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Assessment of genetically modified cotton GHB614 × T304-40 × GHB119 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2014-122)

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Abstract

The three-event stack cotton GHB614 × T304-40 × GHB119 was produced by conventional crossing to combine three single events, GHB614, T304-40 and GHB119. The genetically modified organisms (GMO) Panel previously assessed the three single cotton events and did not identify safety concerns. No new data on the single cotton events that could lead to modification of the original conclusions on their safety were identified. Based on the molecular, agronomic, phenotypic and compositional characteristics, the combination of the single cotton events and of the newly expressed proteins in the three-event stack cotton did not give rise to food and feed safety concern. The GMO Panel considers that the three-event stack cotton GHB614 × T304-40 × GHB119 has the same nutritional impact as its comparator and the non-GM reference varieties tested. The GMO Panel concludes that the three-event stack cotton GHB614 × T304-40 × GHB119, as described in this application, is nutritionally equivalent to and as safe as its comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of accidental release of viable GHB614 × T304-40 × GHB119 cottonseeds into the environment, this three-event stack 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 seeds. The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is as safe as its comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Keywords: GMO, cotton GHB614 × T304-40 × GHB119, Regulation (EC) 1829/2003, PAT, Cry1Ab, Cry2Ae, 2mEPSPS

Requestor: Competent Authority of the Netherlands

Question number: EFSA-Q-2014-00721

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Summary

Following the submission of an application (EFSA-GMO-NL-2014-122) under Regulation (EC) No 1829/2003[1] from Bayer CropScience N.V., the Panel on Genetically Modified Organisms of the European Food Safety Authority (GMO Panel) was asked to deliver a scientific opinion on the safety of insect-resistant and herbicide-tolerant genetically modified (GM) cotton GHB614 × T304-40 × GHB119. The scope of application EFSA-GMO-NL-2014-122 is for import, processing and food and feed uses but excludes cultivation in the European Union (EU) and covers the three-event stack cotton GHB614 × T304-40 × GHB119 in cotton species *Gossypium hirsutum* and *Gossypium barbadense*.

In delivering its scientific opinion, the GMO Panel considered the data available on the single events, the information presented in application EFSA-GMO-NL-2014-122, additional information provided by the applicant, the scientific comments submitted by the Member States and relevant scientific publications. The three-event stack cotton GHB614 × T304-40 × GHB119 was produced by conventional crossing to combine three single events: GHB614 (expressing the modified 5-enolpyruvyl-shikimate-3-phosphate synthase (2mEPSPS) protein)), T304-40 (expressing Cry1Ab and phosphinothricin acetyltransferase (PAT)) proteins) and GHB119 (expressing Cry2Ae and PAT proteins) to confer resistance to certain lepidopteran pests and tolerance to glyphosate- and glufosinate ammonium-based herbicides.

The GMO Panel evaluated cotton GHB614 × T304-40 × GHB119 with reference to the scope and appropriate principles described in Regulation (EU) 503/2013[2] and EFSA guidelines for the risk assessment of GM plants and derived food and feed, the environmental risk assessment of GM plants and the post-market environmental monitoring (PMEM) of GM plants. These establish the principle that where all single events have been assessed, the risk assessment of stacked events should focus mainly on issues related to (a) stability of the inserts, (b) expression of the introduced genes and their products and (c) potential synergistic or antagonistic effects resulting from the combination of the events.

For application EFSA-GMO-NL-2014-122, the previous assessment of the three single cotton events (GHB614, T304-40 and GHB119) provided the basis to evaluate the three-event stack cotton. Cotton GHB614, T304-40 and GHB119 were previously assessed by the GMO Panel and no safety concerns were identified. No safety issue was identified by updated bioinformatics analyses nor reported by the applicant for any of the three single cotton events since the publication of the respective scientific opinions. In line with Regulation (EU) 503/2013, the GMO Panel assessed the three 90-day toxicity studies on the whole food/feed from the single events GHB614, T304-40 and GHB119 and concludes that no adverse effects were noted in these studies. Consequently, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid.

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. In the context of PMEM, the applicant should improve the literature searches according to the GMO Panel recommendations.

The evaluation addressed the following components of the risk assessment: the molecular characterisation of the inserted DNA and analysis of the expression of the corresponding proteins; the comparative analyses of compositional, agronomic and phenotypic characteristics; the safety of the newly expressed proteins and the whole food/feed with respect to potential toxicity, allergenicity and nutritional characteristics; and the environmental risk assessment and the PMEM.

