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Assessment of genetically modified cotton COT102 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2017-141)

EFSA Panel on Genetically Modified Organisms (GMO),
Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Hanspeter Naegeli, Francisco Javier Moreno, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann, Fabio Veronesi, Michele Ardizzone, Giacomo De Sanctis, Antonio Fernández, Andrea Gennaro, José Ángel Gómez Ruiz, Tilemachos Goumperis, Dafni Maria Kagli, Paolo Lenzi, Aleksandra Lewandowska, Ana M Camargo, Franco Maria Neri, Nikoletta Papadopoulou and Tommaso Raffaello

Abstract

Genetically modified cotton COT102 was developed to confer resistance against several lepidopteran species. The molecular characterisation data and bioinformatic analyses do not identify issues requiring food/feed safety assessment. None of the differences in the agronomic-phenotypic and compositional characteristics between cotton COT102 and its non-GM comparator needs further assessment, except for levels of acid detergent fibre, which do not raise safety or nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Vip3Aa19 and APH4 proteins as expressed in cotton COT102 and finds no evidence that the genetic modification would change the overall allergenicity of cotton COT102. In the context of this application, the consumption of food and feed from cotton COT102 does not represent a nutritional concern for humans and animals. The GMO Panel concludes that cotton COT102 is as safe as the non-GM comparator and non-GM cotton varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of accidental release of viable cotton COT102 seeds into the environment, this would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of cotton COT102. The GMO Panel concludes that cotton COT102 is as safe as its non-GM comparator and the tested non-GM cotton varieties with respect to potential effects on human and animal health and the environment.

Keywords: GM, genetic engineering, cotton (*Gossypium hirsutum*), COT102, Vip3Aa19, APH4, import and processing

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Correspondence: nif@efsa.europa.eu

Panel members: Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Hanspeter Naegeli, Francisco Javier Moreno, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann, and Fabio Veronesi.

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Summary

Following the submission of application EFSA-GMO-DE-2017-141 under Regulation (EC) No 1829/2003 from Syngenta ('the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority ('GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) insect resistant cotton (*Gossypium hirsutum* L.) COT102 according to Regulation (EU) No 503/2013. The scope of application EFSA-GMO-DE-2017-141 is for import, processing and food and feed uses of cotton COT102 within the European Union (EU) and does not include cultivation in the EU.

In this scientific opinion, the GMO Panel reports on the outcome of its risk assessment of cotton COT102 according to scope of application EFSA-GMO-DE-2017-141. The GMO Panel conducted the assessment of cotton COT102 in line with the principles described in Regulation (EU) No 503/2013 and its applicable guidelines for the risk assessment of GM plants. The molecular characterisation data establish that cotton COT102 contains a single insert consisting of one copy of the *vip3Aa19* and *aph4* expression cassettes. The quality of the sequencing methodology and datasets was assessed by the EFSA GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note. Updated bioinformatics analyses of the sequences encoding the newly expressed proteins and open reading frames (ORFs) present within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concern. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Vip3Aa19 and APH4 proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant- and microbe-produced Vip3Aa19 and APH4 proteins indicate that these proteins are equivalent and the microbial derived proteins can be used in the safety studies.

Based on the selection of test materials, on the field trial sites and associated management practices and on the agronomic-phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the differences identified in the agronomic/phenotypic and compositional characteristics between cotton COT102 and its non-GM comparator needs further assessment, except for levels of ADF, which do not raise safety or nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Vip3Aa19 and APH4 proteins as expressed in cotton COT102 and finds no evidence that the genetic modification would change the overall allergenicity of cotton COT102. In the context of this application, the consumption of food and feed from cotton COT102 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that cotton COT102 is as safe as the non-GM comparator and non-GM cotton varieties tested, and no post-market monitoring of food/feed is considered necessary.

Considering the introduced trait, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure, cotton COT102 would not raise safety concerns in the case of accidental release of viable GM cotton seeds into the environment. The post-market environmental monitoring (PMEM) plan and reporting intervals are in line with the intended uses of cotton COT102.

The GMO Panel considered the overall quality of the performed literature searches acceptable. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the intended uses of cotton COT102.

The GMO Panel concludes that cotton COT102 is as safe as its non-GM comparator and the tested non-GM cotton reference varieties with respect to potential effects on human and animal health and the environment. The GMO Panel considers that the risk assessment may need to be updated in case products containing hygromycin B or other substrates of the APH4 enzyme obtain future market approval in the EU.

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1. Introduction

The scope of the application EFSA-GMO-DE-2017-141 is for food and feed uses, import and processing of cotton COT102 and does not include cultivation in the European Union (EU). Cotton COT102 was developed to confer resistance against several lepidopteran pests of cotton.

1.1. Background

On 12 April 2017, the European Food Safety Authority (EFSA) received from the Competent Authority of Germany application EFSA-GMO-DE-2017-141 for authorisation of cotton COT102 (unique identifier SYN-IR1Ø2–7), submitted by Syngenta (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1] Following receipt of application EFSA-GMO-DE-2017-141, EFSA informed EU Member States (MS) and the European Commission (EC), and made the application available to them. Simultaneously, EFSA published a summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013,[3] with the EFSA guidance documents, and, when needed, asked the applicant to supplement the initial application. On 24 July 2017, EFSA declared the application valid.

From validity date, EFSA and the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') endeavoured to respect a time limit of six months to issue a scientific opinion on application EFSA-GMO-DE-2017-141. Such time limit was extended whenever EFSA and/or the GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and European Commission (for further details, see Section 5). In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.[4] The EU Member States had three months to make their opinion known on application EFSA-GMO-DE-2017-141 as of date of validity.

1.2. Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of cotton COT102 in the context of the scope defined in application EFSA-GMO-DE-2017-141.

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. In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them because they pertain to risk management.[5]

2. Data and Methodologies

2.1. Data

The GMO Panel based its scientific assessment of cotton COT102 on the valid application EFSA-GMO-DE-2017-141, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU MS and relevant scientific publications. As part of this comprehensive information package, the GMO Panel received additional unpublished studies submitted by the applicant in order to comply with the specific provisions of Regulation (EU) No 503/2013. A list of these additional unpublished studies is provided in Appendix A.

[1] 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, 18.10.2003, p. 1–23.
[2] Available online: https://open.efsa.europa.eu/study-inventory/EFSA-Q-2017-00271
[3] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.
[4] 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 L 106, 12.3.2001, p. 1–38.
[5] These details are available online at: https://open.efsa.europa.eu/study-inventory/EFSA-Q-2017-00271

2.2. Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 1829/2003, the applicable guidelines (i.e. EFSA GMO Panel, 2010a, 2011a,b, 2015; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA, 2010, 2014, 2017, 2018, 2019a,b; EFSA GMO Panel, 2010b, 2018) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/02, OC/EFSA/GMO/2018/04, EOI/EFSA/SCIENCE/2020/01 – CT02GMO and OC/EFSA/GMO/2021/06 the contractors performed preparatory work for the evaluation of the methods applied for the statistical analysis of field trials, the applicant's literature search, the toxicity studies and the bioinformatic analysis, respectively.

3. Assessment

3.1. Introduction

Cotton COT102 expresses Vip3Aa19, a vegetative insecticidal protein derived from the native Vip3Aa1 protein found in *Bacillus thuringiensis* strain AB88. The Vip3Aa19 protein has insecticidal activity against several lepidopteran species, including cotton bollworm and fall armyworm. Cotton COT102 also expresses the APH4 protein, used as an antibiotic resistance marker gene (ARMG). The APH4 enzyme catalyses the phosphorylation of the 4-hydroxyl group of hygromycin B, inactivating its antibiotic activity.

3.2. Systematic literature review[6]

The GMO Panel assessed the applicant's literature searches on cotton COT102, which include a scoping review, according to the guidelines given in EFSA (2010, 2019b).

A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application EFSA-GMO-DE-2017-141. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for cotton COT102 at present.

The GMO Panel considered the overall quality of the performed literature searches acceptable. The literature searches identified 16 relevant peer reviewed and non-peer reviewed publications on cotton COT102 (Appendix B). Based on the relevant publications, the GMO Panel does not identify any safety issues pertaining to the intended uses of cotton COT102.

3.3. Molecular characterisation[7]

3.3.1. Transformation process and vector constructs

Cotton COT102 was developed by *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation. Hypocotyls of cotton (*Gossypium hirsutum* L.) cv. Coker 312 were co-cultured with a disarmed *A. tumefaciens* strain EHA101 containing the vector pCOT1. A non-oncogenic helper plasmid pEHA101 was also used in the transformation process.

The plasmid pCOT1 used for the transformation contains two expression cassettes between the right and left borders of the T-DNA, containing the following genetic elements:

- The *vip3Aa19* expression cassette consists of: the promoter and intron from the actin-2 gene from *Arabidopsis thaliana*; the plant codon-optimised *vip3Aa19* from *B. thuringiensis* strain AB88; and the terminator sequence of the nopaline synthase gene from *A. tumefaciens*.
- The *aph4* expression cassette consists of: the promoter and first intron of the ubiquitin 3 gene from *A. thaliana*; the hygromycin B phosphotransferase (*aph4*) gene from *Escherichia coli* strain K12; and the terminator sequence of the nopaline synthase gene of *A. tumefaciens*.

The vector backbone contained elements necessary for the maintenance and selection of the plasmid in bacteria.

[6] Dossier: Part II – Section 7; additional information: 1/7/2019 and 27/9/2022.
[7] Dossier: Part II – Section 1.2; additional information: 6/4/2018, 22/3/2019, 25/6/2020, 2/7/2020, 17/2/2021, 13/9/2021, 3/6/2022, 28/6/2022, 5/8/2022 and 22/2/2023.

3.3.2. Transgene constructs in the GM plant

Molecular characterisation of cotton COT102 was performed by Southern analysis, polymerase chain reaction (PCR) and DNA sequence analysis, in order to determine insert copy number, size and organisation of the inserted sequences and to confirm the absence of plasmid backbone sequences. The approach used is acceptable both in terms of coverage and sensitivity. Overall, the quality of the sequencing methodology and datasets was assessed by the GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note (2018).

