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SCIENTIFIC OPINION

Assessment of genetically modified cotton GHB614 × T304-40 × GHB119 × COT102 (application EFSA-GMO-ES-2017-147)

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

Genetically modified cotton GHB614 × T304-40 × GHB119 × COT102 was developed by crossing to combine four single events: GHB614, T304-40, GHB119 and COT102. The four-event-stack cotton expresses 2mEPSPS, Cry1Ab, Cry2Ae, Vip3Aa19 and PAT/bar to confer herbicide tolerance and insect resistance. Furthermore, event COT102 expresses the antimicrobial APH4 protein used during its molecular development. The GMO Panel previously assessed the four single cotton events and did not identify safety concerns. Since then, no new data on the single cotton events were identified that would require modification of the original conclusions on their safety. The molecular characterisation, comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment does not indicate interactions which would give rise to new food and feed safety and nutritional concerns. The GMO Panel concludes that the four-event stack cotton, as described in this application, is as safe as its non-GM comparator and non-GM cotton varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of release of processed cotton GHB614 × T304-40 × GHB119 × COT102 or accidental spillage of viable GM cotton 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 GHB614 × T304-40 × GHB119 × COT102. The GMO Panel concludes that four-event stack cotton 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

COT102, cotton (*Gossypium hirsutum*), genetic engineering, GHB119, GHB614, GM, *Gossypium barbadense*, import and processing, T304-40

CONTENTS

SUMMARY

Following the submission of application EFSA-GMO-ES-2017-147 under Regulation (EC) No 1829/2003 from Bayer CropScience N.V. and transferred to BASF Agricultural Solutions Seed US LLC (referred to hereafter as 'the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) herbicide tolerant and insect-resistant cotton (*Gossypium hirsutum* and *Gossypium barbadense*) GHB614 × T304-40 × GHB119 × COT102 (referred to hereafter as 'fourr-event stack cotton'). The scope of application EFSA-GMO-ES-2017-147 is for import, processing and food and feed uses within the European Union (EU) of the fourr-event stack cotton, and does not include cultivation in the EU. The four-event stack cotton was produced by crossing to combine four single cotton events: GHB614, expressing 2mEPSPS to confer tolerance to glyphosate-based herbicides, T304-40 expressing Cry1Ab to confer resistance to lepidopteran pests and PAT/bar to confer tolerance to glufosinate-ammonium-based herbicides, GHB119 expressing Cry2Ae to confer resistance to lepidopteran pests and PAT/*bar* to confer tolerance to glufosinate-ammonium-based herbicides and COT102 expressing Vip3Aa19 to confer resistance to lepidopteran pests. There is also expression of APH4 which was used as selectable marker (hygromycin resistance) used during the molecular development of this event.

The GMO Panel evaluated the four-event stack cotton with reference to the scope and appropriate principles described in its applicable guidelines for the risk assessment of GM plants and the post-market environmental monitoring (PMEM). The GMO Panel considered the information submitted in application EFSA-GMO-ES-2017-147, additional information provided by the applicant during the risk assessment, the scientific comments submitted by the Member States and the relevant scientific literature. For application EFSA-GMO-ES-2017-147, previous assessments of the four single events (GHB614, T304-40, GHB119 and COT102), provided a basis for the assessment of the four-event stack cotton. No safety concerns were identified by the GMO Panel in the previous assessments. No safety issue concerning the four single cotton events was identified by the updated bioinformatic analyses, nor reported by the applicant since the publication of the previous GMO Panel scientific opinions. Therefore, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid.

For the four-event stack cotton, the risk assessment included the molecular characterisation of the inserted DNA and analysis of protein expression. An evaluation of the comparative analysis of agronomic, phenotypic and compositional characteristics was carried out, and the safety of the newly expressed proteins and the whole food and feed were evaluated with respect to potential toxicity, allergenicity and nutritional characteristics. Environmental impacts and PMEM plan provided by the applicant were also evaluated. The molecular characterisation data establish that the events GHB614, T304-40, GHB119 and COT102 combined in the four-event stack cotton have retained their integrity. Protein expression analysis showed that the levels of the newly expressed proteins are similar in the four-event stack cotton and in the single events.

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 was considered by the GMO Panel with the conclusion that the field trials are appropriate to support the comparative analysis. The comparative analysis of agronomic and phenotypic characteristics and seeds composition identified no differences between cotton GHB614 × T304-40 × GHB119 × COT102 and the non-GM comparator (referred to hereafter as comparator) that required further assessment. The molecular characterisation, the comparative analysis and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single cotton events and of the newly expressed proteins in the four-event stack cotton does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119 × COT102, is as safe as the comparator and the selected commercial non-GM cotton reference varieties (referred to hereafter as non-GM reference varieties). Considering the combination of the single events and their potential interactions, the outcome of the comparative analysis and the routes and levels of exposure, the GMO Panel concludes that cotton GHB614 × T304-40 × GHB119 × COT102 would not raise safety concerns in the case of the release of GM cotton material, including viable seeds, into the environment.

The GMO Panel considers that post-market monitoring (PMM) of cotton GHB614 × T304-40 × GHB119 × COT102 is not necessary. The PMEM plan submitted and the suggested reporting intervals are in line with the intended uses of the four-event stack cotton.

Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issue pertaining to the intended uses of cotton GHB614 × T304-40 × GHB119 × COT102.

Considering the close genetic relationship and extensive bidirectional introgression between *G. hirsutum* and *G. barbadense*, along with the fact that the inserted traits, driven by well-characterised regulatory elements ensuring stable expression, are not expected to affect species-specific metabolic pathways, the GMO panel considers that the information collected in application EFSA-GMO-BE-2017-147 allows, for this specific application, to conclude on the safety of cotton GHB614 × T304-40 × GHB119 × COT102 in *G. hirsutum* and *G. barbadense*.

The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119 × COT102, as described in this application, is as safe as its comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment. However, in accordance with the previous assessment of the single event COT102, 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.

1 | INTRODUCTION

The scope of the application EFSA-GMO-ES-2017-147 is for food and feed uses, import and processing of the genetically modified (GM) herbicide tolerant and insect-resistant cotton GHB614 × T304-40 × GHB119 × COT102 and does not include cultivation in the European Union (EU).

To obtain cotton stack GHB614 × T304-40 × GHB119 × COT102, the four single events in *Gossypium hirsutum* L. were combined by conventional crosses; however, the scope of applications EFSA-GMO-ES-2017-147 also covers *G. barbadense*. *G. hirsutum* and *G. barbadense* are genetically close cotton species which have been sympatric over the last several millennia leading to pervasive genome-wide bidirectional introgression (Yuan et al., 2021). In addition, intentional interspecific hybridisation has occurred many times in both directions, with the goal of producing commercial varieties that possess the traits of both *G. hirsutum* and *G. barbadense* species (Jareczek et al., 2023).

1.1 | Background

On 20 June 2017, the European Food Safety Authority (EFSA) received from the Competent Authority of Spain application EFSA-GMO-ES-2017-147 for authorisation of cotton GHB614 × T304-40 × GHB119 × COT102 (Unique Identifier BCS-GHØØ2-5 × BCS-GHØØ4-7 × BCS-GHØØ5-8 × SYN-IR1Ø2–7), submitted by Bayer CropScience N.V. and transferred to BASF Agricultural Solutions Seed US LLC on 1 August 2018 (hereafter referred to as 'the applicant'), according to Regulation (EC) No 1829/2003.[1] Following receipt of application EFSA-GMO-ES-2017-147, 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 asked the applicant to supplement the initial application, when needed. On 22 September 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 6 months to issue a scientific opinion on application EFSA-GMO-ES-2017-147. This time limit was extended whenever EFSA and/or 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 the Section 5, below). 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 3 months to make their opinion known on application EFSA-GMO-ES-2017-147 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 GHB614 × T304-40 × GHB119 × COT102 in the context of its scope as defined in application EFSA-GMO-ES-2017-147.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation and thus will be part of the EFSA overall opinion in accordance with Articles 6(5) and 18(5). 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 four-event stack cotton on the valid application EFSA-GMO-ES-2017-147, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU MS and relevant peer-reviewed scientific publications. As part of this comprehensive information package, the GMO

[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, pp. 1–23.

[2]Available online: https://open.efsa.europa.eu/questions/EFSA-Q-2017-00505?search=EFSA-GMO-ES-2017-147+.

[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, pp. 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, pp. 1–38.

[5]These particulars are available online at: https://open.efsa.europa.eu/study-inventory/EFSA-Q-2017-00505.

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.

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, 2011b, 2015) and explanatory notes and statements (i.e. EFSA, 2010a, 2017a, 2017b, 2019a, 2019b; EFSA GMO Panel, 2010b) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/04 and OC/EFSA/GMO/2021/06, the contractors performed preparatory work for the evaluation of the applicant's literature search and bioinformatics analyses on cotton GHB614 × T304-40 × GHB119 × COT102, respectively.