The molecular data establish that the events stacked in cotton GHB614 × T304-40 × GHB119 have retained their integrity. Protein expression analysis shows that the levels of the newly expressed proteins are similar in the three-event stack cotton and in the single events. PAT shows the expected higher level in the stack resulting from the combination of the single cotton events T304-40 and GHB119. Therefore, there is no indication of an interaction that may affect the integrity of the events and the levels of the newly expressed proteins in this stack.

Given the magnitude of the differences observed in the comparative assessment, the nature of the endpoints and the outcome of the equivalence test, the GMO Panel considers that none of the agronomic and phenotypic differences between cotton GHB614 × T304-40 × GHB119 and the comparator needs further assessment for its potential environmental impact except the percentage of lint. This difference was further assessed and found not to affect the ability of cotton GHB614 × T304-40 × GHB119 to

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[2] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.

survive until subsequent seasons or to establish occasional feral plants under European environmental conditions. The GMO Panel does not identify the need for further food/feed safety assessment on the composition characteristics except for total fat, dihydrosterculic acid and α-linolenic acid. These differences were assessed and do not raise concerns with regard either food and feed safety or nutritional relevance.

The proteins 2mEPSPS, PAT, Cry1Ab and Cry2Ae newly expressed in the three-event stack cotton do not raise concerns for human and animal health and no interactions between these proteins relevant for food and feed safety were identified. Similarly, the GMO Panel does not identify indications regarding the overall allergenicity of the three-event stack cotton. The nutritional impact of cotton GHB614 × T304-40 × GHB119-derived food and feed is expected to be the same as those derived from the comparator and non-GM reference varieties.

The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is nutritionally equivalent to and as safe as the comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

Considering the combined 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 would not raise safety concerns in the case of accidental release of viable GM cottonseeds into the environment. The PMEM plan and reporting intervals are in line with the intended uses of cotton GHB614 × T304-40 × GHB119.

The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is as safe as its comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Table of contents

1. Introduction

The scope defined by the applicant at the time of submission is for import, processing and food and feed uses but excludes cultivation in the European Union (EU) and covers the three-event stack cotton GHB614 × T304-40 × GHB119 in cotton species *Gossypium hirsutum* and *Gossypium barbadense*.

1.1. Background

On 8 October 2014, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands application EFSA-GMO-NL-2014-122 for placing on the market of genetically modified (GM) cotton GHB614 × T304-40 × GHB119, submitted by Bayer CropScience N.V. within the framework of Regulation (EC) No 1829/2003 on GM food and feed.

After receiving application EFSA-GMO-NL-2014-122, and in accordance with Articles 5(2)(b) and 17(2)(b) of the Regulation (EC) No 1829/2003, EFSA informed the Member States and the European Commission, and made the summary of the application publicly available on the EFSA website.[3] EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of the Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013. On 23 March 2015 and on 9 April 2015, EFSA received additional information requested under completeness check on 4 December 2014. On 30 April 2015, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the European Commission and consulted nominated risk assessment bodies of Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[4] following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Member States had 3 months after the date of receipt of the valid application (until 21 February 2017) to make their opinion known.

The genetically modified organisms (GMO) Panel requested additional information from the applicant on 27 September 2016, 16 December 2016, 23 February 2017, 12 April 2017, 11 May 2017, 1 August 2017, 1 September 2017, 7 December 2017, 15 February 2018 and 28 March 2018. The applicant provided information on 13 December 2016, 14 February 2017, 27 March 2017, 30 March 2017, 28 July 2017, 15 September 2017, 2 February 2018, 20 February 2018 and 15 May 2018. The applicant provided spontaneous information on 13 December 2016.

In the frame of the contracts OC/EFSA/GMO/2013/01 and OC/EFSA/GMO/2014/01, contractors performed preparatory work and delivered reports on the methods applied by the applicant in performing bioinformatics, toxicological and statistical analyses, respectively.

In giving its scientific opinion on cotton GHB614 × T304-40 × GHB119 to the European Commission, Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of 6 months from the acknowledgement of the valid application. As additional information was requested by the GMO Panel, the time limit of 6 months was extended accordingly, in line with Articles 6(1), 6(2), 18(1) and 18(2) of Regulation (EC) No 1829/2003.

According to Regulation (EC) No 1829/2003, 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).