Southern analyses indicated that cotton COT102 contains a single insert, which consists of a single copy of the T-DNA in the same configuration as in the pCOT1 transformation vector. The insert and copy number were confirmed by multiple restriction enzyme/probe combinations covering the T-DNA region and flanking regions. PCR analyses confirmed the results obtained by the Southern analyses. The absence of vector backbone sequences was demonstrated by Southern analysis using a backbone-specific probe.

The nucleotide sequence of the entire insert of cotton COT102 together with 1129 base pair (bp) of the 5′ and 1302 bp of the 3′ flanking regions were determined. The insert of 7475 bp is identical to the T-DNA of pCOT1, with the exception of 24 bp deleted from the T-DNA RB and 19 bp deleted from the T-DNA LB.

A comparison with the pre-insertion locus indicated that 86 bp of the parental genomic sequence had been deleted upon transformation and 4 bp of filler DNA were added. Moreover, an additional 690 bp are present at the 3′ insert to genome junction that did not align to sequence at the genomic site. The possible interruption of known endogenous cotton genes by the insertion in cotton COT102 was evaluated by bioinformatic analyses of the pre-insertion locus and of the genomic sequences flanking the insert. The results of these analyses do not indicate the interruption of any known endogenous gene in the cotton COT102.

The results of segregation (see Section 3.3.5) and bioinformatics analyses are compatible with a single insertion in the nuclear genome.

Updated bioinformatic analyses of the amino acid sequences of the newly expressed APH4 and Vip3Aa19 proteins reveal no significant similarities to known toxins and allergens. Updated bioinformatic analyses of the newly created open reading frames (ORFs) within the insert and spanning the junctions between the insert and genomic DNA indicated that four ORFs (COT102_insert_150, COT102_insert_165, COT102_insert_175 and COT102_insert_189) exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach, and one ORF (COT102_insert_147) presents eight identical contiguous amino acids with allergen sequences. These ORFs are found within the transcriptional unit of Vip3Aa19 coding sequence, in the same orientation, but in a different reading frame and do not contain start codons. In conclusion, the updated bioinformatic analyses indicated that the expression of any ORF showing significant similarities to toxins or allergens in cotton COT102 is unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for cotton COT102 to microbial DNA. The likelihood and potential consequences of plant to bacteria gene transfer are described in Section 3.6.1.2.

3.3.3. Protein characterisation and equivalence

Cotton COT102 expresses two new proteins: Vip3Aa19 and APH4. Given the technical restraints in producing large enough quantities for safety testing from plants, these proteins were recombinantly produced in *E. coli*. A set of biochemical methods was employed to demonstrate the equivalence between the cotton- and microbe-derived proteins. Purified proteins from these two sources were characterised and compared in terms of their biochemical, structural and functional properties.

Vip3Aa19 characterisation and equivalence

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both cotton- and microbe-derived Vip3Aa19 proteins had the expected molecular weight of ~ 89 kDa and were comparably immunoreactive to Vip3Aa19-specific antibodies. Glycosylation detection analysis demonstrated that none of the Vip3Aa19 proteins were glycosylated. Amino acid sequence analysis by mass spectrometry (MS) methods showed that both proteins matched the deduced sequence as defined by the inserted *vip3Aa19* gene. Functional equivalence was

demonstrated by an insect feeding bioassay which showed that the insecticidal activity of the plant-produced Vip3Aa19 was similar to the activity of the microbial-produced Vip3Aa19.

APH4 characterisation and equivalence

The applicant has provided two batches of microbe-derived APH4 protein to be used for safety studies:

1) *E. coli*-derived protein used for *in vitro* and acute toxicity studies (Sections 3.5.1.2.3 and 3.5.2.1.2). Protein characterisation study for this batch of recombinant protein showed that the plant-produced APH4 in cotton COT102 was undetectable by SDS-PAGE, western blot or ELISA analyses. However, sufficient amounts of APH4 were present in crude protein extract from pollen which was used to carry out a western blot analysis. This analysis showed that plant- and microbe-derived APH4 proteins had the expected molecular weight of ~ 38 and 39 kDa respectively, and were comparably immunoreactive to APH4 protein specific antibodies. The slight molecular weight difference was due to the presence of an additional 14 amino acid residues: 11 from the T7 tag and three from the pET-3a vector polylinker. Due to the low expression in cotton COT102, APH4 could not be extracted at sufficient quantity to further experimentally demonstrate its equivalence with the microbial produced APH4. In addition, *in silico* analysis of the APH4 amino acid sequence for potential glycosylation sites indicated the absence of any glycosylation consensus sequences for either for *N*- or *O*-linked glycosylation. Enzymatic activity of the microbe-derived APH4 protein was confirmed by an *in vitro* activity assay.
2) *E. coli*-derived protein used for 28-day toxicity studies (Section 3.5.2.1.2). SDS-PAGE and western blot analysis showed that both cotton- and microbe-derived APH4 proteins had the expected molecular weight of ~ 38 kDa and were comparably immunoreactive to APH4-specific antibodies. Glycosylation detection analysis demonstrated that neither of the APH4 proteins were glycosylated. Amino acid sequence analysis by MS methods showed that both proteins matched the deduced sequence as defined by the inserted *aph4* gene. Functional equivalence was demonstrated by an *in vitro* assay which showed that plant- and microbe-derived APH4 proteins had comparable enzymatic activity.

The data provided by the applicant confirmed that the two recombinantly produced APH4 protein batches were equivalent and can be used in the safety studies.

The protein characterisation data comparing the structural, biochemical and functional properties of plant- and microbe-produced Vip3Aa19 and APH4 proteins indicate that these proteins are equivalent, and the microbial derived proteins can be used in the safety studies.

3.3.4. Information on the expression of the insert

Protein levels of Vip3Aa19 and APH4 were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from replicated field trials across four locations in the US during the 2018 growing season. Samples analysed included leaves (BBCH 14–16, BBCH 60, BBCH 80), bolls (BBCH 65–67), flower (BBCH 65–67), pollen (BBCH 65–67), root (BBCH 14–16, BBCH 85), fuzzy seeds (BBCH 99), squares (BBCH 60), and whole plants (BBCH 14–16, BBCH 85). The mean values, standard deviations and ranges of protein expression levels in fuzzy seeds (N = 20) and pollen (N = 4) of the Vip3Aa19 and APH4 proteins used to estimate human and animal dietary exposure (see Section 3.4.5) are summarised in Table 1.

Table 1: Protein expression data for the Vip3Aa19 and APH4 proteins in fuzzy seeds [μg/g dry weight (dw) and μg/g fresh weight (fw)] from cotton COT102[(a)]

Fuzzy seeds	**μg/g dry weight (dw)**	**μg/g fresh weight (fw)**
Vip3Aa19	2.16[(b)] ± 0.470[(c)]	1.83 ± 0.468
	(1.01–2.99)[(d)]	(0.725–2.62)
APH4	< LOQ[(e)]	< LOQ[(e)]
Pollen		
Vip3Aa19	< LOD[(f)]	< LOD[(f)]

Fuzzy seeds	µg/g dry weight (dw)	µg/g fresh weight (fw)
APH4[(g)]	10.2 ± 1.44	4.34 ± 0.787
	(8.38–11.7)	(3.69–5.48)

(a): Number of samples n = 20 in fuzzy seeds and n = 4 for in pollen.
(b): Mean.
(c): Standard deviation.
(d): Range.
(e): For APH4 in fuzzy seeds 12 samples were below the limit of detection (LOD = 0.125 µg/g dw) and 8 were below the limit of quantification (LOQ = 0.250 µg/g dw).
(f): For Vip3Aa19 all samples were below the limit of detection (LOD = 3.20 µg/g dw).
(g): The concentration of APH4 in pollen represents the sum of all iterations collected through sequential exhaustive extractions, therefore no further adjustment for extraction efficiency is applicable.

3.3.5. Inheritance and stability of inserted DNA

Genetic stability of cotton COT102 insert was assessed by Southern analysis of genomic DNA from five consecutive generations (BC3F3, BC3F4, BC3F5, BC3F6 and BC3F7) and PCR-based segregation analysis of both traits of cotton COT102 from three generations (F1, BC1F1 and BC4F1). For the Southern analysis, the restriction enzyme/probe combinations used were sufficient to conclude that all the plants tested retained the single copy of the insert and flanking regions, which were stably inherited in subsequent generations.

Phenotypic stability was assessed by measuring the concentration of Vip3Aa19 and APH4 proteins in leaves and seeds collected from three generations (T7, T9 and T11). The expression of the Vip3Aa19 and APH4 proteins was confirmed in the tested tissue. The results support the presence of a single insertion, segregating in a Mendelian fashion.

3.3.6. Conclusion on molecular characterisation

The molecular characterisation data establish that cotton COT102 contains a single insert consisting of one copy of the *Vip3Aa19 and APH4* expression cassettes. Bioinformatics analyses of the sequences encoding the newly expressed proteins and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Vip3Aa19 and APH4 proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Vip3Aa19 and APH4 proteins, indicate that these proteins are equivalent and the microbially derived proteins can be used in the safety studies.

3.4. Comparative analysis[8]

3.4.1. Overview of studies conducted for the comparative analysis

Application EFSA-GMO-DE-2017-141 presents data on agronomic and phenotypic characteristics as well as on seed composition of cotton COT102 (Table 2).

Table 2: Overview of the comparative analysis studies to characterise cotton COT102 provided in application EFSA-GMO-DE-2017-141

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic, phenotypic and compositional analysis	Field study, US, 2018, 10 field trial sites[(a)]	01W34	6[(b)]

GM: Genetically modified.
(a): The field trials were located in: Perquimans, NC; St. Landry Parish, LA; Barnwell, SC; Tift, GA; Uvalde, TX; Washington, MS; Waller, TX, Caddo, OK; Tulare, CA; Tom Green, TX; and Rapides Parish, LA. An additional site in Waller, TX was excluded from the study because poor germination resulted in inadequate plant stands.
(b): The non-GM cotton reference varieties were LA 122, AM UA 45, UA 222, HQ212CT, UA 114 and HQ21 OCT.