3 | ASSESSMENT

3.1 | Introduction

Cotton GHB614 × T304-40 × GHB119 × COT102 was produced by crossing to combine four single cotton events: GHB614 expressing 2mEPSPS conferring tolerance to glyphosate-based herbicides, T304-40 expressing Cry1Ab and PAT/bar conferring resistance to lepidopteron pests and tolerance to glufosinate-ammonium-based herbicides, GHB119 expressing PAT/bar and Cry2Ae conferring tolerance to glufosinate-ammonium-based herbicides and resistance to lepidopteran pests and COT102 expressing APH4 and Vip3Aa19 conferring resistance to hygromycin B (used as a selectable marker during its molecular development) and lepidopteran pests.

All four single events were assessed previously (Table 1) and no safety concerns were identified.

TABLE 1 Single cotton events previously assessed by the GMO Panel and publication date of the corresponding EFSA scientific opinions.

Events	Application or mandate	References of scientific opinion
GHB614	EFSA-GMO-NL-2008-51	EFSA GMO Panel (2009)
	EFSA-GMO-RX-018	EFSA GMO Panel (2021a)
T304-40	EFSA-GMO-NL-2011-97	EFSA GMO Panel (2013)
	GMFF-2024-23010	EFSA GMO Panel (2025)
GHB119	EFSA-GMO-NL-2011-96	EFSA GMO Panel (2016)
COT102	EFSA-GMO-DE-2017-141	EFSA GMO Panel (2023a)

3.2 | Updated information on single events[6]

Since publication of the scientific opinions on the single cotton events by the GMO Panel (Table 1), no safety issue concerning the four single events has been reported by the applicant.

Updated bioinformatic analyses of the junction regions for cotton events GHB614, T304-40, GHB119 and COT102, using up-to-date sequence databases and methodology specified in EFSA guidance (EFSA GMO Panel, 2011a), confirmed that no known endogenous genes were disrupted by any of the inserts.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed 2mEPSPS, PAT/bar, Cry1Ab, Cry2Ae, VIP3Aa19 and APH4 proteins confirmed previous results indicating no significant similarities to known toxins and allergens. Updated bioinformatic analyses of the newly created open reading frames (ORFs) within the inserts or spanning the junctions between the insert and the flanking regions for events GHB614, T304-40, GHB119 and COT102 indicated that the production of new peptides showing significant similarities to toxins or allergens for any of the events in cotton GHB614 × T304-40 × GHB119 × COT102 is highly unlikely, which confirmed previous analyses (Table 1).

In order to update the bioinformatic analyses to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis with microbial DNA for cotton events GHB614, T304-40, GHB119 and COT102. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.4.4.2.

Based on the above information, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid. In accordance with the previous assessment of the single event COT102, the GMO Panel considers that the risk assessment of cotton GHB614 × T304-40 × GHB119 × COT102 may need to be updated in case products

[6]Additional information: 23/2/2024, 12/9/2024, 3/9/2025.

containing hygromycin B, or other substrates of the APH4 enzyme, for therapeutic, prophylactic or any other medical uses in humans or animals obtain future market approval in the EU.

3.3 | Systematic literature review[7]

The GMO Panel assessed the applicant's literature searches on cotton GHB614 × T304-40 × GHB119 × COT102, which include a scoping review, according to the guidelines given in EFSA (2010a, 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-ES-2017-147. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for cotton GHB614 × T304-40 × GHB119 × COT102 at present.

The GMO Panel considered the overall quality of the performed literature searches acceptable. The literature searches identified 34 relevant publications on cotton GHB614 × T304-40 × GHB119 × COT102 from online bibliographic databases. The relevant publications are listed in Appendix B.

None of the relevant publications identified through the literature searches reported information pointing to safety issues associated with cotton GHB614 × T304-40 × GHB119 × COT102 and its subcombinations relevant to the scope of this application.

3.4 | Risk assessment of the four-event stack cotton GHB614 × T304-40 × GHB119 × COT102

3.4.1 | Molecular characterisation[8]

In line with the requirements laid down by Regulation (EU) 503/2013, the possible impact of the combination of the events on the integrity of the events, the expression levels of the newly expressed proteins or the biological functions conferred by the individual inserts are considered below.

3.4.1.1 | *Genetic elements and biological function of the inserts*

Cotton events GHB614, T304-40, GHB119 and COT102 were combined by crossing to produce the four-event stack cotton GHB614 × T304-40 × GHB119 × COT102. The structures of the inserts introduced into the four-event stack cotton are described in detail in the respective EFSA scientific opinions (Table 1) and no new genetic modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 2.

Intended effects of the inserts in cotton GHB614 × T304-40 × GHB119 × COT102 are summarised in Table 3. Based on the known biological function of the newly expressed proteins (Table 3), the only foreseen interactions identified by the GMO panel at the biological level are between the Cry proteins or between the Vip3Aa19 and the Cry proteins in susceptible insects, which will be dealt with in Section 3.4.4. Furthermore, the potential for a functional interaction between the newly expressed proteins with impact on the safety of cotton GHB614 × T304-40 × GHB119 × COT102 for humans and animals is addressed in Section 3.4.3.1.1.

TABLE 2 Genetic elements in the expression cassettes of the events stacked in cotton GHB614 × T304-40 × GHB119 × COT102.

Event	Promoter	5′ UTR	Transit peptide	Coding region	Terminator
GHB614	*Ph4a748At* (histone H4) (*Arabidopsis thaliana*)	First intron of gene II of histone H3.III variant (*A. thaliana*)	Optimised to (*Zea mays* and *Helianthus annuus*)	*2mepsps* (*Zea mays*)	3′ histone H4 (*A. thaliana*)
T304-40	Ps7s7 (*Subterranean clover stunt virus* genome segment 7)	Tapetum-specific E1 (5′ *e1* from *Oryza sativa*)	–	*cry1Ab* (*Bacillus thuringiensis* subsp. *berliner* 1715)	3′ NADP-me1 (*Flaveria bidentis*)
	35S (*Cauliflower mosaic virus* P35S3)	–	–	*bar* (*Streptomyces hygroscopicus*)	3′ nos (*Agrobacterium tumefaciens*)
GHB119	35S (*Cauliflower mosaic virus*)	5′ cab22L (*Petunia hybrida*)	TPssuAt (*ats1A* gene of *A. thaliana*)	*cry2Ae* (*Bacillus thuringiensis* subsp. *dakota* 1715)	3′ 35S (*Cauliflower mosaic virus*)
	PCsVMX XYZ (*Cassava Vein Mosaic Virus*)	–	–	*bar* (*Streptomyces hygroscopicus* strain ATCC21705)	3′ nos (*A. tumefaciens*)

(Continues)

[7]Additional information: 13/7/2023, 7/11/2023, 29/5/2024, 31/7/2024, 12/9/2024, 30/9/2025.

[8]Dossier: Part II – Section 1.2; additional information: 13/7/2023, 7/11/2023, 12/4/2024, 12/9/2024, 18/4/2025, 3/9/2025.

TABLE 2 (Continued)

Event	Promoter	5′ UTR	Transit peptide	Coding region	Terminator
COT102	Ubq3 (*A. thaliana*)	–	–	*aph4* (*Escherichia coli*)	NOS (*A. tumefaciens*)
	Act2 (*A. thaliana*)	–	–	*vip3Aa19** (*B. thuringiensis* strain AB88)	NOS (*A. tumefaciens*)

Note: –, when no element was specifically introduced to optimise expression.
*Codon optimised.

TABLE 3 Characteristics and intended effects of the events stacked in cotton GHB614 × T304-40 × GHB119 × COT102.