1.2. Terms of Reference as provided by the requestor

The GMO Panel was requested to carry out a scientific assessment of cotton GHB614 × T304-40 × GHB119 (*G. hirsutum and G. barbadense*) for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003.

Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environments and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

[3] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionDocumentsLoader?question=EFSA-Q-2014-00721

[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.

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

2. Data and methodologies

2.1. Data

In delivering its scientific opinion, the GMO Panel took into account application EFSA-GMO-NL-2014-122, additional information provided by the applicant, scientific comments submitted by the Member States and relevant scientific publications.

2.2. Methodologies

The GMO Panel carried out a scientific risk assessment of cotton GHB614 × T304-40 × GHB119 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. The GMO Panel took into account Regulation (EU) No 503/2013 and the appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed (EFSA GMO Panel, 2011a), the conducting of repeated-dose 90-day oral toxicity study in rodents on whole food/feed (EFSA Scientific Committee, 2011), the environmental risk assessment (ERA) of GM plants (EFSA GMO Panel, 2010), and the post-market environmental monitoring (PMEM) of GM plants (EFSA GMO Panel, 2011b). The GMO Panel took into account the criteria included in the 'Explanatory statement for the applicability of the Guidance of the EFSA Scientific Committee on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed for GMO risk assessment' (EFSA, 2014), to perform the assessment of the 90-day feeding studies provided.

According to Regulation (EU) 503/2013 and as described in EFSA guidance 'For GM plants containing a combination of transformation events (stacked events) the primary concern for risk assessment is to establish that the combination of events is stable and that no interactions between the stacked events, that may raise safety concerns compared to the single events, occur. The risk assessment of GM plants containing stacked events focuses on issues related to: (a) stability of the inserts, (b) expression of the introduced genes and their products and (c) potential synergistic or antagonistic effects resulting from the combination of the events' (EFSA GMO Panel, 2011a).

The GMO Panel also assessed the applicant's systematic literature searches in accordance with the guidelines on literature searching given in EFSA (2010, 2017). The comments raised by the Member States are addressed in Annex G of EFSA's overall opinion and were taken into consideration during the scientific risk assessment.

3. Assessment

3.1. Introduction

Cotton GHB614 × T304-40 × GHB119 was developed by conventional crossing to combine three single events: GHB614 (expressing the modified 5-enolpyruvyl-shikimate-3-phosphate synthase (2mEPSPS) protein)), T304-40 (expressing Cry1Ab and phosphinothricin acetyltransferase (PAT)) protein) and GHB119 (expressing Cry2Ae and PAT proteins) to confer resistance to certain lepidopteran pests and tolerance to glyphosate- and glufosinate ammonium-based herbicides.

All three single events were assessed previously (see Table 1) and no concerns for human and animal health or environmental safety were identified.

Table 1: Single cotton events already assessed by the GMO Panel

Event	Application	EFSA Scientific Opinion
GHB614	EFSA-GMO-NL-2008-51	EFSA (2009)
T304-40	EFSA-GMO-NL-2010-97	EFSA GMO Panel (2013)
GHB119	EFSA-GMO-UK-2010-96	EFSA GMO Panel (2016)

3.2. Updated information on single events[5]

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

Updated bioinformatic analyses for cotton events GHB614, T304-40 and GHB119 confirms 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, Cry1Ab and Cry2Ae proteins confirm previous results indicating no significant similarities to 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 and GHB119 confirms that the production of a new peptide showing significant similarity to toxins or allergens is highly unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for events GHB614, T304-40 and GHB119 to microbial DNA. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.7.2.

Based on the above information, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid.

3.3. Systematic literature review[6]

The GMO Panel assessed the applicant's literature searches on cotton GHB614 × T304-40 × GHB119, which include a scoping review, according to the guidelines given in EFSA (2010).

A systematic literature review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of the application EFSA-GMO-NL-2014-122. 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 at present.

Although the overall quality of the performed literature searches is acceptable, the GMO Panel considers that future searches on cotton GHB614 × T304-40 × GHB119 should be improved. The GMO Panel therefore recommends the applicant to:

- provide the search strategies that were actually run (the search history) – line by line with the number of publications identified per line;
- ensure that enough search term variation is used (covering possible synonyms, related terms, acronyms, spelling variants, old and new terminology, brand and generic names, lay and scientific terminology, common typos, translation issues);
- use truncation consistently;
- include controlled vocabulary (subject indexing) in the searches when available, and where subject headings are available use both free-text terms and controlled vocabulary in the searches;
- assess the relevance and risk assessment implications of publications retrieved via searches beyond electronic bibliographic databases.