[8] Dossier: Part II – Section 1.3; additional information: 25/6/2020, 13/10/2020, 23/4/2021 and 13/9/2021.

3.4.2. Experimental field trial design and statistical analysis

At each field trial site, the following materials were grown in a randomised complete block design with four replicates: cotton COT102, the comparator 01W34 and four non-GM reference varieties.

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a,b). This includes the application of a difference test (between the GM cotton and the comparator) and an equivalence test (between the GM cotton and the set of non-GM commercial reference varieties). The results of the equivalence test are categorised into four possible outcomes (I-IV, ranging from equivalence to non-equivalence).[9]

3.4.3. Suitability of selected test materials

3.4.3.1. Selection of the test materials

Cotton variety Coker 312 was transformed to obtain cotton COT102, which was then backcrossed with the variety 01W34 to produce the GM cotton used in the comparative analysis.

The comparator used in the field trials is the non-GM cotton variety 01W34, which has a genetic background similar to cotton COT102 (as documented by the pedigree) and is considered to be an acceptable non-GM comparator.

Cotton COT102 and the non-GM comparator belong to the mid-season maturity group, which is considered appropriate for growing in environments across the US, where the field trials were conducted.

Commercial non-GM reference varieties with a maturity group ranging from early to early-mid season were selected by the applicant and, at each selected site, four reference varieties were tested (see footnote of Table 2). Based on the information provided, the GMO Panel considers the selected non-GM reference varieties acceptable for the comparative assessment.

3.4.3.2. Seed production and quality

Seeds of cotton COT102 and the non-GM comparator used in the 2018 field trials were produced from plants free of diseases, harvested and stored under similar conditions, before being sown in the field trial sites. The seed lots were verified for their identity via event specific quantitative polymerase chain reaction analysis.

The seeds were tested for their germination capacity under warm and cold temperature conditions.[10] The results[11] indicate that the seed germination of cotton COT102 was not different than that of its non-GM comparator.

3.4.3.3. Conclusion on suitability

The GMO Panel concludes that cotton COT102, the non-GM comparator and the non-GM reference varieties were properly selected and are of sufficient quality. The test materials are considered acceptable for the comparative analysis.

3.4.4. Representativeness of the receiving environments

3.4.4.1. Selection of field trial sites

The field trials sites were located in commercial cotton-growing regions of the US.

The soil and climatic characteristics of the selected fields were diverse,[12] corresponding to optimal, near-optimal and sub-optimal conditions for cotton cultivation (Sys et al., 1993).

The GMO Panel considers that the selected sites reflect commercial cotton-growing regions in which the test materials are likely to be grown.

[9] In detail, the four outcomes are: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence).

[10] The seed germination test reports were produced by the North Carolina Department of Agriculture, following the germination test in line with the Association of Official Seed Analysts (AOSA, 2010).

[11] Cotton COT102 showed a mean germination of 97% and 18% while the non-GM comparator showed a mean of 97% and 22% under warm and cold temperature conditions respectively.

[12] Soil types of the field trials were sand, loamy sand, sandy loam, clay loam, clay and silt loam; soil organic matter ranged from 0.4% to 3.6%; pH ranged from 5.1 to 8.0; average temperatures and sum of precipitations during the usual crop growing season ranged respectively from 27.6°C to 20.9°C and from 592 mm to 1,305 mm.

3.4.4.2. Meteorological conditions

Maximum and minimum mean temperatures and sum of precipitations were provided on a weekly basis.

An exceptional weather condition was reported at one of the selected sites.[13] However, due to the lack of major impacts on plant growth at this site, the GMO Panel considers that the exceptional weather condition did not invalidate the selection of the field trial sites for the comparative analysis.

3.4.4.3. Management practices

The field trials included plots containing cotton COT102, plots with the non-GM comparator and plots with non-GM cotton reference varieties, mostly managed according to local agricultural practices.

At some field trial sites[14] sowing occurred relatively later than usual, resulting in a shifted growing cycle. The additional information indicated that the shifted growing cycle was unlikely to affect the representativeness of field trial conditions. The GMO Panel considers that the management practices, including sowing, harvesting and application of plant protection products were acceptable for the selected receiving environments.

3.4.4.4. Conclusion on representativeness

The GMO Panel concludes that the geographical locations, soil characteristics, meteorological conditions and most of the management practices are typical for receiving environments where the tested materials could be grown.

3.4.5. Agronomic and phenotypic analysis

Nine agronomic and phenotypic endpoints[15] plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trials (see Table 2). The endpoints were analysed as described in Section 3.3.2, with the following results:

- Statistically significant differences between cotton COT102 and the non-GM comparator were identified for plant height, fruit body count, final stand count and seed weight. All these endpoints fell under equivalence category I or II.

3.4.6. Compositional analysis

Cotton COT102 fuzzy seeds harvested from the field trials (Table 2) were analysed for 57 constituents, including those recommended by OECD (OECD, 2009). The statistical analysis was not applied to 13 constituents because their concentration in more than half of the samples was below the limit of quantification.[16]

The statistical analysis was applied to a total of 44 constituents;[17] a summary of the outcome of the test of difference and the test of equivalence is presented in Table 3.

For cotton COT102 seeds, statistically significant differences with the non-GM comparator were found for 12 endpoints. All the endpoints for which significant differences were found fell under equivalence category I or II, except for acid detergent fibre (ADF) which fell under equivalence category III.

The equivalence test was not done for methionine because of the lack of variation among the non-GM reference varieties; however, no statistically significant differences were observed between cotton COT102 and the non-GM comparator.

[13] Severe thunderstorms were registered at the field trial in St. Landry Parish, LA.
[14] Two field trials located in Tom Green, TX and Rapides Parish, LA.
[15] Early stand count, days to 50% flowering, plant height, fruiting body count, final stand count, lodging, days to maturity, seedcotton yield and seed weight.
[16] Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecenoic acid (C17:1), gamma linolenic acid (18:3), stearidonic acid (C18:4), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5).
[17] Moisture, crude protein, crude fat, ash, total carbohydrates (calculated), total dietary fibre, acid detergent fibre, neutral detergent fibre, alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0), dihydrosterculic acid, malvalic acid, sterculic acid, calcium, phosphorus, α-tocopherol, total gossypol and free gossypol.

Table 3: Outcome of the comparative compositional analysis in fuzzy seeds for cotton COT102. The table shows the number of endpoints in each category

		Test of difference[a]	
		Not different	Significantly different
Test of equivalence[b]	Category I/II	31	11[c]
	Category III/IV	–	1[d]
	Not categorised	1[e]	–
	Total endpoints	44	

(a): Comparison between cotton COT102 and its non-GM comparator.
(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.
(c): Endpoints with significant differences between cotton COT102 and its non-GM comparator falling in equivalence category I-II: ash, calcium, phosphorus, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), malvalic acid, α-tocopherol, free gossypol and total gossypol.
(d): Endpoint with significant differences between the cotton COT102 and its non-GM comparator and falling in equivalence category III-IV: ADF. Estimated means are reported for these endpoints in Table 4.
(e): Endpoint not categorised for equivalence and without significant differences between the cotton COT102 and its non-GM comparator: methionine.

The GMO Panel assessed all significant differences between cotton COT102 and its non-GM comparator, taking into account potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. Quantitative results for the endpoint showing a significant difference between cotton COT102 and its non-GM comparator and falling under equivalence category III are given in Table 4.

Table 4: Quantitative results (estimated means and equivalence limits) for the compositional endpoint that is further assessed based on the results of the statistical analysis

Endpoint	Cotton COT102[a]	Comparator	Non-GM reference varieties	
			Mean	Equivalence limits
ADF (% dw)	36.9*	35.4	34.6	32.9–36.5

dw: dry weight; ADF: acid detergent fibre.
(a): For cotton COT102, significantly different values are marked with an asterisk, while the light grey background is used to show that the outcome of the test of equivalence is category III. Means and equivalence limits were calculated on a log-transformed scale; the values shown in the table are back-transformed to the original scale.

3.4.7. Conclusion on comparative analysis

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic-phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis.

Taking into account the natural variability observed for the set of non-GM reference varieties, the GMO Panel concludes that:

- None of the differences identified in agronomic and phenotypic characteristics between cotton COT102 and the non-GM comparator needs further assessment for potential environmental impact.
- None of the differences identified in seed composition between cotton COT102 and the non-GM comparator needs further assessment regarding food and feed safety except for ADF, which is further assessed in Sections 3.5.4 and 3.5.5.

3.5. Food/feed safety assessment[18]

3.5.1. Overview of overarching information for food/feed assessment

3.5.1.1. Compositional analysis

The compositional analysis of cotton COT102 and the non-GM comparator provided by the applicant and assessed by the GMO Panel is described in Section 3.4.6.

3.5.1.2. Newly expressed proteins

The Vip3Aa19 and APH4 proteins newly expressed in cotton COT102 have never been previously assessed by the GMO Panel. However, a similar Vip3Aa protein, the Vip3Aa20 protein, was previously assessed by the GMO Panel and no safety concerns for humans and animals (i.e. farmed and companion animals) were identified (EFSA GMO Panel, 2012).

3.5.1.2.1. Molecular characterisation

The protein characterisation of the newly expressed Vip3Aa19 and APH4 proteins provided by the applicant and assessed by the GMO Panel is described in Section 3.3.3.

3.5.1.2.2. Substrate specificity

APH4 substrate specificity was tested for 14 aminoglycosides and among those only hygromycin B was recognised by the enzyme (Stogios et al., 2011). This ability was attributed to APH4 structure containing a pocket that accepts the molecular structure of hygromycin B but restricts access of other aminoglycosides to the active site (Stogios et al., 2011).