Event	Protein	Donor organism and biological function	Intended effects in GM plant
GHB614	2mEPSPS	Based on a gene from *Zea mays*, 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Lebrun et al., 2003)	The amino acid sequence of the cotton EPSPS enzyme was modified by two substitutions to render it tolerant to glyphosate-based herbicides. Expression of 2mEPSPS confers tolerance to glyphosate-ammonium-based herbicides
T304-40	Cry1Ab	Based on genes from *Bacillus thuringiensis*, subsp. *berliner* 1715, Cry1Ab confers resistance to insect pests of the lepidopteran family; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Schnepf et al., 1998)	Cotton T304-40 expresses a chimeric, truncated cry1Ab gene. Cry1Ab is a chimeric protein toxic to certain lepidopteran larvae feeding on cotton
	PAT/bar	Based on the bar gene from *Streptomyces hygroscopicus*, Phosphinothricin-acetyltransferase (PAT) enzyme confers resistance to the antibiotic bialaphos (Eckes et al., 1989)	Cotton T304-40 expresses the PAT/bar protein, which acetylates L-glufosinate-ammonium and thereby confers tolerance to glufosinate-ammonium-based herbicides
GHB119	Cry2Ae	Based on genes from *Bacillus thuringiensis* subsp. *dakota* 1715, Cry1Ae confers resistance to insect pests of the lepidopteran family; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Schnepf et al., 1998)	Cotton GHB119 expresses a cry2Ae gene. Cry2Ae is a chimeric protein toxic to certain lepidopteran larvae feeding on cotton
	PAT/bar	Based on the bar gene from *Streptomyces hygroscopicus* strain ATCC21705, Phosphinothricin-acetyl-transferase (PAT) enzyme confers resistance to the antibiotic bialaphos (Eckes et al., 1989)	Cotton GHB119 expresses the PAT/bar protein, which acetylates L-glufosinate-ammonium and thereby confers tolerance to glufosinate-ammonium-based herbicides
COT102	APH4	Based on gene *aph4* from *E. coli* strain K-12, phosphotransferase enzyme which catalyses the phosphorylation of hygromycin and some related aminoglycosides (Waldron, 1997)	Cotton COT102 expresses the APH4 protein, which phosphorylates hygromycin. The expression of APH4 allows growth and thereby enables selection of the transformed cells in the presence of hygromycin
	Vip3Aa19	Based on a gene from *B. thuringiensis* strain AB88 (Estruch et al., 1996). In addition to Cry proteins, *B. thuringiensis* also produces insecticidal proteins during its vegetative growth stage. These are referred to as vegetative insecticidal proteins (Vip) (Fang et al., 2007)	Cotton COT102 expresses a modified version of the *B. thuringiensis vip3Aa1* gene, and encodes Vip3Aa19, a protein toxic to certain lepidopteran larvae feeding on cotton

3.4.1.2 | *Integrity of the events in the four-event stack*

The genetic stability of the inserted DNA over multiple generations in the single cotton events GHB614, T304-40, GHB119 and COT102 was previously demonstrated (Table 1 and Section 3.2). The integrity of these events in cotton GHB614 × T304-40 × GHB119 × COT102 was demonstrated by Southern analyses and by PCR followed by DNA analysis demonstrating that the sequences of the events (inserts and their flanking regions) in the four-event cotton stack are identical to the sequences already assessed (Table 1 and Section 3.2), thus confirming that the integrity of these events was maintained in the four-event stack cotton.

3.4.1.3 | *Information on the expression of the insert*

Protein levels of 2mEPSPS, PAT/bar, Cry1Ab, Cry2Ae, Vip3Aa19 and APH4 were analysed by an enzyme-linked immunosorbent assay (ELISA) in material harvested in a field trial across three locations in the USA during the 2013 growing season. Samples analysed included leaves (BBCH 14–16, BBCH 51–55, BBCH 60–67), roots (BBCH 14–16), pollen (BBCH 60–69), pre-candle squares (BBCH 60–67), immature bolls (BBCH 60–67), whole plant (BBCH 60–67) and fuzzy seed (BBCH 83–97) from plants treated and not treated with glyphosate- and/or glufosinate-ammonium-based herbicide. Since seeds and pollen are the main raw commodities used for food and feed purposes, their protein levels are summarised in Appendix C (Table C.1).

In order to assess the changes in protein expression levels which may result from interactions between the events, protein levels were determined for the four-event stack and the corresponding single events in different parts of the plant.

The levels of all the newly expressed proteins in the four-event stack cotton and the corresponding singles were comparable in all tissues, except for the higher PAT/bar protein level in the stack, which was expected because of the combination of events T304-40 and GHB119, both producing PAT/bar protein in the four-event stack cotton (Appendix C). Therefore, there is no indication of an interaction that may impact on the levels of the newly expressed proteins in this stack.

3.4.1.4 | *Conclusion on molecular characterisation*

The molecular data establish that the events stacked in cotton GHB614 × T304-40 × GHB119 × COT102 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the four-event stack and in the single events, except for the higher PAT/bar protein level in the stack, which was expected because of the combination of events T304-40 and GHB119, both producing PAT/bar protein in the four-event stack cotton. Therefore, there is no indication of an interaction that may affect the integrity of the events or the levels of the newly expressed proteins in this stack.

Based on the known biological function (Table 3) of the newly expressed proteins, the only foreseen interactions at the biological level are between the Cry proteins or between the Vip3Aa19 and the Cry proteins, which will be dealt with in Section 3.4.4.

3.4.2 | Comparative analysis[9]

3.4.2.1 | *Overview of studies conducted for the comparative analysis*

Application EFSA-GMO-ES-2017-147 presents data on agronomic and phenotypic characteristics, as well as seed composition of the four-event stack cotton GHB614 × T304-40 × GHB119 × COT102 (Table 4).

TABLE 4 Main comparative analysis studies to characterise the four-event stack cotton provided in the application EFSA-GMO-ES-2017-147.

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, USA, 2013, eight sites[a]	FM966	6[b]
Compositional analysis			

[a]Three field trials were located in Texas, one field trial in each of Oklahoma, California, Mississippi, Louisiana, Georgia, South Carolina and North Carolina. Additional sites in North Carolina and South Carolina were excluded from the statistical analysis due to weather related issues.

[b]The non-GM cotton reference varieties were FM958, FM989, ST457, Deltapine DP491, ST468, Acala Maxxa.

3.4.2.2 | *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: the four-event stack cotton not exposed to the intended herbicides (not treated), the four-event stack cotton exposed to the intended herbicides (treated), the comparator FM966 and three commercial non-GM cotton reference varieties (hereafter, 'non-GM reference varieties').

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010a, 2011b). This includes, for each of the two treatments of the four-event stack cotton, the application of a difference test (between the GM cotton and the non-GM comparator) and an equivalence test (between the GM cotton and the set of non-GM reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[10]

3.4.2.3 | *Suitability of selected test materials*

3.4.2.3.1 | *Selection of the test materials*

To obtain the four-event stack cotton, the single events GHB614, T304-40, GHB119 and COT102 were transferred and stabilised in the genetic background of the non-GM cotton FM966 variety. The comparator used in the field trials is the non-GM cotton variety FM996, which has a similar genetic background as cotton GHB614 × T304-40 × GHB119 × COT102 (as documented by the pedigree and by the additional information). The GMO panel considered this to be a suitable comparator. The four-event stack cotton and its comparator were considered appropriate for growing in environments across USA where the comparative field trials were conducted.

[9]Dossier: Part II – Section 1.3; additional information: 13/7/2023, 7/11/2023, 12/4/2024.

[10]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).

Commercial non-GM reference varieties were selected by the applicant and, at each selected site, three reference varieties were tested (Table 4). On the basis of the information provided, the GMO Panel considers the selected non-GM reference varieties acceptable for the comparative assessment.

3.4.2.3.2 | *Seed production and quality*

Seeds of the four-event stack cotton and its comparator used in the 2013 field trials were produced in Arizona and Puerto Rico respectively. The seed lots were verified for their identity by event specific PCR. The germination capacity of the GM four-event stack cotton was compared with that of its comparator and the results indicated similar seed germination.

The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies was of suitable quality.

3.4.2.3.3 | *Conclusion on suitability*

The GMO Panel is of the opinion that the four-event stack cotton, the comparator and the non-GM cotton reference varieties were properly selected and are of sufficient quality. Therefore, the test materials are considered acceptable for the comparative analysis.

3.4.2.4 | *Representativeness of the receiving environments*

3.4.2.4.1 | *Selection of field trial sites*

The selected field trials sites were located in commercial cotton-growing regions of USA. The soil and climatic characteristics of the selected fields[11] correspond 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.

3.4.2.4.2 | *Meteorological conditions*

Data on monthly maximum and minimum mean temperatures and sum of precipitations were provided for each site. Some exceptional weather conditions were reported at two of the selected sites.[12] However, due to the lack of major impacts on plant growth, the GMO Panel considers that these exceptional weather conditions did not invalidate the selection of the field trial sites for the comparative analysis.

3.4.2.4.3 | *Management practices*

The field trials included plots containing four-event stack cotton, plots with the comparator and plots with non-GM reference varieties, mostly managed according to local agricultural practices. In addition, the field trials included plots containing four-event stack cotton managed following the same agricultural practices and exposed to the intended herbicides. The glyphosate-based herbicide was applied at the BBCH[13] 12–16 growth stages, while glufosinate-ammonium-based herbicide was applied at the BBCH 12–14 and at the BBCH 31–65 growth stages.

At some field trial sites,[14] sowing occurred later than usual, resulting in a shifted growing cycle. The analysis of agronomic and phenotypic endpoints indicated that this 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.

3.4.2.4.4 | *Conclusion on representativeness*

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

[11]Soil types of the field trials were sand, loamy sand, sandy loam, silt loam, loam and clay loam; soil organic matter ranged from 0.6% to 2.0%; pH ranged from 5.8 to 8.0; average temperatures and sum of precipitations during the usual crop growing season ranged respectively from 27.8 to 18.3°C and from 491 to 1060 mm.