None of the relevant publications identified through the literature searches report information pointing to safety issues associated with the scope of application EFSA-GMO-NL-2014-122.

[5] Dossier: Part II – Section 1.2.2.2; additional information: 13/12/2016 and 2/2/2018.
[6] Dossier: Part II – Section 7, including relevant Appendices; additional information: 28/7/2017 and 15/9/2017.

3.4. Risk assessment of the three-event stack cotton GHB614 × T304-40 × GHB119

3.4.1. Molecular characterisation

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 functions of the inserts[7]

Cotton events GHB614, T304-40 and GHB119 were combined by conventional crossing to produce the three-event stack cotton GHB614 × T304-40 × GHB119. The structure of the inserts introduced into the three-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 are summarised in Table 3.

Based on the known biological function (Table 3) of the newly expressed proteins, the only foreseen interactions at the biological level are between the two Cry proteins in susceptible insects.

Table 2: Genetic elements in the expression cassettes of the events stacked in cotton GHB614 × T304-40 × GHB119

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*)	Optimized *tp (Zea mays* and *Helianthus annuus)*	2m*epsps (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 (A. tumefaciens*)
GHB119	35S (*Cauliflower Mosaic virus*)	5′ *cab*22L (*Petunia hybrida*)	TpssuAt (*ats1A* gene of *A. thaliana*)	*cry2Ae (Bacillus thuringiensis subsp. dakota 1715)*	3′ 35S (*Cauliflower Mosaic virus*)
	Pcsvmv XYZ (*Cassava Vein Mosaic Virus*)	–	–	*bar (Streptomyces hygroscopicus strain ATCC21705)*	*3′ nos* (*A. tumefaciens*)

–: when no element was specifically introduced to optimise expression.

Table 3: Characteristics and intended effects of the events stacked in cotton GHB614 × T304-40 × GHB119

Event	Protein	Donor organism and 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 maize EPSPS enzyme was modified by two substitutions to render it tolerant to glyphosate. Expression of 2mEPSPS confers tolerance to glyphosate-containing herbicides

[7] Dossier: Part II –Section 1.2.1.2.

Event	Protein	Donor organism and function	Intended effects in GM plant
T304-40	Cry1Ab	Based on genes from *Bacillus thuringiensis,* subsp *berliner* 1715, Cry1Ab confers resistance to insect pests of the leptidopteran 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	Based on the *bar* gene from *Streptomyces hygroscopicus*, Phosphinothricin-acetyl-transferase (PAT) enzyme confers resistance to the antibiotic bialaphos (Eckes et al., 1989)	Cotton T304-40 expresses the PAT 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 leptidopteran 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	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 protein, which acetylates L-glufosinate-ammonium and thereby confers tolerance to glufosinate ammonium-based herbicides

3.4.1.2. Integrity of the events in the three-event stack[8,9]

The genetic stability of the inserted DNA over multiple generations in single cotton events GHB614, T304-40 and GHB119 was demonstrated previously (see Table 1). Integrity of these events in cotton GHB614 × T304-40 × GHB119 was demonstrated by Southern analyses. In addition, the sequence of the events (inserts and their flanking regions) was determined in the three-event stack cotton GHB614 × T304-40 × GHB119 and compared to the sequences originally reported for the three single events. The sequences of the events in the three-event stack and in the single events were found to be identical, thus confirming that the integrity of these events was maintained in the three-event stack cotton.

3.4.1.3. Information on the expression of the inserts[10]

2mEPSPS, PAT, Cry1Ab and Cry2Ae protein levels were analysed by enzyme-linked immunosorbent assay (ELISA), in material harvested in a field trial across three locations in the USA in 2013. Samples analysed included leaf (4–6 leaf, square initiation, 2 weeks after flowering), root (4–6 leaf), pollen (flowering), squares (2 weeks after flowering), bolls (2 weeks after flowering), whole plant (2 weeks after flowering) and fuzzy seed (maturity) both those treated and not treated with glyphosate- and/or glufosinate ammonium-containing herbicides. Since seeds are the main raw commodities used for food and feed purposes, protein levels in seeds from GHB614 × T304-40 × GHB119 (the highest mean values, regardless the treatment) are summarised in Table 4.