The applicant performed a search in the Dictionary of Natural Products[19] and scientific literature databases to investigate for structural analogues to hygromycin B occurring in nature. Seven analogues were identified, all of them belonging to the phylum of prokaryotic Actinobacteria and none of them were of plant origin. Rao et al. (1983) reported phosphorylation by APH4 of three of these analogues with structures similar to hygromycin B (i.e. destomycin A and B, and SS-56 C).

Hygromycin B was used as growth promotor for poultry in the EU until 1976 (Castanon, 2007). Hygromycin B and destomycin A were reported to be used as anthelmintics in animals in the past in the EU (EMA, 2018).

The GMO Panel sought advice from the European Medical Agency (EMA) regarding the current uses of hygromycin B in humans and animals in the European Union. EMA confirmed[20] that there are no products containing hygromycin B authorised for therapeutic, prophylactic or any other medical uses in humans or animals in the EU Member States and there are no central authorisations for human or veterinary use for medicinal products that contain hygromycin B.

3.5.1.2.3. History of safe use for consumption as food/feed of the NEPs/new constituent

a) Information on the source organism.

The *vip3Aa19* gene was isolated from *B. thuringiensis* strain AB88 and encodes the Vip3Aa19 protein that exhibits insecticidal activity against several lepidopteran pests of cotton.

The *aph4* gene was isolated from *E. coli* K-12 strain and encodes the APH4 protein that inactivates hygromycin B activity. Members of the genus *Escherichia* are ubiquitous in the environment and found in the digestive tract of vertebrates, including humans and animals. The K-12 strains from *E. coli* are commonly used as protein production systems in many commercial applications.

b) Information on structure, function, and mode of action of the newly expressed protein.

Vip3Aa19 shows a similar mechanism to Cry proteins, conferring insecticidal activity against several lepidopteran species. Vip3Aa19 is ingested and cleaved by specific proteases under the alkaline conditions of the insect midgut thereby leaving an active toxin core. The active toxin core binds to specific targets on the epithelial cell brush border of susceptible insect species. This binding initiates the formation of ion channels within the epithelial membranes. Opening of these channels causes an

[18] Dossier: Part II – Section 1, 2, 3, 4; additional information: 13/10/2020, 14/10/2020, 4/5/2021, 5/8/2022, 2/9/2022, 22/2/2023, 7/3/2023 and 5/8/2022.
[19] https://dnp.chemnetbase.com/chemical/ChemicalSearch.xhtml?dswid=9908
[20] Correspondence between EFSA and EMA is available online https://open.efsa.europa.eu/study-inventory/EFSA-Q-2017-00271

aberrant flow of ions across the cell membrane, leading to gut paralysis, cell lysis and insect mortality in target species (Chakroun et al., 2016; Bel et al., 2017).

The APH4 enzyme catalyses the ATP dependent phosphorylation of the 4-hydroxyl group of hygromycin B 2-deoxystreptamine ring, inactivating its antibiotic activity. Because of its narrow substrate specificity to hygromycin B, APH4 is used as a selectable marker for the transformation of COT102. The gene *aph4*, when transformed into plant cells, enables the transgenic cells to produce APH4, which de-toxifies hygromycin B and allows for growth and selection of the transformed cells in its presence.

c) Information on identity/homology of NEPs to other proteins in conventional food and feed sources.

No information on identity/homology of Vip3Aa19 and APH4 proteins to other proteins in conventional food and feed sources was provided by the applicant.

d) Overall conclusion of the history of safe use.

The GMO Panel considers the above information not sufficient to duly document the history of safe use for consumption of the Vip3Aa19 and APH4 proteins.

3.5.1.2.4. Stability of the NEPs

Protein stability is one of several relevant parameters to consider in the weight-of-evidence approach in protein safety assessment (EFSA GMO Panel, 2010c, 2011a, 2017, 2021). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability, proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, one of the most prominent traits attributed to food allergens is protein stability (Helm, 2001; Breiteneder and Mills, 2005; Foo and Mueller, 2021; Costa et al., 2022).

As indicated in Section 3.3.3, protein stability studies were conducted on a recombinant microbial APH4 protein containing 14 extra amino acids at the *N*-terminus as compared to the NEP expressed in cotton COT102. Based on the assessment of the amino acid sequence of the 14-mer peptide and the outcome of the APH4 protein stability and enzymatic activity studies, the GMO Panel concluded that the extra amino acids present in the recombinant APH4 protein do not have an impact on the structural and enzymatic properties of the APH4 as expressed in cotton COT102 (without the 14-mer peptide).

a) Effect of temperature and pH on NEPs.

The applicant provided information on the effects of temperature on Vip3Aa19 and APH4 proteins. Vip3Aa19 protein samples were incubated for 30 min at 25°C, 37°C, 65°C or 95°C followed by ELISA or by a bioassay measuring its activity. The studies showed that the Vip3Aa19 protein is unstable, as evidenced by loss of functional activity and protein degradation, at temperatures ≥ 65°C. Furthermore, APH4 protein samples were incubated for 30 min at 25°C, 37°C, 65°C, 95°C or 120°C followed by ELISA or by enzymatic activity. The studies showed that the APH4 protein is unstable, as evidenced by loss of enzymatic activity and protein degradation at 120°C.

In relation to the effect of pH on the Vip3Aa19, the molecular mass and immunoreactivity of the protein was unchanged at pH 1.2 and 7.5. In the case of the APH4 protein, maximum enzymatic activity was identified at pH 8.5, while the half-maximal activities were reported at approximately pH 7.0 and pH 9.4.

b) *In vitro* protein degradation by proteolytic enzymes.

The resistance to degradation by pepsin of the Vip3Aa19 and APH4 proteins was investigated in independent solutions at pH ~ 1.2. The integrity of the test proteins in samples of the incubation mixture taken at various timepoints was analysed by SDS–PAGE followed by protein staining or by western blotting. The Vip3Aa19 and APH4 proteins were degraded by pepsin within 1 min of incubation. In addition, transient low molecular weight fragments of ~ 3–4 kDa in samples of Vip3Aa19 and APH4 proteins were observed at different timepoints. Following a western blot analysis, no intact Vip3Aa19 and APH4 proteins or fragments thereof were observed after 1 min incubation.

3.5.1.2.5. Synergistic or antagonistic interactions

The potential for a functional interaction among the Vip3Aa19 and APH4 proteins has been assessed with regard to human and animal health. Based on current scientific knowledge on the biological function of the two proteins (Table 5), no synergistic or antagonistic interactions between these two proteins are expected which could raise safety concerns for food and feed from cotton COT102.

Table 5: Intended effects of the NEPs in cotton COT102

Protein	Intended effect in GM plant
Vip3Aa19	The Vip3Aa19 protein is secreted by *B. thuringiensis* during its vegetative phase acting in target insects (several lepidopteran species) via a mechanism similar to that of Cry proteins (Chakroun et al., 2016; Bel et al., 2017)
APH4	The APH4 enzyme catalyses the phosphorylation of the 4-hydroxyl group of hygromycin B, inactivating its antibiotic activity, with a highly-specific and well-defined mechanism of action. The APH4 protein is used as a selection marker in the production of the GM plant and performs no function in the cultivated plant.

3.5.1.3. Effect of processing

Cotton COT102 will undergo existing production processes used for conventional cotton. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing of this GM cotton into food and feed products is not expected to result in products being different from those of conventional non-GM cotton varieties.

3.5.2. Toxicology assessment

The strategies to assess the toxicological impact of any changes on the whole genetically modified food and feed resulting from the genetic modification focus on the assessment of (i) newly expressed proteins; (ii) new constituents other than NEPs; (iii) altered levels of food and feed constituents; and (iv) the whole genetically modified food and feed.

3.5.2.1. Assessment of newly expressed proteins

A weight-of-evidence approach was followed by the GMO Panel to assess the toxicological profile of the newly expressed Vip3Aa19 and APH4 proteins, taking into account all of the information relevant for their hazard assessment, including molecular characterisation, substrate specificity, history of safe use for consumption as food and feed of the NEPs, stability of the NEPs and synergistic or antagonistic interactions (Section 3.5.1.2), updated bioinformatic analyses for similarity to toxins and in vivo toxicity studies.

To further integrate the assessment of the Vip3Aa19 protein, the GMO Panel took into account its previous assessment on the highly similar Vip3Aa20 protein expressed in maize MIR162 (EFSA GMO Panel, 2012), for which no safety concerns were identified for humans and animals. The two proteins are highly similar (99%), differing only by one amino acid, which is unlikely to lead to safety concerns because the activity of Vip3Aa19 in cotton COT102 is retained, and the nature and the position of the amino acid difference was predicted not to cause major changes in its structure.

3.5.2.1.1. Bioinformatic analyses

Updated bioinformatic analyses of the amino acid sequences of the Vip3Aa19 and APH4 proteins revealed no relevant similarities to known toxins.

3.5.2.1.2. In vivo toxicity studies

For the assessment of the Vip3Aa19 protein, the applicant provided an acute toxicity study with Vip3Aa19, and cross referred to an acute and a 28-day toxicity study with the Vip3Aa20 protein (Appendix A), previously assessed in the frame of the Scientific Opinion on maize MIR162, for which no safety concerns were identified (EFSA GMO Panel, 2012). For the assessment of the APH4 protein, the applicant provided an acute and a 28-day toxicity study with APH4. The outcome of the in vivo toxicity studies with the Vip3Aa19 and APH4 proteins is described below.

a) Acute toxicity study with Vip3Aa19 protein

An acute toxicity study in Crl-1(ICR)BR mice, administered the *E. coli*-produced Vip3Aa19 (test substance VIP3A-0100, containing ca. 73.5% Vip3Aa19 protein (w/w)), by gavage at the dose of 5,000 mg/kg bw, which is equivalent to ca. 3,675 mg Vip3Aa19 protein/kg bw, showed no adverse effects.

b) Acute toxicity study with APH4 protein

An acute toxicity study in AP_fCD-1 strain mice, administered the *E. coli*-produced APH4-0102 test substance, containing ca. 42.6% APH4 protein (w/w), by gavage at the dose of 1,828 mg/kg bw, which is equivalent to ca. 779 mg APH4 protein/kg bw, showed no adverse effects.

c) 28-day toxicity study with APH4 protein

Upon EFSA request to further corroborate information on the history of safe use for consumption as food and feed of the APH4 protein, the applicant provided a 28-day repeated dose toxicity study which was assessed by the GMO Panel.