[12]Heavy rainfall immediately after sowing occurred at one field trial in Texas and early frost at one field trial in Oklahoma.

[13]BBCH scale describes phenological stages (Meier, 2001).

[14]Four field trials located in Georgia, Louisiana, California and Mississippi.

3.4.2.5 | *Agronomic and phenotypic analysis*

Forty-one agronomic and phenotypic endpoints plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trials (Table 4). Of those, nine endpoints (including information on biotic and abiotic stressors) were measured on a categorical scale and analysed with the Cochran–Mantel–Haenszel (CMH) test.[15]

The remaining 32 endpoints[16] were analysed as described in Section 3.4.2.2, with the following results:

- For the four-event stack cotton (not treated) the test of difference identified statistically significant differences with the comparator for 12 endpoints.[17] All these endpoints fell under equivalence category I.
- For the four-event stack cotton (treated) the test of difference identified statistically significant differences with the comparator for 16 endpoints.[18]All these endpoints fell under equivalence category I or II.

The CMH test identified statistically significant differences between the four-event stack cotton and the comparator for plant lodging (for both GM treatments) and plant vigour (for the not treated GM). However, the values for plant lodging were very low for all the test materials and the ranges of plant vigour for the GM were comparable with those for the reference varieties.

3.4.2.6 | *Compositional analysis*

Fuzzy seeds of four-event stack cotton harvested from eight sites (Table 4) were analysed for 71 constituents, including those recommended by OECD (OECD, 2009). The statistical analysis as described in Section 3.4.2.2 was not applied to 19 constituents[19] because their concentration in at least one third of the samples were below the limit of quantification.

The statistical analysis was applied to a total of 52 constituents[20]; a summary of the outcome of the test of difference and the test of equivalence is presented in Table 5:

- For four-event stack cotton not treated with the intended herbicides, statistically significant differences with the comparator were found for 24 endpoints. All these endpoints fell under equivalence category I or II. The equivalence test could not be done for sterculic acid because of the lack of variation among the non-GM reference varieties.
- For four-event stack cotton treated with conventional herbicides, statistically significant differences with the comparator were found for 24 endpoints. All these endpoints fell under equivalence category I or II. The equivalence test could not be done for sterculic acid because of the lack of variation among the non-GM reference varieties.

[15]These included plant lodging, plant vigour, boll type, health rating (at three stages: early, mid-season and late season) and disease incidence (at two stages: early and late season).

[16]Days to emergence, early stand count, final stand count, days to flowering, days to first open bolls, % open bolls, total plot weight, total seed cotton yield, lint weight per plot, lint yield, weight of 25 bolls (lint, seed and lint + seed), average boll weight, % lint, 100 seed weight, number of seeds per boll, plant height, number of nodes per plant, height to node ratio, average first fruiting branch, number of fruiting branch bolls, number of potential fruiting sites, % fruit retention, % harvestable bolls and fibre properties (micronaire, length, length uniformity, strength, elongation, colour/greyness and colour/yellowness). The applicant provided the analysis of total number of bolls per plant, but the GMO Panel did not consider this endpoint as the data were generated from seven field trial sites, less than the minimum required eight sites.

[17]Weight of 25 bolls (lint, seed and lint + seed), 100 seed weight, average boll weight, nodes per plant, height to node ratio, number of fruiting branch bolls and the following fibre endpoints: micronaire, length, strength and elongation.

[18]Days to emergence, final stand count, weight of 25 bolls (lint, seed and lint + seed), 100 seed weight, average boll weight, number of seeds per boll, number of nodes per plant, height to node ratio, average first fruiting branch, number of fruiting branch bolls and the following fibre endpoints: micronaire, length, strength and elongation.

[19]Sodium, 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), γ-linolenic acid (C18:3), octadecatetraenoic acid (C18:4), eicosenoic acid (C20:1), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5), erucic acid (C22:1), n3-docosapentaenoic acid (C22:5), n6-docosapentaenoic acid (C22:5), docosahexaenoic acid (C22:6).

[20]Moisture, crude protein, crude fat, ash, total carbohydrates (by calculation), 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), behenic acid (C22:0), lignoceric acid (C24:0), total gossypol, free gossypol, dihydrosterculic acid, malvalic acid, sterculic acid, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, zinc, α-tocopherol, γ-tocopherol and total tocopherols.

TABLE 5 Outcome of the comparative compositional analysis in fuzzy seeds for four-event stack cotton. The table shows the number of endpoints in each category.

		Test of difference[a]			
		Not treated[b]		Treated[b]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[c]	Category I/II	27	24[d]	27	24[d]
	Category III/IV	–	–	–	–
	Not categorised	1[e]	–	1[e]	–
	Total endpoints	52		52	

[a]Comparison between four-event stack cotton and its comparator.

[b]Treated/not treated with the intended herbicide.

[c]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.

[d]Endpoints with significant differences between four-event stack cotton and its conventional counterpart falling in equivalence category I-II. Not treated only: lysine, tryptophan, linoleic acid (C18:2); treated only: phosphorus, zinc, α-tocopherol; both treated and not treated: crude fat, total carbohydrates (by calculation), acid detergent fibre, neutral detergent fibre, histidine, myristic acid (C14:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), linolenic, acid (18:3), arachidic acid (C20:0), behenic acid (C22:0), total gossypol, free gossypol, dihydrosterculic acid, malvalic acid, copper, manganese, potassium, γ-tocopherol and total tocopherols.

[e]Endpoint not categorised for equivalence and without significant differences between the four-event stack cotton and its comparator: sterculic acid.

The GMO Panel assessed all the significant differences between the four-event stack cotton and the comparator, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. No endpoints with outcomes under category III/IV were identified.

3.4.2.7 | *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 tested between the four-event stack cotton and the comparator needs further assessment regarding potential environmental impact.
- None of the differences identified in seed composition between the four-event stack cotton and the comparator needs further assessment regarding food and feed safety.

3.4.3 | Food/feed safety assessment[21]

The four single events included in this stack cotton application have been previously assessed and no safety concerns were identified by the EFSA GMO Panel (Table 1). The four-event stack cotton 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 the four-event stack cotton into food and feed products is not expected to result in products differing from those of conventional non-GM cotton varieties currently in the EU market.[22]

In accordance with Regulation (EU) No 503/2013 for the risk assessment of genetically modified food or feed containing stacked events, the GMO Panel evaluated the potential for interactions resulting from the combination of the single transformation events impacting on toxicity (Section 3.4.3.1), allergenicity (Section 3.4.3.2) and nutritional assessment (Section 3.4.3.3) described below.

3.4.3.1 | *Toxicology*

3.4.3.1.1 | *Testing of newly expressed proteins*

Six proteins (2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4) are newly expressed in the four-event stack cotton (Section 3.4.1). The GMO Panel has previously assessed these proteins in the context of the single events (Table 1), and

[21]Dossier: Part II – Sections 1.4, 1.5, 1.6, 2; additional information: 12/4/2024.

[22]On-going assessment of novel food applications (https://food.ec.europa.eu/food-safety/novel-food/authorisations/summary-applications-and-notifications_en).

no safety concerns were identified for humans, farmed and companion animals. The GMO Panel is not aware of any new information that would change its previous conclusions on the safety of these proteins. The potential for a functional interaction among the proteins newly expressed in four-event stack cotton has been assessed by the applicant and evaluated by the GMO Panel with regard to human and animal health.

TABLE 6 Intended effects of the newly expressed proteins (NEPs) in cotton GHB614 × T304-40 × GHB119 × COT102.

Protein	Intended effect in GM plant
Cry1Ab, Cry2Ae	The two insecticidal proteins Cry1Ab and Cry2Ae are delta-endotoxins acting through cellular receptors found in lepidopteran species. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high specific affinity to Cry proteins (Hammond et al., 2013; Jurat-Fuentes & Crickmore, 2017; Koch et al., 2015)
Vip3Aa19	The Vip3Aa19 protein is secreted by *B. thuringiensis* during its vegetative phase acting in lepidopterous species via a mechanism similar to that of Cry proteins (Bel et al., 2017; Chakroun et al., 2016)
2mEPSPS	The 2mEPSPS protein confers tolerance to glyphosate-based herbicides acting on the shikimic acid pathway for the biosynthesis of aromatic amino acids in plants, showing high substrate specificity
PAT/bar	The PAT/bar protein confers tolerance to glufosinate-ammonium-based herbicides acting by acetylation of glufosinate-ammonium
APH4	The APH4 protein catalyses the highly specific phosphorylation of the 4-hydroxyl group of hygromycin B, inactivating its antibiotic activity. The APH4 protein is used as a selection marker in the molecular development of the GM plant and shows no agriculturally relevant property in the cultivated plant

The three enzymatic proteins catalyse distinct biochemical reactions, acting on unrelated substrates and are not expected to interact, while the insecticidal proteins Cry1Ab, Cry2Ae and Vip3Aa19 act through cellular receptors found in target insect species (Table 6). On the basis of the known biological function of the individual newly expressed proteins, there is no expectation for possible interactions relevant to the food and feed safety of this four-event stack cotton (Table 3).