[8] Dossier: Part II–Section 1.2.2.2.a
[9] Dossier: Part II–Section 1.2.2.4
[10] Dossier: Part II–Section 1.2.3; additional information: 27/3/2017, 30/3/2017 and 28/7/2017.

Table 4: Highest mean values and corresponding standard deviation and ranges of protein levels (μg/g dry weight) in seed (n = 11 or n = 12) from cotton GHB614 × T304-40 × GHB119 either treated with glyphosate- and glufosinate ammonium-containing herbicides or not treated

Tissue/Developmental stage	2mEPSPS	PAT	Cry1Ab	Cry2Ae
Seed/maturity	160.35[(a)] ± 26.61[(b)] (132.13–227.84)[(c)]	219.46 ± 30.14 (179.53–265.46)	6.37 ± 1.80 (4.07–9.34)	29.15 ± 6.14 (19.10–39.14)
	(T)	(T)	(NT)	(NT)

(a): Mean.
(b): Standard deviation.
(c): Range.
(T): treated with intended herbicides; (NT): not treated with intended herbicides.

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

The levels of all the proteins newly expressed in the three-event cotton stack and the corresponding singles were similar in all tissues, except for PAT protein levels expected to be different because of the combination of events T304-40 and GHB119 both producing PAT protein in the three-event stack cotton (Appendix A). Therefore, there is no indication of an interaction that may affect the levels of the newly expressed proteins in this stack.

3.4.1.4. Conclusion of the molecular characterisation

The molecular data establish that the events stacked in cotton GHB614 × T304-40 × GHB119 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the three-event stack cotton and in the single events. PAT shows the expected higher level in the stack resulting from the combination of events T304-40 and GHB119. Therefore, there is no indication of an interaction that may affect the integrity of the events and 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 two Cry proteins, which will be dealt with in Sections 3.6 and 3.7.

3.5. Comparative analyses[11]

3.5.1. Choice of comparator and production of material for the comparative analysis

Application EFSA-GMO-NL-2014-122 presents data on agronomic and phenotypic characteristics as well as on cottonseed composition of the three-event stack cotton GHB614 × T304–40 × GHB119 derived from field trials performed in the US in 2012 (Table 5).

Table 5: Overview of comparative assessment studies with cotton GHB614 × T304–40 × GHB119

Study focus	Study details	Comparator	Non-GM reference varieties[(a)]
Agronomic and phenotypic characteristics and compositional analysis	Field trials, 2012, US, eight locations	Coker 312	Six[(b)]
Agronomic and phenotypic analysis	Seed germination study – controlled conditions, two temperature regimes	Coker 312	–

(a): Three non-GM reference varieties were grown at each location.
(b): The six non-GM commercial reference varieties that were included in the field trials are: FM958, FM989, ST457, DP491, ST468 and Acala Maxxa.

[11] Dossier: Part II–Section 1.3

The field trial study was conducted at eight sites representative of major cotton-growing areas of the US,[12] with diverse agronomic practices and environmental conditions. At each site, the following materials were grown in a randomised complete block design with four replicates: cotton GHB614 × T304-40 × GHB119, a comparator (Coker 312) and three non-GM reference varieties, all treated (sprayed) with plant protection products according to local requirements, and cotton GHB614 × T304–40 × GHB119 treated with the intended herbicides (glyphosate- and glufosinate ammonium-containing herbicides) in addition to the other plant protection products. A total of six[12] non-GM cotton reference varieties were included in the field trial study. The non-GM cotton line (Coker 312) used as comparator had a genetic background similar to that of the three-event stack cotton as documented by the pedigree[13] and was considered to be a suitable comparator by the GMO Panel.

The statistical analysis of the agronomic, phenotypic and compositional data followed the recommendations of the GMO Panel (EFSA GMO Panel, 2011a) and complied with Regulation (EU) 503/2013. This included, for each of the two treatments of cotton GHB614 × T304–40 × GHB119, the application of a difference test (between the GM cotton and the comparator) and an equivalence test (between the GM cotton and the set of non-GM reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[14]

3.5.2. Agronomic and phenotypic analysis

3.5.2.1. Agronomic and phenotypic characteristics tested under field conditions[15]

Twenty-seven agronomic and phenotypic endpoints were evaluated in the 2012 field trial study.[16] The endpoints were analysed applying the test of difference and equivalence with the exception of disease reaction, boll type and stalk lodged plants. The latter endpoints were analysed with the Cochran–Mantel–Haenszel test (CMH test).