The 28-day toxicity study in mice with the APH4 protein was conducted in accordance with OECD TG 407 (2008) and with the principles of good laboratory practice.

Crl:CD1 (ICR) mice (10/sex/group), ~ 8-week-old at the initiation of dosing, were allocated to three groups. Groups were administered by oral gavage: the test substance (APH4-0119) at targeted nominal dose of 1,000 mg/kg bw per day (test group); the control substance (bovine serum albumin, BSA) at targeted nominal dose of 1,000 mg/kg bw per day (BSA control group); and the vehicle (1% methylcellulose in deionised water) (vehicle control group).

Mice were randomised to treatment groups (males and females separately) using a stratified randomisation scheme designed to achieve similar group mean body weights (20% of the mean for each sex). Animals were singly housed owing to behavioural characteristics. The GMO Panel considers this justification acceptable.

The APH4 protein used in this study was produced by a *E. coli* recombinant system. The purified protein was lyophilized and the resulting powder, designated test substance batch APH4-0119, was reported to contain 52.7% of the active ingredient (APH4 protein).

The amino acid sequence analysis of the *E. coli*-produced APH4 protein used in this 28-day toxicity study matched the deduced sequence as defined by the *aph4* gene. This protein had the expected molecular weight and immunoreactivity to APH4 specific antibodies, was not glycosylated and showed functional activity (equivalence study RIR-0009070) (Section 3.3.3).

The APH4 dose formulations were prepared daily, based on the content of APH4 protein in the APH4 (corrected for 52.7% of APH4 protein in the APH4 lyophilised powder) and were administered to the animals within 4 h of preparation. Test substance APH4 was removed from the refrigerator and allowed to equilibrate to room temperature in a desiccator before the container was opened, and the formulation was gently magnetically stirred before dosing. The APH4 dose formulation was confirmed to be homogeneous and stable for the purpose of the present study. Analysed concentrations of APH4 in the dosing suspensions were within the acceptable range (> 85% of the nominal value) with one exception (81%); this result is considered not to impact on the acceptability of the study.

The first 10 animals per group were subject to in-life procedures and observations and terminal procedures in accordance to OECD TG 407 (2008), except coagulation analysis; the remaining 10 animals per group were used to evaluate coagulation parameters only.

Deviations to the protocol reported in the study were considered minor deviations with no impact on the study results.

An appropriate range of statistical tests was performed on the results of the study and a detailed description of the methodology and of statistically significant findings identified in mice is reported in Appendix C.

There were five deaths during the study (four in APH4 dosed mice (three males, one female) and one in a BSA dosed female); there was no pattern to the date of the deaths in APH4 dosed mice (dosing days 6, 19, 22 and 23) and post-mortem examinations indicated they were likely associated with the gavage dosing procedure. These deaths did not compromise the overall interpretation of the study. No APH4-related clinical observations or ophthalmology findings were seen. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- they were within the normal variation[21] for the parameter in mice of this age;
- they were of small magnitude;
- they were identified at only a small number of time intervals with no impact on the overall value;
- they exhibited no consistent pattern with related parameters or endpoints;
- they exhibited no consistency with increasing dose levels.

No gross pathology findings related to the administration of the test item were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test item compared to the controls.

The GMO Panel concludes that no adverse effects were observed in this 28-day mice toxicity study on the *E. coli*-produced APH4 protein, at a targeted nominal dose of 1,000 mg/kg bw per day.

d) Overall conclusion of the toxicological assessment of the NEPs

Based on the above information, the GMO Panel did not identify indications that the Vip3Aa19 and APH4 proteins raise food and feed safety concerns in humans and animals.

3.5.2.2. Assessment of new constituents other than NEPs

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, no new constituents other than the newly expressed proteins have been identified in seed from cotton COT102. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

3.5.2.3. Assessment of altered levels of food and feed constituents

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, no altered levels of food and feed constituents have been identified in seed from cotton COT102, except for ADF. This change is considered not to represent a toxicological concern, considering the biological role of the affected constituent and the magnitude of the change, therefore, no further toxicological assessment is needed. Further information on the relevance of these findings is provided in Section 3.5.5 (nutritional assessment of endogenous constituents).

3.5.2.4. Assessment of the whole genetically modified food and feed

Based on the outcome of the molecular characterisation, comparative analysis and toxicological assessment, no indication of findings relevant to food and feed safety have been identified for cotton COT102 related to the stability and expression of the inserts or to modifications of toxicological concern in the composition of cotton COT102 (see Sections 3.4.1, 3.4.2 and 3.4.3.3). Therefore, animal studies on food/feed derived from cotton COT102 are not necessary (EFSA GMO Panel, 2011a). In accordance with Regulation (EU) No 503/2013, the applicant provided a 90-day feeding study in rats fed diets containing meal derived from cotton COT102.

In this study, pair-housed Han Wistar rats (RccHan:WIST) (10/sex per group; 2 rats/cage) were allocated to four groups using a randomised complete block design with five replications.

Groups were fed diets containing cotton COT102 meal or the non-GM comparator meal, at inclusion levels of 10% and 3%. The upper level of 10% is justified based on the content of gossypol in cottonseed.

The study was adapted from OECD test guideline 408 (OECD, 1998), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP) with some deviations not impacting the study results and interpretation.

The stability of the test and control materials was not verified; however, in accordance to product expiration information declared by the diet manufacturer, the constituents of the diets are considered

[21] Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is 'adverse' account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (historical control data).

stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them.

Event-specific PCR analysis confirmed the presence of event COT102 in both the GM meal and diets and excluded the presence of the event in the respective controls.

Both GM and control meal and diets were analysed for nutrients, antinutrients and potential contaminants. Balanced diets were formulated based on the specifications for SDS CT5 diets.

Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD test guideline 408 (OECD, 1998).

An appropriate range of statistical tests were performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given diets containing meal derived from cotton COT102 is reported in Appendix C.

There were no test diet-related incidents of mortality or clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted but these were not considered adverse effects of treatment for one or more of the following reasons:

- they were within the normal variation[22] for the parameter in rats of this age;
- they were of small magnitude;
- they were identified at only a small number of time intervals with no impact on the overall value;
- they exhibited no consistent pattern with related parameters or end-points;
- they exhibited no consistency with increasing dietary incorporation level.

The GMO Panel concludes that this study is in line with the requirements of Regulation (EU) No 503/2013 and that no treatment related adverse effects were observed in rats after feeding diets containing COT102 meal at 3% or 10% of inclusion level for 90 days.

3.5.3. Allergenicity

The strategies to assess the potential risk of allergenicity focus: (i) on the source of the recombinant protein; (ii) on the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons; and (iii) on whether the transformation may have altered the allergenic properties of the modified plant. Furthermore, the assessment also takes into account potential adjuvant properties of the newly expressed proteins, which is defined as the ability to enhance an allergic reaction.

3.5.3.1. Assessment of allergenicity of the newly expressed proteins

A weight-of-evidence approach was followed, taking into account all of the information obtained on the newly expressed protein, as no single piece of information or experimental method yielded sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a,b, 2017; Regulation (EU) No 503/2013).

The *vip3Aa19* and *aph4* genes originate from *B. thuringiensis* and *E. coli*, respectively, none of which are considered allergenic sources. It is noted that a highly similar Vip3Aa protein has been assessed by the GMO Panel and no safety concerns were identified (see Section 3.5.2.1).

Updated bioinformatic analyses of the amino acid sequences of the Vip3Aa19 and APH4 proteins, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens. The studies on protein stability of the Vip3Aa19 and APH4 proteins have been described in Section 3.5.1. Furthermore, the GMO Panel did not find an indication that the Vip3Aa19 and APH4 proteins at the levels expressed in cotton COT102 might be adjuvants.

Furthermore, the applicant provided information on the safety of the Vip3Aa19 and APH4 proteins regarding their potential hazard to cause a celiac disease response. For such assessment, the applicant

[22] Although animal used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is 'adverse' account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (historical control data).

followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). The assessment of the Vip3Aa19 protein identified no perfect or relevant partial matches with known celiac disease peptide sequences. The assessment of the APH4 protein revealed partial matches containing the Q/E-X1-P-X2 motif and required further investigations. The applicant followed a stepwise approach and considered the position and nature of amino acids flanking the ELPA motif, as well as the preDQ tool[23] for peptide binding prediction to HLA-DQ2.5 and HLA-DQ8.1 molecules. No indications of safety concerns were identified by the GMO Panel as the peptides failed to mimic gluten sequences.

In the context of this application, the GMO Panel considers that there are no indications that the newly expressed Vip3Aa19 and APH4 proteins in cotton COT102 may be allergenic.

3.5.3.2. Assessment of allergenicity of the whole GM plant or crop

The GMO Panel regularly reviews the available publications on food allergy to cottonseed-derived products. However, cotton is not considered a common allergenic food[24] (OECD, 2009). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM cotton.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.3, 3.4 and 3.5), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from cotton COT102 with respect to that derived from the non-GM comparator.