During the assessment of the COT102 event, the GMO Panel sought advice from the European Medicines Agency (EMA) regarding the uses of hygromycin B in humans and animals in the European Union (EFSA GMO Panel, 2023a). EMA confirmed[23] that there were 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 were no central authorisations for human or veterinary use for medicinal products that contain hygromycin B.

The GMO Panel concludes that there are no safety concerns for human and animal health related to the newly expressed proteins 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 and their combination in the four-event stack cotton.

3.4.3.1.2 | *Testing of new constituents other than proteins*

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 the four-event stack cotton. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

3.4.3.1.3 | *Information on altered levels of food and feed constituents*

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, none of the differences identified between the four-event stack cotton and its non-GM comparator in seed composition require further assessment (Section 3.4.2.7).

3.4.3.1.4 | *Testing of the whole genetically modified food and feed*

The GMO Panel considers animal studies with food/feed derived from the four-event stack cotton unnecessary based on the outcome of molecular characterisation, comparative analysis and toxicological assessment (EFSA GMO Panel, 2011a). There were no concerns regarding the stability and expression of the inserts or interaction between the four events, and no toxicological concerns regarding the composition of the four-event stack cotton were identified (Sections 3.4.1, 3.4.2.6 and 3.4.3.1).

The GMO Panel had previously concluded that the 90-day feeding studies in rodents on whole food/feed from cotton GHB614, T304-40, GHB119 and COT102 are in line with Regulation (EU) No 503/2013 and do not show adverse effects related to diets incorporating the respective single events (EFSA GMO Panel, 2018a, 2023a).

[23]Correspondence between EFSA and EMA is available online https://open.efsa.europa.eu/study-inventory/EFSA-Q-2017-00271.

3.4.3.2 | *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.

3.4.3.2.1 | *Assessment of allergenicity of the newly expressed proteins*

The GMO Panel has previously evaluated the safety of 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 proteins individually, and no evidence of allergenicity was identified in the context of the single event applications assessed (Table 1). No new information on allergenicity of the proteins newly expressed in this four-event stack cotton that might change the previous conclusions of the GMO Panel has become available. Based on the current knowledge, and as there is no evidence of allergenicity of the newly expressed proteins, there are no expected concerns of allergenicity as a consequence of their simultaneous presence in this four-event stack cotton.

The GMO Panel has previously evaluated the safety of the newly expressed proteins, and no evidence of adjuvanticity was identified in the context of the applications assessed (Table 1). This aspect has been discussed in detail by EFSA (EFSA, 2018; Parenti et al., 2019). To date, there is no evidence for adjuvanticity in the GMOs assessed by the Panel. This four-event stack cotton has similar levels of the individual Bt proteins as those in the respective single cotton events (see Section 3.4.1). The GMO Panel did not find indications that the Bt proteins at the levels expressed in this four-event stack cotton might be adjuvants able to enhance an allergic reaction.

The applicant also provided information on the safety of the 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 proteins regarding their potential to cause a celiac disease response. For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). No indications of safety concern were identified by the GMO Panel as the relevant peptides containing the motif fail to mimic gluten sequences.

The GMO Panel considers that there are no indications that the 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and/or APH4 proteins in this four-event stack cotton may be allergenic.

3.4.3.2.2 | *Assessment of allergenicity of the whole GM plant*

The GMO Panel regularly reviews the available publications on food allergy to cotton. 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 3.4.1–3.4.3), the GMO Panel found no indications of a potentially increased allergenicity of food and feed derived from this four-event stack cotton with respect to that derived from the non-GM comparator and the non-GM reference varieties tested.

3.4.3.3 | *Dietary exposure assessment to new constituents*

In line with Regulation (EU) No 503/2013 the applicant provided dietary exposure estimates to 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 proteins newly expressed in the four-event stack cotton. Dietary exposure was estimated based on protein expression levels reported in this application for the four-event stack cotton treated with the intended herbicides, the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

For the purpose of estimating dietary exposure, the levels of newly expressed proteins in the four-event stack cotton seeds and pollen were derived from field trials (three locations, four replicates each) in 2013 in the USA (see Section 3.4.1.3). Table 7 presents the protein expression levels used to estimate both human and animal dietary exposure.

[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.

TABLE 7 Mean values ($n = 12$[a], µg/g dry weight and µg/g fresh weight) for newly expressed proteins in seeds and pollen from four-event stack cotton treated with the intended herbicides.[b]

	Tissue/developmental stage	
Protein	**Fuzzy seeds (µg/g dry weight)**	**Pollen (µg/g fresh weight)[c]**
Cry1Ab	4.68	0.20
Cry2Ae	24.47	0.17
2mEPSPS	143.37	7.18
PAT/bar	282.61	1.66
Vip3Aa19	8.56	0.39
APH4	< LOQ[d]	< LOQ[d]

[a]For Cry1Ab, 2mEPSPS and PAT/bar in pollen, $n = 10$; for Cry2Ae and Vip3Aa19 in pollen, $n = 11$.

[b]Intended herbicides: glyphosate- and glufosinate-ammonium-based herbicides.

[c]Concentrations values in pollen were adjusted to 6% moisture content before using them to estimate dietary exposure to the different newly expressed protein via the consumption of pollen supplements.

[d]APH4 values were reported below the Limit of Quantification in fuzzy seeds (1.0 µg/g dw) and pollen (1.2 µg/g fw).

3.4.3.3.1 | *Human dietary exposure*

The applicant considered the dietary exposure to 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, 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[25]) 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.[26]

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 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 proteins is expected from the consumption of these products derived from the four-event stack cotton. Dietary exposure to the newly expressed 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 considered by the GMO Panel for consumers of pollen supplements under the assumption that these supplements might be made of pollen from the four-event stack cotton. Consumption data on pollen supplements are available for few consumers across seven different European countries.[27] The low number of consumers available adds uncertainty to the exposure estimations which should be carefully interpreted, and only allows the estimation of dietary exposure for average consumers. Expression levels in pollen as described in Table 7 were used to derive concentrations in pollen supplements considering around 6% moisture content in these products. Since the expression levels of APH4 protein were reported as below the LOQ for all pollen samples, the reported LOQ was used for the exposure estimations.[28] The highest mean acute dietary exposure would be between 0.16 µg/kg bw per day for Cry2Ae and 6.7 µg/kg bw per day for 2mEPSPS, in the elderly population. Similarly, the highest mean chronic dietary exposure in consumers of pollen supplements would be between 0.11 µg/kg bw per day for Cry2Ae and 4.4 µg/kg bw per day for 2mEPSPS, also in the elderly population.

3.4.3.3.2 | *Animal dietary exposure*

Dietary exposure to Cry1Ab, Cry2Ae, 2mEPSPS, PAT/bar and Vip3Aa19 proteins in the four-event stack cotton was estimated by the applicant across different animal species as below described, assuming the consumption of cotton products commonly entering the feed supply chain (i.e. undelinted seeds and meal). The GMO Panel observed that the applicant had not provided an estimate of dietary exposure to APH4 in the four-event stack cotton because all samples were below LOQ, and therefore the GMO Panel covered this aspect within this opinion.

A conservative scenario with 100% replacement of conventional cotton products by the four-event stack cotton products was considered.

[25]Short cellulose fibres left on the seed after the staple cotton is removed by ginning.

[26]https://www.efsa.europa.eu/en/applications/gmo/tools. EFSA consumption database: version 1.0 (updated March 2022).

[27]https://www.efsa.europa.eu/en/data-report/food-consumption-data. Latest update: 18 December 2024.

[28]Left-censored data were treated by the substitution method (WHO, 2009) and, as also indicated in the EFSA scientific report 'Management of left-censored data in dietary exposure assessment of chemical substances' (EFSA, 2010b). For all samples reported as < LOD or as < LOQ, LB and UB values were derived by assigning a value of zero and LOD/LOQ, respectively. For the dietary exposure estimations, only the UB scenario (worst-case) was used; uncertainties linked to this approach will be considered should the exposure estimations be used in the safety assessment of the NEPs. This approach is also mentioned in the corresponding documents on animal and human dietary exposure (EFSA, 2019a; EFSA GMO Panel, 2023b).

Mean levels (dry weight) of the newly expressed proteins in undelinted seeds from four-event stack event cotton treated with the intended herbicide used for animal dietary exposure are listed in Table 7.

All seed samples analysed in the four-event stack cotton for the presence of APH4 protein were below the limit of quantification (LOQ = 1.00 μg/g dry weight); for the purpose of estimating daily dietary intake (DDE), the determined values of LOQ were used as substitutes to compute an average concentration of APH4 in seed to be used for the exposure calculations.[28]

Mean levels of Cry1Ab, Cry2Ae, 2mEPSPS, PAT/bar, 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, 2023b).

