, providing the additional information requested.

36. Letter from EFSA to applicant, dated 16 May 2011, restarting the clock.

37. Letter from applicant to the European Commission, dated 16 June 2011, asking for extension of the scope of application EFSA-GMO-RX-MON531 to include renewal of authorisation of existing foods produced from MON 531 cotton (cottonseed oil) that was previously notified according to Article 8(1)(a) of Regulation (EC) 1829/2003 on genetically modified food and feed.

38. Letter from the European Commission to applicant, dated 30 June 2011, acknowledging the receipt of the request for the extension of the scope.

39. Letter from EFSA to applicant, dated 5 July 2011, requesting to submit an updated Cartagena Protocol (Part III), Labelling proposal (Part IV), Post-market environmental monitoring plan and stopping the clock.

40. Letter from applicant to EFSA, received 11 July 2011, providing the updated Cartagena Protocol and Labelling proposal.

41. Letter from the European Commission to EFSA, dated 19 July 2011, providing the updated Summary of application EFSA-GMO-RX-MON531.

42. Letter from EFSA to applicant, dated 2 August 2011, restarting the clock.

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