3.5.4. Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to the proteins Vip3Aa19 and APH4 newly expressed in cotton COT102. Dietary exposure was estimated based on the protein expression levels reported in this application for cotton COT102 (see Table 1), the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

3.5.4.1. Human dietary exposure

The applicant considered the dietary exposure to Vip3Aa19 and APH4 newly expressed proteins as negligible in the European population. The GMO Panel identified different cottonseed-derived products such as flour and oil as well as by-products (cottonseed linters) used for human consumption, with the refined bleached deodorised (RBD) oil being currently the most relevant. The GMO Panel confirmed that no consumption data of cottonseed, cottonseed oil or other cottonseed derived products were available in the EFSA Comprehensive European Food Consumption Database.[25] Cottonseed oil might be consumed as an ingredient in the production of a wide variety of food products such as dressings, mayonnaise, fine bakery wares, chocolate spreads and chips. However, considering that RBD cottonseed oil and cottonseed linters are free from proteins, no dietary exposure to Vip3Aa19 and APH4 proteins is expected from the consumption of these products derived from cotton COT102. Dietary exposure to Vip3Aa19 and APH4 proteins cannot be excluded via the consumption of cottonseed flour, although as indicated above this product seems not to be consumed (or in very small amounts) in Europe at present.

An *ad hoc* dietary exposure scenario was carried out by the GMO Panel for consumers of pollen supplements under the assumption that these supplements might be made of pollen from cotton COT102. Vip3Aa19 and APH4 levels in pollen as described in Table 1 were used to derive concentrations in pollen supplements considering around 6% moisture content in these products. For Vip3Aa19 all samples were below the limit of detection (LOD = 3.20 μg/g dw); the LOD was used to estimate dietary exposure to this protein. Consumption data on pollen supplements are available for few consumers across eight different European countries.[25] The low number of consumers available adds uncertainty to the exposure estimations which should be carefully interpreted, and it prevents from estimating exposure for high consumers of pollen supplements. In average consumers of pollen

[23] https://r4eu.efsa.europa.eu/app/predq [Accessed: February 2023].

[24] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

[25] https://www.efsa.europa.eu/en/food-consumption/comprehensive-database [Accessed: February 2023].

supplements, the highest acute dietary exposure would be 2.2 and 7.1 μg/kg bw per day in the elderly population for Vip3Aa19 and APH4 proteins, respectively. Similarly, the highest chronic dietary exposure in average consumers would be 1.5 and 4.7 μg/kg bw per day also in the elderly population for Vip3Aa19 and APH4 proteins, respectively.

3.5.4.2. Animal dietary exposure

Upon EFSA request to provide further clarifications on the animal dietary exposure (ADE) originally provided in the main dossier, the applicant submitted a new ADE study which was assessed by the GMO Panel. The recommendations outlined in the GMO Panel statement on ADE, recently published (EFSA GMO Panel, 2023), were followed.

Dietary exposure to Vip3Aa19 and APH4 proteins in cotton COT102 was estimated across different animal species, as described below, assuming the consumption of cotton products commonly entering the feed supply chain (i.e. undelinted seeds and meal). A conservative scenario with 100% replacement of conventional cotton products by the cotton COT102 products was considered.

Mean levels (dry weight) of Vip3Aa19 and APH4 proteins in undelinted seeds from cotton COT102 used for dietary exposure are listed in Table 1. All seed samples analysed in cotton COT102 for the presence of APH4 protein were below the limit of detection (LOD = 0.125 μg/g dry weight) or the limit of quantification (LOQ = 0.250 μg/g dry weight); for the purpose of estimating DDE, the determined values of LOQ or LOD were used as substitutes to compute an average concentration of APH4 in seed to be used for the exposure calculations.

Mean levels of Vip3Aa19 and APH4 proteins in cotton meal were calculated to be respectively 1.75-fold than in seed, based on factors that take into account the protein content in these feed materials relative to cotton seed, and assuming that no protein is lost during their production/processing (EFSA GMO Panel, 2023).

The applicant estimated dietary exposure to Vip3Aa19 and APH4 proteins via the consumption of undelinted seeds in dairy cow, dairy sheep and dairy goat and cottonseed meal in dairy cow, beef cattle, dairy sheep, dairy goat, rabbit, fattening pig, lactating sow, broiler, laying hens, turkey and horse.

Estimations were based on default values for animal body weight, daily feed intake and inclusion rates (percentage) of cottonseed meal and undelinted seeds in rations, as provided for the EU by EFSA GMO Panel (2023).

Estimated dietary exposure in the concerned animals is reported in Appendix D.

3.5.5. Nutritional assessment of endogenous constituents

The intended trait of cotton COT102 is protection against feeding damage caused by lepidopteron insect pests with no intention to alter nutritional parameters. However, in cotton COT102, the levels of ADF in the fuzzy seeds were significantly different from the non-GM comparator and showed a lack of equivalence with the set of non-GM reference varieties (Section 3.4.6). The biological relevance of ADF, the role of cotton COT102 as contributor to its total intake and the magnitude and direction of the observed change were considered during the nutritional assessment.

3.5.5.1. Human nutrition

As mentioned in Section 3.5.4.1, RBD oil is currently the most and almost only cottonseed-derived product consumed by humans, and ADF is not expected to be present in RBD oil. Cotton COT102 might also enter the food chain through the consumption of cottonseed linters, a by-product produced during cottonseed processing, with more than 99% fibre content, and used in different food products such as baked goods, dressings, snacks and processed meat, among others. However, the relatively small change observed in ADF levels in the fuzzy seeds does not represent any nutritional concern.

3.5.5.2. Animal nutrition

Cotton seeds are used mainly in the nutrition of ruminants, because of the high content in fibre. Cotton seeds are also a good source of protein and lipid. The ruminant diet consists of feed materials of plant origin or their by-products which contain variable amounts of fibre which ruminants may use as energy source through degradation by rumen microbes (e.g. anaerobic bacteria, protozoa and fungi). In contrast, monogastric animals and poultry cannot use the fibre as energy source, because they lack gastric bacterial fermentation and endogenous enzymes capable to digest fibre, although, up to certain amount, microbial digestion may happen in the large intestine. ADF is a fraction of the total fibre of the feed, and it is used to formulate balanced rations for animals. On this basis, the increase

of ADF percentage in GM cotton seeds and its magnitude do not represent a safety issue for animals, and the nutritional impact in feeds is considered negligible.

3.5.6. Post-market monitoring of GM food/feed

The GMO Panel concluded that cotton COT102, as described in this application, does not raise any nutritional concern and is as safe as the non-GM comparator and the non-GM reference varieties tested. Therefore, the GMO Panel considers that post-market monitoring of food and feed from this GM cotton, as described in this application, is not necessary.

3.5.7. Conclusions on the food/feed safety assessment

The proteins Vip3Aa19 and APH4 newly expressed in cotton COT102 do not raise safety concerns for human and animal health. No interactions between the newly expressed proteins relevant for food and feed safety were identified. Moreover, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in cotton COT102. The GMO Panel found no evidence that the genetic modification impacts the overall safety of cotton COT102. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of cotton COT102 does not represent any nutritional concern, in the context of the scope of this application. The GMO Panel concludes that cotton COT102, as described in this application, is as safe as the non-GM comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

3.6. Environmental risk assessment and monitoring plan[26]

3.6.1. Environmental risk assessment

Considering the scope of application EFSA-GMO-DE-2017-141, which excludes cultivation, the environmental risk assessment (ERA) of cotton COT102 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of GM material, including viable cotton COT102 seeds during transportation and/or processing (EFSA GMO Panel, 2010a).

3.6.1.1. Persistence and invasiveness of the GM plant

In Southern Europe, *Gossypium herbaceum*, *G. barbadense* and *G. hirsutum* have been grown since the 19th century and led to transient or locally naturalised cotton plants in the same area (Davis, 1967; Tutin et al., 1992;Sarno et al., 1993 ; Celesti-Grapow et al., 2010). However, survival of cottonseeds outside cultivation areas in Europe is limited due to the absence of a seed dormancy phase. Even if seeds from spillage germinate, the resulting cotton plants are unlikely to survive due to factors such as cold climatic conditions, the susceptibility to diseases and their low competitiveness (Eastick and Hearnden, 2006). For example, after the end of cotton cultivation in Italy in 1950s, no feral cotton was reported in southern Italy, except in some restricted areas (Sarno et al., 1993; Celesti-Grapow et al., 2010). Also, in other cotton-growing regions, such as in Australia, surveys showed that feral GM cotton established infrequently along transportation routes and mostly as transient populations (Addison et al., 2007). Field observations indicate that cottonseed may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Charles et al., 2013). However, cotton volunteers have been shown to rarely yield as well as newly planted seeds due to seedling diseases and early emergence in cool conditions. Thus, the establishment and survival of feral and volunteer cotton plants in the EU is currently limited and transient.

It is unlikely that the intended trait of cotton COT102 will provide a selective advantage to cotton plants, except when they are infested by insect pests that are susceptible to the Vip3Aa19 protein. However, if this was to occur this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors (described above) limiting plant persistence and invasiveness. Therefore, the presence of the intended trait will not affect the persistence and invasiveness of the GM plant.

[26] Dossier: Part II – Section 5, 6; additional information: 3/6/2022 and 5/8/2022.

In conclusion, the GMO Panel considers that cotton COT102 will be equivalent to conventional cotton varieties in their ability to survive until subsequent seasons, or to establish occasional feral plants under European environmental conditions in case of accidental release into the environment of viable cotton COT102 seeds.

3.6.1.2. Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through HGT of DNA, or through vertical gene flow via cross-pollination from feral plants originating from spilled seeds.

Plant-to-microorganism gene transfer

Genomic DNA can be a component of food and feed products derived from cotton. It is well documented that such DNA becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, bacteria in the digestive tract of humans and animals, and in other environments, may be exposed to fragments of DNA, including the recombinant fraction of such DNA.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally-located DNA fragments between unrelated organisms (such as from plants to bacteria) is not likely to occur at detectable frequencies under natural conditions (for further details, see EFSA, 2009).

Homologous recombination is known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes. This requires the presence of at least two stretches of DNA sequences that are similar in the recombining DNA molecules. In the case of sequence identity with the transgene itself, recombination would result in gene replacement. In the case of identity with two or more regions flanking recombinant DNA, recombination could result in the insertion of additional DNA sequences in bacteria and thus confer the potential for new properties.