The applicant estimated dietary exposure to Cry1Ab, Cry2Ae, 2mEPSPS, PAT/bar, 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 (2023b).

Estimated dietary exposure in the concerned animals is reported in Appendix D (Tables D.1–D.12).

3.4.3.4 | *Nutritional assessment of endogenous constituents*

The intended traits of this four-event stack cotton are herbicide tolerance and insect resistance, with no intention to alter nutritional parameters. Comparison of the composition of this four-event stack cotton with the non-GM comparator and the non-GM reference varieties did not identify differences that would require further safety assessment. From these data, the GMO Panel concludes that this four-event stack cotton is nutritionally equivalent to the non-GM comparator and non-GM reference varieties used.

3.4.3.5 | *Conclusions on the food/feed safety assessment*

The newly expressed proteins 2mEPSPS, Cry1Ab, PAT/bar, Cry2Ae, Vip3Aa19 and APH4 in the four-event stack cotton 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, and no overall toxicological concerns on the four-event stack cotton were identified. Similarly, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in this four-event stack cotton, or regarding its overall allergenicity. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of cotton GHB614 × T304-40 × GHB119 × COT102 does not represent any nutritional concern, in the context of the scope of this application. It should be noted that for the overall assessment of this four-stack cotton, the GMO Panel also evaluated the additional unpublished studies, as listed in Appendix A and the relevant scientific publications listed in Appendix B.

3.4.4 | Environmental risk assessment[29]

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

3.4.4.1 | *Persistence and invasiveness of the GM plant*

In Southern Europe, *G. 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 (Celesti-Grapow et al., 2010; Davis, 1967; Sarno et al., 1993; Tutin et al., 1992). 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 & 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 (Celesti-Grapow et al., 2010; Sarno et al., 1993). 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

[29]Dossier: Part II – Section 5; additional information: 3/9/2025.

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 traits of four-event stack cotton will provide a selective advantage to cotton plants, except when they are exposed to glyphosate- and/or glufosinate-ammonium-based herbicides or infested by insect pests that are susceptible to the Cry1Ab, Cry2Ae and/or Vip3Aa19 proteins. 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's persistence and invasiveness. Therefore, the presence of the intended traits will not affect the overall persistence and invasiveness of the GM plant.

The results of an additional study provided by the applicant on seed germination (Appendix A) provided no evidence that four-event stack cotton GHB614×T304-40×GHB119×COT102 has an increased risk of persistence and invasiveness than its conventional counterpart.

The GMO Panel concludes that it is very unlikely that the four-event stack cotton will differ from conventional cotton varieties in its 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 four-event stack cotton seeds.

3.4.4.2 | *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 grains.

Plant-to-microorganism gene transfer

The probability and potential adverse effects of HGT of the recombinant DNA have been assessed in previous GMO Panel Scientific Opinions for the single events (Table 1). This assessment included consideration of homology-based recombination processes, as well as non-homologous end joining and microhomology-mediated end joining. Possible fitness advantages that the bacteria in the receiving environments would gain from acquiring recombinant DNA were considered. No concern as a result of an unlikely, but theoretically possible, HGT of the recombinant genes to bacteria in the gut of domesticated animals and humans fed GM material or in other receiving environments was identified.

The applicant submitted an updated bioinformatic analysis for each of the single events to assess the possibility for HGT by homologous recombination. This information confirms the assessments provided in the context of previous Scientific Opinions of the single events (EFSA GMO Panel, 2018a, 2023a).

Synergistic effects of the recombinant genes, for instance due to combinations of recombinogenic sequences, which would cause an increase in the likelihood for HGT or a selective advantage were not identified.

Therefore, the GMO Panel concludes that the unlikely, but theoretically possible, horizontal transfer of recombinant genes from this four-event stack cotton to bacteria does not raise any environmental safety concern.

Plant-to-plant gene transfer

The potential for occasional feral cotton GHB614×T304-40×GHB119×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 3.4.4.1). Therefore, the likelihood/frequency of cross-pollination between occasional feral GM cotton plants resulting from seed spillage, and weedy or cultivated *Gossypium* plants is 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 3.4.4.1.

3.4.4.3 | *Interactions of the GM plant with target organisms*

Taking the scope of application EFSA-GMO-ES-2017-147 into account (no cultivation), potential interactions of occasional feral four-event stack cotton plants arising from seed import spills with the target organisms are not considered a relevant issue.

3.4.4.4 | *Interactions of the GM plant with non-target organisms*

The environmental risk assessment considers potential effects of the GM plant on populations of non-target organisms, defined as all those species directly or indirectly exposed to the GM plant and which are not targets of the newly expressed metabolite(s) it expresses. The GMO Panel evaluated the potential hazards of the NEPs and considered that the environmental exposure of non-target organisms to spilled GM cotton material or occasional feral GM cotton plants arising from spilled cotton GHB614 × T304-40 × GHB119 × COT102 seeds will be limited. Additionally, ingested proteins are typically degraded before entering the environment through faecal material of animals fed with GM cotton (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of protein stability in the context of the assessment of the single events and also for this application (Section 3.4.3.1.1 and Appendix A) supports that also the NEPs will be degraded. As compared to non-GM cotton, the GMO Panel considers that potential interactions of cotton GHB614 × T304-40 × GHB119 × COT102 with non-target organisms do not raise any additional environmental safety concern. Interactions that may occur between the insecticidal proteins Cry1Ab, Cry2Ae and/or Vip3Aa19 will not alter this conclusion.

3.4.4.5 | *Interactions with biogeochemical cycles*

Biogeochemical cycles encompass the microbiologically mediated movement, transformation and storage of carbon, nitrogen and other compounds that are considered here for the receiving environments. The GMO Panel evaluated the potential hazards of the NEPs and considered that the environmental exposure to spilled GM cotton material or occasional feral GM cotton plants arising from spilled cotton GHB614 × T304-40 × GHB119 × COT102 seeds will be limited, whereas exposure to manure and faeces of animals fed with cotton GHB614 × T304-40 × GHB119 × COT102 material is expected to be higher. However, ingested proteins are typically degraded before entering the environment through manure and faeces of animals fed with GM cotton (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of protein stability in the context of the assessment of the single events and also for this application (Section 3.4.3.1.1 and Appendix A) supports that also the NEPs will be degraded. As compared to non-GM cotton, the GMO Panel considers that potential interactions of cotton GHB614 × T304-40 × GHB119 × COT102 with biogeochemical cycles do not raise any environmental safety concern.

3.4.4.6 | *Conclusion of the environmental risk assessment*

The GMO Panel concludes that it is unlikely that four-event stack cotton GHB614 × T304-40 × GHB119 × COT102 would differ from conventional cotton varieties in its ability to persist under European environmental conditions. Taking into account the scope of application EFSA-GMO-ES-2017-147, interactions of occasional feral four-event stack cotton plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from four-event stack cotton to bacteria does not indicate a safety concern. Therefore, considering the introduced traits, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that four-event stack cotton would not raise safety concerns in the event of release of processed GM cotton GHB614 × T304-40 × GHB119 × COT102 or the accidental spillage of viable GM cotton seeds into the environment.

3.5 | Post-market monitoring[30]

3.5.1 | Post-market monitoring of GM food/feed

The GMO Panel concludes that the four-event stack cotton, as described in this application, does not raise any nutritional concern and is as safe as the comparator and the non-GM reference varieties tested (Section 3.4.3). Therefore, the GMO Panel does not see the necessity for post-market monitoring of food and feed from the four-event stack cotton, as described in this application.

3.5.2 | Post-market environmental monitoring

The objectives of a PMEM plan, according to Annex VII of Directive 2001/18/EC, are: (i) 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 (ii) 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.

[30]Dossier: Part II – Section 6; additional information: 23/2/2024.

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 four-event stack cotton GHB614×T304-40×GHB119×COT102, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for four-event stack cotton GHB614×T304-40×GHB119×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 the four-event stack cotton and agrees with the proposed reporting intervals.

3.5.3 | Conclusions on post-market monitoring

No post-market monitoring of food and feed is necessary. The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of the four-event stack cotton.

3.6 | Cotton species covered by the scope of the application[31]

G. hirsutum and *G. barbadense* are genetically close cotton species which have been sympatric over the last several millennia leading to pervasive genome-wide bidirectional introgression (Yuan et al., 2021). In addition, intentional interspecific hybridisation has occurred many times in both directions, with the goal of producing commercial varieties that possess the traits of both *G. hirsutum* and *G. barbadense* species (Jareczek et al., 2023). The events present in cotton GHB614×T304-40×GHB119×COT102 are not predicted to interact with metabolic pathways controlling any traits that differ between *G. hirsutum* and *G. barbadense*. Moreover, the combined events contain promoters and terminators widely used for constitutive expression of proteins (Brooks et al., 2023; de Paes Melo et al., 2021; Kummari et al., 2020; Lepetit et al., 1992; Villao-Uzho et al., 2023) and are expected to lead to similar expression of the newly expressed proteins in both species. Based on these observations, the GMO panel considers that the information collected in application EFSA-GMO-BE-2017-147 allows, for this specific application, to conclude on the safety of cotton GHB614×T304-40×GHB119×COT102 in *G. hirsutum* and *G. barbadense*.

4 | OVERALL CONCLUSIONS

The GMO Panel was asked to carry out a scientific assessment of cotton GHB614×T304-40×GHB119×COT102 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

No new information was identified on the four single cotton events (GHB614, T304-40, GHB119 and COT102) that would lead to a modification of the original conclusions on their safety.

The molecular characterisation, the comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single cotton events and of the newly expressed proteins in the four-event stack cotton does not give rise to food/feed safety and nutritional concerns. The GMO Panel concludes that the four-event stack cotton, as described in this application, does not raise any nutritional concern and is as safe as its comparator and the selected non-GM reference varieties.

The GMO Panel concludes that additional environmental effects as compared to conventional cotton resulting from the release of the four-event stack cotton into the environment are unlikely.

Based on the relevant publications identified through the literature searches, the GMO Panel did not identify any safety issues pertaining to the intended uses of cotton GHB614×T304-40×GHB119×COT102.

In addition, the GMO Panel considered the additional unpublished studies listed in Appendix A, which did not raise any concern for human and animal health and the environment. Given the absence of safety and nutritional concerns for foods and feeds from the four-event stack cotton, the GMO Panel considers that PMM of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the four-event stack cotton. In conclusion, the GMO Panel considers that cotton GHB614×T304-40×GHB119×COT102, as described in this application, is as safe as the comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

[31]Additional information: 12/9/2024, 20/5/2025.

Considering the close genetic relationship and extensive bidirectional introgression between *G. hirsutum* and *G. barbadense*, along with the fact that the inserted traits, driven by well-characterised regulatory elements ensuring stable expression, are not expected to affect species-specific metabolic pathways, the GMO panel considers that the information collected in application EFSA-GMO-BE-2017-147 allows, for this specific application, to conclude on the safety of cotton GHB614 × T304-40 × GHB119 × COT102 in *G. hirsutum* and *G. barbadense*.

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 Competent Authority of Spain received on 20 June 2017 concerning a request for authorisation of placing on the market of genetically modified cotton GHB614 × T304-40 × GHB119 × COT102, submitted in accordance with Regulation (EC) No1829/2003 by Bayer CropScience N.V. and transferred to BASF Agricultural Solutions Seed US LLC on 1 August 2018 (EFSA Ref. EFSA-GMO-BE-2017-147; EFSA-Q-2017-00505).
- The application was made valid on 22 September 2017.
- Risk assessment stopped on 25 September 2017 due to "single event" principle for COT102.
- Risk assessment resumed on 10 May 2023.
- Additional Information (2) was requested on 26 May 2023.
- Additional Information (2) was received on 13 July 2023.
- Additional Information (3) was requested on 8 August 2023.
- Additional Information (3) was received on 7 November 2023.
- Additional Information (4) was requested on 6 December 2023.
- Additional Information (4) was received on 23 February 2024 partial; on 12 April 2024 partial; 12 September 2024 complete.
- Additional Information (5) was requested on 4 April 2024.
- Additional Information (5) was received on 29 May 2024 partial; on 31 July 2024 complete.
- Additional Information (6) was requested on 16 July 2024.
- Additional Information (6) was received on 18 April 2025.
- Additional Information (7) was requested on 19 August 2024.
- Additional Information (7) was received on 12 September 2024.
- Additional Information (8) was requested on 4 September 2024.
- Additional Information (8) was received on 12 September 2024.
- Additional Information (9) was requested on 20 March 2025.
- Additional Information (9) was received on 20 May 2025.
- Additional Information (10) was requested on 16 July 2025.
- Additional Information (10) was received on 3 September 2025.
- Additional Information (11) was requested on 23 September 2025.
- Additional Information (11) was received on 30 September 2025.
- Spontaneous information received on 15 July 2020.

ABBREVIATIONS

bw	body weight
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
fw	fresh weight
GM	genetically modified
GMO	genetically modified organisms
HGT	horizontal gene transfer
HR	homologous recombination
NEPs	newly expressed proteins
ORFs	open reading frames
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
PMM	post-market monitoring

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REQUESTOR

Competent Authority of Spain

QUESTION NUMBER

EFSA-Q-2017-00505

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

List of additional studies performed by or on behalf of the applicant with regard to the evaluation of the safety of cotton GHB614 × T304-40 × GHB119 × COT102 for humans, animal or the environment

Study identification	Title
M-498312-01-1	Comparative Evaluation of the Germination Potential of GHB614 × T304-40 × GHB119 × COT102 and FM966 cotton
M-287164-01-1	GEM2 protein: In vitro digestibility study in simulated gastric fluid
M-508050-01-1	Cry2Ae protein – Acute toxicity study by oral gavage in mice
M-521141-01-1	Additional analysis on the structural stability of Gossypium hirsutum GHB614 × T304-40 × GHB119 × COT102
M-537788-01-1	GHB614 × T304-40 × GHB119 × COT102 Cotton – Protein expression analyses of field samples grown in Brazil during 2013–2014
M-538152-01-1	GHB614 × T304-40 × GHB119 × COT102 cotton – Protein expression analyses of field samples grown in Australia during 2014–2015
M-553376-01-1	GHB614 × T304-40 × GHB119 × COT102 cotton – Production, processing and composition analyses of field grown samples. USA, 2012
M-085589-02-1[a]	PAT/bar protein: Heat stability study
M-208793-04-1[a]	PAT/bar protein: in vitro digestibility study in human simulated intestinal fluid
M-217195-04-1[a]	PAT/bar protein: in vitro digestibility study in human simulated gastric fluid
M-251592-01-1[a]	GEM2 protein – Heat stability study
M-293053-02-1[a]	2mEPSPS protein – Heat stability study
M-345445-01-1[a]	Absence of newly created conformational epitopes after double mutation of the maize 2mEPSPS
M-353455-01-1[a]	Cry1Ab protein – In vitro digestibility study in human simulated gastric fluid
M-406126-01-1[a]	2mEPSPS protein: In vitro digestibility study in human simulated gastric fluid
M-411947-01-1[a]	Analysis of the heat stability of the Cry2Ae protein
M-429308-01-1[a]	Analysis of the heat stability of the Cry1Ab protein
M-461494-01-1[a]	Recombinant PAT/bar protein: Acute toxicity by oral gavage in female mice
M-475319-01-1[a]	PAT/bar protein – Acute toxicity by oral gavage in mice
M-535903-01-1[b]	The effect of temperature on 2mEPSPS as assessed by SDS-PAGE and western blot
M-549236-01-1[b]	The effect of temperature on 2mEPSPS as assessed by the EPSPS quantitative activity assay – Amended final report
M-554703-01-1[c]	The effect of temperature on PAT/bar as assessed by the PAT quantitative activity assay
M-557508-01-1[c]	The effect of temperature on PAT/bar as assessed by ELISA
M-497630-01-1[d]	2mEPSPS protein – Acute toxicity study by oral gavage in mice
M-500886-01-1[d]	The effect of temperature on microbially-produced 2mEPSPS as assessed by ELISA

[a]Study previously assessed in EFSA GMO Panel (2018a).
[b]Study previously assessed in EFSA GMO Panel (2021a).
[c]Study previously assessed in EFSA GMO Panel (2018b).
[d]Study previously assessed in EFSA GMO Panel (2021b).