In addition to homology-based recombination processes, at a lower transformation rate, the non-homologous end joining and microhomology-mediated end joining are theoretically possible (Hülter and Wackernagel, 2008; EFSA, 2009).

The recombinant DNA of cotton COT102 contains the gene *aph4* (see Section 3.3.1). The protein encoded by this gene inactivates the antibiotic hygromycin B (see Section 3.5.1.2). Therefore, this gene may confer resistance to recipient microorganisms and thus, in presence of hygromycin B a selective advantage. As reported in Section 3.5.1.2 no products containing hygromycin B are authorised for therapeutic, prophylactic or any other medical uses in humans or animals in the EU Member States and there are no authorisations for medicinal products for human or veterinary use that contain hygromycin B.

The bioinformatic analysis of cotton COT102 reveals, as expected, high DNA sequence identity of the *aph4* gene with other bacteria known to carry the *aph4* gene, including *E. coli* and *Klebsiella pneumoniae*. Recombination events would only replace natural variants (i.e. substitutive recombination) and are therefore unlikely to provide any new property connected to a selective advantage for the recipient organisms (EFSA, 2009). The bioinformatic analysis also identified at the 5′ and 3′ ends of the COT102 event, a ~ 250 bp-long DNA sequence (NOS terminator) of sufficient identity to a single site on an *A. tumefaciens* nopaline Ti plasmid. This would not increase the potential double homologous recombination but could facilitate a possible transfer of the *aph4* gene from COT102 to *A. tumefaciens* nopaline Ti plasmid. However, the EFSA GMO Panel considers that the *aph4* gene is already widely distributed (Kim et al., 2015; Elbehery et al., 2017, UniRef100[27]) among soil and enteric bacteria and that is highly unlikely that cotton COT102 will contribute to increase its prevalence of this gene. More in particular, Agrobacterium species, including *A. tumefaciens*, or its close relatives from the genus Rhizobium, are not expected to be prevalent in the gastrointestinal tract. However, occurrence of the recombinant genes outside the immediate receiving environment (through faecal material), in habitats where *A. tumefaciens* could be more abundant, cannot be ruled out (Hart et al., 2009) and is therefore also taken into account for concluding on the risks associated with a HGT from DNA of cotton COT102 to bacteria in the context of its intended use.

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from cotton COT102 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identified no safety concern linked to an unlikely but theoretically possible HGT.

[27] https://www.uniprot.org/uniref/UniRef100_P00557 [Accessed: 2 May 2023].

Plant-to-plant gene transfer

The potential for occasional feral cotton COT102 plants originating from seed import spills to transfer recombinant DNA to sexually compatible plants and the environmental consequences of this transfer were considered.

For plant-to-plant gene transfer to occur, imported GM cotton seeds need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated cotton with synchronous flowering and environmental conditions favouring cross-pollination.

Cotton is an annual predominantly self-pollinating crop, although cross-pollination can occur at low frequencies in the presence of insect pollinators (such as wild bees, honeybees, bumblebees) (OECD, 2008). For cotton, no wild relatives have been reported in Europe; therefore, any vertical gene transfer is limited to *G. hirsutum*, *G. barbadense* and *G. herbaceum* cotton plants. However, gene transfer to *G. herbaceum* is considered unlikely due to the difference in ploidy level.

The potential of spilled cotton seeds to establish, grow and produce pollen is extremely low and transient (see Section 3.5.1.1). Therefore, the likelihood/frequency of cross-pollination between occasional feral GM cotton plants resulting from seed spillage, and weedy or cultivated *Gossypium* plants is considered extremely low. Even if cross-pollination would occur, the GMO Panel is of the opinion that the likelihood of environmental effects as a consequence of the spread of genes from occasional feral GM cotton plants in Europe will not differ from that of conventional cotton varieties for the reasons given in Section 3.5.1.1.

3.6.1.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-DE-2017-141 into account (no cultivation), potential interactions of occasional feral cotton COT102 plants arising from seed import spills with the target organisms are not considered a relevant issue.

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

Given that environmental exposure of non-target organisms to spilled GM material or occasional feral GM cotton plants arising from spilled cotton COT102 seeds is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM cotton, the GMO Panel considers that potential interactions of cotton COT102 with non-target organisms do not raise any environmental safety concern.

3.6.1.5. Interactions with abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled GM material or occasional feral cotton COT102 plants arising from seed import spills is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM cotton, potential interactions with the abiotic environment and biogeochemical cycles the GMO Panel considers that potential interactions with the abiotic environment and biogeochemical cycles do not raise any environmental safety concern.

3.6.2. Post-market environmental monitoring

The objectives of a post-market environmental monitoring (PMEM) plan, according to Annex VII of Directive 2001/18/EC, are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific rationale of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA did not identify potential adverse environmental effects from cotton COT102, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for cotton COT102 includes: (1) the description of a monitoring approach involving operators (federations involved in 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 CropLife Europe for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis for the duration of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of cotton COT102. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

3.6.2.1. Conclusion of the environmental risk assessment and monitoring plan

The GMO Panel concludes that it is unlikely that cotton COT102 would differ from conventional cotton varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-DE-2017-141, interactions of occasional feral cotton COT102 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from cotton COT102 to bacteria does not indicate a safety concern. Therefore, considering the introduced trait, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that cotton COT102 would not raise safety concerns in the event of accidental release of viable GM cotton seeds into the environment.

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of cotton COT102.

4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of cotton COT102 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data establish that cotton COT102 contains a single insert consisting of one copy of the *vip3Aa19 and aph4* expression cassettes. Bioinformatics analyses of the sequences encoding the newly expressed proteins and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Vip3Aa19 and APH4 proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant- and microbe-produced Vip3Aa19 and APH4 proteins indicate that these proteins are equivalent and the microbial-derived proteins can be used in the safety studies.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic-phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the differences identified in the agronomic-phenotypic and compositional characteristics between cotton COT102 and its non-GM comparator needed further assessment, except for the levels of ADF which do not raise nutritional and safety concerns.

The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Vip3Aa19 and APH4 proteins as expressed in cotton COT102 and finds no evidence that the genetic modification would change the overall allergenicity of cotton COT102. In the context of this application, the consumption of food and feed from cotton COT102 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that cotton COT102 is as safe as the non-GM comparator and non-GM cotton reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable seeds from cotton COT102 into the environment. The PMEM plan and reporting intervals are in line with the intended uses of cotton COT102. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the uses of cotton COT102.

The GMO Panel concludes that cotton COT102 is as safe as its non-GM comparator and the tested non-GM cotton reference varieties with respect to potential effects on human and animal health and the environment. The GMO Panel considers that the risk assessment may need to be updated in case products containing hygromycin B or other substrates of the APH4 enzyme obtain future market approval in the EU.

5. Documentation as provided to EFSA

- Letter from the Competent Authority of Germany received on 12 April 2017 concerning a request for authorization of the placing on the market of genetically modified cotton COT102, submitted in accordance with Regulation (EC) No 1829/2003 by Syngenta Crop Protection NV/SA (EFSA Ref.: EFSA-GMO-DE-2017-141; EFSA-Q-2017-00271).

- The application was made valid on 24 July 2017.
- Additional information (1) was requested on 2 August 2017.
- Additional information (1) was received on 25 June 2020.
- Additional information (2) was requested on 5 December 2017.
- Additional information (2) was received on 6 April 2028 partial; 17 April 2018 complete.
- Additional information (3) was requested on 7 June 2018.
- Additional information (3) was received on 22 March 2019.
- Additional information (4) was requested on 18 September 2018.
- Additional information (4) was received on 1 July 2019.
- Additional information (5) was requested on 1 September 2020.
- Additional information (5) was received on 13 October 2020.
- Additional information (6) was requested on 29 October 2020.
- Additional information (6) was received on 23 April 2021.
- Additional information (7) was requested on 17 December 2020.
- Additional information (7) was received on 17 February 2021.
- Additional information (8) was requested on 7 April 2021.
- Additional information (8) was received on 3 June 2022 partial; 28 June 2022 partial; 5 August 2022 complete.
- Additional information (9) was requested on 12 July 2021.
- Additional information (9) was received on 13 September 2021.
- Additional information (10) was requested on 29 July 2022.
- Additional information (10) was received on 2 September 2022.
- Additional information (11) was requested on 23 November 2022.
- Additional information (11) was received on 22 February 2023.
- Additional information (12) was requested on 23 February 2023.
- Additional information (12) was received on 7 March 2023.
- Supplementary information was provided on voluntary basis on 25 June 2020; 2 July 2020; 14 October 2020 and 5 August 2022.