APPENDIX B

List of relevant publications identified by the applicant through literature searches (January 2006–June 2025)

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APPENDIX C

Protein expression data

TABLE C.1 Mean, standard deviation and range of protein levels in fuzzy seeds (μg/g dry weight and μg/g of fresh weight) and pollen (μg/g of fresh weight) from cotton GHB614 × T304-40 × GHB119 × COT102 (not treated) and GHB614, TK304-40, GHB119 and COT102 (not treated), from field trials performed across three locations in the USA in 2013 ($n = 12$).[a]

Protein	Event(s)	Fuzzy seeds μg/g dry weight (dw)	Fuzzy seeds μg/g fresh weight (fw)	Pollen μg/g fresh weight (fw)
2mEPSPS	**GHB614 × T304-40 × GHB119 × COT102**	141.34 ± 10.93 (120.92 ± 152.80)	122.46 ± 9.10 (109.05–137.24)	8.42 ± 5.13 (1.94–17.93)
	GHB614	159.36 ± 13.06 (131.33 ± 180.65)	141.06 ± 14.44 (112.81–163.16)	8.95 ± 6.69 (2.43–23.14)
PAT/bar	**GHB614 × T304-40 × GHB119 × COT102**	279.97 ± 29.62 (220.34–317.70)	242.34 ± 23.86 (198.70–272.61)	1.23 ± 0.92 (0.30–2.91)
	T304-40	110.95 ± 12.80 (85.41–130.41)	96.82 ± 10.40 (77.19–109.88)	0.75 ± 1.00 (0.03–3.31)
	GHB119	96.10 ± 12.37 (74.80–117.54)	82.17 ± 10.71 (61.93–99.51)	1.53 ± 1.00 (0.29–3.06)
Cry1Ab	**GHB614 × T304-40 × GHB119 × COT102**	4.59 ± 0.86 (3.47–6.20)	3.97 ± 0.69 (2.98–5.05)	0.22 ± 0.14 (0.07–0.47)
	T-304-40	6.56 ± 1.25 (5.24–9.05)	5.75 ± 1.23 (4.57–8.26)	0.35 ± 0.23 (0.12–0.82)
Cry2Ae	**GHB614 × T304-40 × GHB119 × COT102**	29.78 ± 3.30 (25.27–35.29)	25.83 ± 3.02 (21.67–30.37)	0.20 ± 0.09 (0.08–0.45)
	GHB119	26.79 ± 7.53 (15.00–35.10)	22.70 ± 5.75 (13.46–29.72)	0.28 ± 0.41 (0.06–1.56)
Vip3Aa19	**GHB614 × T304-40 × GHB119 × COT102**	6.69 ± 1.25 (< LOD-8.86)[b]	5.79 ± 1.01 (< LOD-7.45)[b]	0.44 ± 0.10 (0.31–0.60)
	COT102	10.81 ± 3.26 (6.99–16.44)	8.97 ± 3.12 (6.04–14.88)	0.58 ± 0.23 (0.18–1.16)
APH4	**GHB614 × T304-40 × GHB119 × COT102**	ND[c] < LOD	ND[c] < LOD	ND[c] < LOD–< LLOQ
	COT102	ND[c] < LOD	ND[c] < LOD	ND[c] < LOD–< LLOQ

[a]Number of samples is $n = 12$ except: for 2mEPSPS $n = 11$ in fuzzy seeds for GHB614 × T304-40 × GHB119 × COT102; for Cry1Ab n = 11 in fuzzy seeds for GHB614 × T304-40 × GHB119 × COT102, for Cry2Ae n = 11 in fuzzy seeds for GHB614 × T304-40 × GHB119 × COT102; for Vip3Aa19 n = 11 in fuzzy seeds and pollen for GHB614 × T304-40 × GHB119 × COT102; for PAT n = 11 in fuzzy seeds for GHB614 × T304-40 × GHB119 × COT102; for APH4 n = 11 in fuzzy seeds for GHB614 × T304-40 × GHB119 × COT102.

[b]LOD = 0.20 μg/g dw for fuzzy seeds and LOD = 0.04 μg/g fw for pollen.

[c]ND: not determined for concentration (LOD = 0.30 μg/g dw for fuzzy seeds and LOD = 0.36 μg/g fw for pollen; LLOQ = 1.0 μg/g dw for fuzzy seeds and LLOQ = 1.20 μg/g fw for pollen).

APPENDIX D

Animal dietary exposure

TABLE D.1 Dietary exposure to **Cry1Ab** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**μg/kg**	**mg/kg**
Dairy cow	650	25	10	18.00	0.02
Dairy sheep	80	2.8	25	40.95	0.04
Dairy goat	60	3.4	20	53.04	0.05

TABLE D.2 Dietary exposure to **Cry1Ab** protein in farmed and companion animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**μg/kg**	**mg/kg**
Dairy cow	650	25	5	15.75	0.02
Beef cattle	500	12	5	9.83	0.01
Dairy sheep	80	2.8	20	57.33	0.06
Dairy goat	60	3.4	15.5	71.94	0.07
Rabbit	2	0.15	43	264.13	0.26
Fattening pig	100	3	5	12.29	0.01
Lactating sow	200	6	10	24.57	0.02
Broiler	2	0.158	5	32.35	0.03
Laying hens	1.9	0.13	5	28.02	0.03
Turkey	7	0.5	10	58.50	0.06
Horse	450	9	10	16.38	0.02

TABLE D.3 Dietary exposure to **Cry2Ae** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**μg/kg**	**mg/kg**
Dairy cow	650	25	10	94.12	0.09
Dairy sheep	80	2.8	25	214.11	0.21
Dairy goat	60	3.4	20	277.33	0.28

TABLE D.4 Dietary exposure to **Cry2Ae** protein in selected animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**μg/kg**	**mg/kg**
Dairy cow	650	25	5	82.35	0.08
Beef cattle	500	12	5	51.38	0.05
Dairy sheep	80	2.8	20	299.74	0.30
Dairy goat	60	3.4	15.5	376.10	0.38
Rabbit	2	0.15	43	1380.95	1.38
Fattening pig	100	3	5	64.23	0.06
Lactating sow	200	6	10	128.46	0.13
Broiler	2	0.158	5	169.14	0.17
Laying hens	1.9	0.13	5	146.49	0.15
Turkey	7	0.5	10	305.86	0.31
Horse	450	9	10	85.64	0.09

TABLE D.5 Dietary exposure to **2mEPSPS** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	10	551.42	0.55
Dairy sheep	80	2.8	25	1254.49	1.25
Dairy goat	60	3.4	20	1624.86	1.62

TABLE D.6 Dietary exposure to **2mEPSPS** protein in farmed and companion animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	5	482.50	0.48
Beef cattle	500	12	5	301.08	0.30
Dairy sheep	80	2.8	20	1756.30	1.76
Dairy goat	60	3.4	15.5	2203.74	2.20
Rabbit	2	0.15	43	8091.53	8.09
Fattening pig	100	3	5	376.35	0.38
Lactating sow	200	6	10	752.70	0.75
Broiler	2	0.158	5	991.06	0.99
Laying hens	1.9	0.13	5	858.34	0.86
Turkey	7	0.5	10	1792.14	1.79
Horse	450	9	10	501.80	0.50

TABLE D.7 Dietary exposure to **PAT/bar** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	10	1086.96	1.09
Dairy sheep	80	2.8	25	2472.84	2.47
Dairy goat	60	3.4	20	3202.91	3.20

TABLE D.8 Dietary exposure to **PAT/bar** protein in farmed and companion animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	5	951.10	0.95
Beef cattle	500	12	5	593.48	0.59
Dairy sheep	80	2.8	20	3461.99	3.46
Dairy goat	60	3.4	15.5	4343.97	4.34
Rabbit	2	0.15	43	15949.88	15.95
Fattening pig	100	3	5	741.86	0.74
Lactating sow	200	6	10	1483.71	1.48
Broiler	2	0.158	5	1953.55	1.95
Laying hens	1.9	0.13	5	1691.95	1.69
Turkey	7	0.5	10	3532.64	3.53
Horse	450	9	10	989.14	0.99

TABLE D.9 Dietary exposure to **Vip3Aa19** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	10	32.92	0.03
Dairy sheep	80	2.8	25	74.90	0.07
Dairy goat	60	3.4	20	97.01	0.10

TABLE D.10 Dietary exposure to **Vip3Aa19** protein in farmed and companion animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	5	28.81	0.03
Beef cattle	500	12	5	17.98	0.02
Dairy sheep	80	2.8	20	104.86	0.10
Dairy goat	60	3.4	15.5	131.57	0.13
Rabbit	2	0.15	43	483.11	0.48
Fattening pig	100	3	5	22.47	0.02
Lactating sow	200	6	10	44.94	0.04
Broiler	2	0.158	5	59.17	0.06
Laying hens	1.9	0.13	5	51.25	0.05
Turkey	7	0.5	10	107.00	0.11
Horse	450	9	10	29.96	0.03

TABLE D.11 Dietary exposure to **APH4** protein in farmed and companion animals, based on the consumption of cotton undelinted seeds.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	10	0.02	0.00002
Dairy sheep	80	2.8	25	0.04	0.00004
Dairy goat	60	3.4	20	0.05	0.00005

TABLE D.12 Dietary exposure to **APH4** protein in farmed and companion animals, based on the consumption of cottonseed meal.

	Body weight	Total daily intake	Inclusion rates	Daily dietary exposure	
Animal species	**kg**	**kg/animal**	**%**	**µg/kg**	**mg/kg**
Dairy cow	650	25	5	0.03	0.00003
Beef cattle	500	12	5	0.02	0.00002
Dairy sheep	80	2.8	20	0.10	0.00010
Dairy goat	60	3.4	15.5	0.13	0.00013
Rabbit	2	0.15	43	0.46	0.00046
Fattening pig	100	3	5	0.02	0.00002
Lactating sow	200	6	10	0.04	0.00004
Broiler	2	0.158	5	0.06	0.00006
Laying hens	1.9	0.13	5	0.05	0.00005
Turkey	7	0.5	10	0.10	0.00010
Horse	450	9	10	0.03	0.00003