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Abbreviations

ADF	acid detergent fibre
ALT	alanine aminotransferase
ANOVA	analysis of variance
ARMG	antibiotic resistance marker gene
bp	base pair
BSA	bovine serum albumin

bw	body weight
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
GLP	good laboratory practice
GM	genetically modified
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HGT	horizontal gene transfer
HR	homologous recombination
LOD	limit of detection
LOQ	limit of quantification
MS	Member States
NEP	newly expressed protein
OECD	Organisation for Economic Co-operation and Development
ORFs	open reading frames
PMEM	post-market environmental monitoring
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SES	standardised effect size

Appendix A – Additional studies

Study identification	Title
120040.11	Protein expression of SYN-IR1Ø2–7 cotton
160565	Compositional and agronomic analysis of cotton event SYN-IR1Ø2–7 according to EFSA guidelines
1781.4133	Evaluation of Event COT102 Transgenic Cottonseed Meal in a Broiler Chicken Feeding Study
AM7543	MIR162 VIP3A-0106 Single Dose Oral Toxicity Study In Mice
520597	Vip3Aa20 - A 28 Day Repeat Dose Toxicity Study by Oral Gavage in Rats
IBI100 – 2002US	Agronomic Performance of COT102 Cotton Grown during 2002 in the USA
J7491-JH-OCT09-Revision 1	Peptide mass mapping of Vip3Aa19 proteins from Event COT102 cotton and *E. coli* – Revision 1
RA-COT102-2	Determination of Vitamin E levels in Acid Delinted Cottonseed from Event COT102 Cotton Grown During 2007 in the USA
RA-COT102-A4	Compositional Analysis of Cottonseed from Event COT102 Cotton Plants
SSB-129-09 A2	Compositional Analysis of Acid Delinted Cottonseed from Event COT102 Cotton Grown During 2007 in the USA
SSB-133-06 A4	Additional Molecular Characterisation of Event COT102 Cotton by Southern Analyses
SSB-138-06 A2	Southern Analysis of Event COT102 Cotton in Support of the Japan Stage 3 Field Trial
SSB-176-09 A3	Molecular Characterisation of Transgenic DNA in Event COT102 Cotton
SSB-194-10 A3	Additional Molecular Characterisation of Transgenic DNA in Event COT102 Cotton
SSB-221-10	N-Terminal Amino Acid Sequence Analysis of a 3 kDa Band Obtained from Simulated Mammalian Gastric Fluid Digestion of Microbially Produced Hygromycin B Phosphotransferase (APH4)
SSB-222-10	N-Terminal Amino Acid Sequence Analysis of a 3 kDa Band Obtained from Simulated Mammalian Gastric Fluid Digestion of Microbially Produced Vip3Aa19
TK0022139	Re-Characterisation of Microbially Produced Test Substance Containing Vip3Aa19 Protein and Certificate of Analysis
TK0225737	Seed Germination and Dormancy Test
TK0252290	Functional Stability of Hygromycin B Phosphotransferase (APH4) in 50 mM2-Amino- 2 (Hydroxymethyl)-1,3- Propanediol Solution
TK0256466	Storage Stability Assessment of Microbially Produced Test Substance APH4-0308 Containing Hygromycin B Phosphotransferase (APH4) Protein

Appendix B – List of relevant publications identified by the applicant through literature searches (January 2007–September 2022)

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Appendix C – Statistical analysis and commentary on the statistically significant findings in *in vivo* toxicity and feeding studies

C.1. Statistical analysis of the 28-day study on the *E. coli*-produced APH4 protein in mice

The following endpoints were statistically analysed: body weights, body weight changes, food consumption, clinical pathology values (as applicable), absolute and relative organ weights, functional observational battery data, locomotor activity and histopathological data. For all continuous endpoints, mean, standard deviation in terms of the standardised effect size (SES) of each dose group for each sex, variable and period or time interval were reported.

The main statistical analysis compared BSA group and protein (APH4-0119) test diet groups separately with the vehicle group. The analysis was performed for male and female mice separately at 5% level of significance. All endpoints were analysed with a one-way analysis of variance (ANOVA) model (factor: diet group and for Motor Activity data: diet, time and interaction term 'dose-time'). Ranges from historical control data were provided to aid the assessment of statistically significant differences between the test and the control diet group. Missing data were considered by the Panel and found not to have an impact on the results.

Table C.1: Statistically significant findings in 28-day study on *E. coli*-produced APH4-0019 protein in mice

Statistically significant parameter/ endpoint	Finding	GMO Panel interpretation
Body weight gain d15–22	Lower in males (minus-0.1 g vs. +0.54 g); and females (+0.24 g vs. +0.99 g) for APH4 versus vehicle controls.	Normal variation with no impact on terminal body weights (within 2%). For males the BSA control group lost 0.7 g in the same period. Not an adverse effect of treatment.
Eosinophil count	Lower (50% in males) versus vehicle control	Within normal variation. Two vehicle control values are unusually high; no difference compared with BSA control group. Not an adverse effect of treatment
Mean cell volume	Higher (3% in male); lower (3% in females) versus vehicle control.	Low magnitude. Within normal variation. Not an adverse effect of treatment.
Mean cell haemoglobin	Higher (2% in male); lower (3% in females) versus vehicle control.	Low magnitude. Within normal variation. Not an adverse effect of treatment.
Serum creatinine	Lower (7% males, 20% females) versus vehicle control.	Not adverse in isolation. Low magnitude, within normal variation, (all APH4 values are within control ranges -0.1 to 0.2). Not an adverse effect of treatment.
Serum cholesterol	Lower (13%) in APH4 males versus vehicle control.	Not adverse in isolation. Low magnitude, within normal variation. Not an adverse effect of treatment.
Serum glucose	Lower (14%) in APH4 females versus vehicle control.	Low magnitude, within normal variation. Not an adverse effect of treatment.
Thyroid/parathyroid weights (absolute, and relative to body wt and brain wt)	Lower (20%) in APH4 males versus vehicle control.	Low magnitude, within normal variation (all APH4 values were within BSA control range). No associated histopathology findings. Not an adverse effect of treatment.

C.2. Statistical analysis of the 90-day study on cotton COT102 in rats

The following endpoints were statistically analysed: body weight (including cumulative body weight gain), food consumption and food utilisation, haematology, coagulation and clinical chemistry, functional observations, motor activity data and organ weights (absolute and relative). For all continuous endpoints, the applicant reported mean, standard deviation in terms of the SES of each dose group for each sex, variable and period or time interval.

In the statistical analysis, for each of the two inclusion rates, rats consuming the test diet were compared with those consuming the respective control diet. The cage was considered the experimental unit, and the analysis was done on the mean values per cage. For continuous parameters, a multi-way ANOVA was conducted for the two sexes combined (factors: treatment, sex, block-within-sex and sex-by-treatment interaction). In case a significant sex-by-treatment interaction was identified, a two-way ANOVA (factors: treatment and block) was performed separately for males and females; the two-way ANOVA was also used to analyse sex-specific parameters. For absolute organ weights, terminal body weight was included as a covariate in the models. Missing data were considered by the Panel and found not impacting the results.

Table C.2: Statistically significant findings in 90-day study on cotton COT102 in rats

Statistically significant parameter/ endpoint	**Finding**	**GMO Panel interpretation**
Body weight and body weight gain	Decreased (5–20%) in the 3% groups, at some timepoints.	No significant change in body weight at the end of the study. No dose response, not present in the 10% groups. Not an adverse effect of treatment.
Food consumption and utilisation	Decreased (5–20%) in the 10% groups, at some timepoints.	No significant change in body weight at the end of the study. Not an adverse effect of treatment.
Motor skills and activity	Increases and decreases at different measurement points in all COT102 groups.	No significant changes in the overall values. Within normal variation for these parameters. Not an adverse effect of treatment.
Mean cell haemoglobin concentration	Decreased (< 5%) in females of the 10% group.	Small magnitude. No impact on total haemoglobin (higher than controls). Not an adverse effect of treatment.
Neutrophil count	Decreased in males and increased in females of the 3% groups.	No consistent pattern. No dose response, not present in the 10% groups. Not an adverse effect of treatment.
Alanine aminotransferase (ALT) activity	Decreased (25%) in the 10% groups.	Decreased ALT is not an adverse finding in isolation. No associated changes (e.g. liver pathology). Not an adverse effect of treatment.
Sodium levels	Increased (2%) in the 3% groups.	No dose response, not present in the 10% groups. Not an adverse effect of treatment.
Ovary weight	Increased (12%) in 3% females.	Small magnitude. No associated pathological changes. No dose response, not present in the 10% group. Not an adverse effect of treatment
Spleen weight	Decreased (5%) in females and increased (15%) in males of the 10% groups.	No consistent pattern across the sexes. Small magnitude with no associated changes in pathology or haematology. Not an adverse effect of treatment.

Appendix D – Animal dietary exposure

Table D.1: Dietary exposure to Vip3Aa19 protein in selected animals, based on the consumption of cotton undelinted seeds

Animal species	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
	kg	kg/animal	%	µg/kg	mg/kg
Dairy cow	650	25	10	8.31	0.00831
Dairy Sheep	80	2.8	25	18.9	0.0189
Dairy Goat	60	3.4	20	24.5	0.0245

Vip3Aa19 in undelinted seed: 2.16 µg/g dw.

Table D.2: Dietary exposure to Vip3Aa19 protein in selected animals, based on the consumption of cottonseed meal

Animal species	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
	kg	kg/animal	%	µg/kg	mg/kg
Dairy cow	650	25	5	7.27	0.00727
Beef cattle	500	12	5	4.54	0.00454
Dairy Sheep	80	2.8	20	26.5	0.0265
Dairy Goat	60	3.4	15.5	33.2	0.0332
Rabbit	2	0.15	43	122	0.122
Fattening pig	100	3	5	5.67	0.00567
Lactating sow	200	6	10	11.3	0.0113
Broiler	2	0.158	5	14.9	0.0149
Laying hens	1.9	0.13	5	12.9	0.0129
Turkey	7	0.5	10	27.0	0.0270
Horse	450	9	10	7.56	0.00756

Vip3Aa19 in meal: 3.78 µg/g dw.

Table D.3: Dietary exposure to APH4 protein in selected animals, based on the consumption of cotton undelinted seeds

Animal species	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
	kg	kg/animal	%	µg/kg	mg/kg
Dairy cow	650	25	10	0.673	0.000673
Dairy Sheep	80	2.8	25	1.53	0.00153
Dairy Goat	60	3.4	20	1.98	0.00198

APH4 in undelinted seed: 0.175 µg/g dw.

Table D.4: Dietary exposure to APH4 protein in selected animals, based on the consumption of cottonseed meal

Animal species	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
	kg	kg/animal	%	µg/kg	mg/kg
Dairy cow	650	25	5	0.588	0.000588
Beef cattle	500	12	5	0.367	0.000367
Dairy Sheep	80	2.8	20	2.14	0.00214
Dairy Goat	60	3.4	15.5	2.69	0.00269
Rabbit	2	0.15	43	9.87	0.00987
Fattening pig	100	3	5	0.459	0.000459
Lactating sow	200	6	10	0.918	0.00092
Broiler	2	0.158	5	1.21	0.00121
Laying hens	1.9	0.13	5	1.05	0.00105
Turkey	7	0.5	10	2.19	0.00219
Horse	450	9	10	0.612	0.000612

APH4 in meal: 0.306 µg/g dw.