- Statistically significant differences between the three-event stack cotton not treated with the intended herbicides and the comparator were identified for early stand count, total cottonseed yield, lint yield, percentage of lint, fibre micronaire, fibre length uniformity, fibre strength, fibre yellowness and total number of nodes.
- Statistically significant differences between the three-event stack cotton treated with the intended herbicides and its comparator were identified for early stand count, days to first open bolls, percentage of lint, fibre micronaire, fibre length, fibre reflectance, fibre yellowness, total number of nodes, total number of bolls per plant and first fruiting branch.

Except for the percentage of lint[17] that fell under category IV (GM cotton not treated with the intended herbicides) and category III (GM treated with the intended herbicide), all these endpoints fell under equivalence categories I and II. For the endpoints analysed with the CMH test, no significant differences were identified between the three-event stack cotton and its comparator, and the observed means fell within the range of the tested non-GM reference varieties.

3.5.2.2. Agronomic and phenotypic characteristics tested under controlled conditions

The applicant tested the germination potential of GHB614 × T304–40 × GHB119 cottonseed compared to its comparator Coker 312 under warm and cold temperature regimes.[18] The endpoints

[12] The sites for the field trials were in Chula (Georgia); West Memphis (Arkansas); Wall (Texas); Seven Springs (North Carolina); Tallassee (Alabama); Uvalde (Texas); Edmonson (Texas); and Levelland (Texas).
[13] Spontaneous information: 13/12/2016 and additional information: 14/2/2017.
[14] 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).
[15] Dossier: Part II – Section A3.4.
[16] Early season observations: days to emergence, early stand count, disease reaction. Mid-season observations: number of days to first flower, number of days to first open bolls, disease reaction, percentage of open bolls, boll type, stalk lodged plants, total seed cotton yield, lint yield. Boll Properties: number of seeds per boll, boll size, seed index, percentage of lint. Fibre properties: length, length uniformity, strength, micronaire, elongation, reflectance, yellowness. Plant Mapping: plant height, total number of nodes, first fruiting branch, total number of bolls per plant, height to node ratio.
[17] Mean values for % lint were as follows: comparator exposed to conventional herbicides: 38.16; GHB614 × T304-40 × GHB119 (exposed to the intended herbicides): 36.55; GHB614 × T304-40 × GHB119 (exposed to conventional herbicides): 36.18. Equivalence limits for % lint from non-GM reference varieties exposed to conventional herbicides: (37.15, 42.44).
[18] Warm germination test conditions: seeds were incubated at 30°C±5°C for 8 days without light. Cold germination test conditions: seeds were incubated at 10±5°C for 7 days and then transferred 30°C±5°C for 8 days without light. For both test conditions, the germination conditions were evaluated at 4 and 8 days after the start of incubation at 30°C±5°C.

analysed were the numbers of normal germinated seeds, abnormal germinated seeds and ungerminated seeds. For all endpoints, no statistically significant differences between GHB614 × T304–40 × GHB119 cottonseed and its comparator at the two tested temperatures were observed.

3.5.3. Compositional analysis

Together with the field trial study conducted in the US in 2012 described in Table 5, two additional studies on composition analysis were made available by the applicant.[19] The GMO Panel did not consider these two additional studies for the risk assessment since they did not comply with EFSA requirements as described in Regulation (EU) 503/2013.

Regarding the field trial study in the US in 2012 (Table 5), fuzzy cottonseed was analysed for 73 different constituents, including the key constituents recommended by the OECD (OECD, 2009). For 20 cottonseed components,[20] more than 50% of the observations were below the limit of quantification and were not analysed.

The statistical analysis was applied to the remaining 53 constituents.[21] The test of equivalence could not be applied to moisture in GHB614 × T304-40 × GHB119 cottonseed (not treated and treated) because of the lack of variation among the non-GM reference varieties. A summary of the outcome of the test of difference and the test of equivalence is presented in Table 6.

- For GHB614 × T304-40 × GHB119 cottonseed (not treated), all the 33 endpoints for which significant differences were found as compared to the comparator fell under equivalence category I or II in the equivalence test, except for total fat, dihydrosterculic acid and α-linolenic acid that fell under category III/IV.
- For GHB614 × T304-40 × GHB119 cottonseed (treated), all the 35 endpoints for which significant differences were found as compared to the comparator fell under equivalence category I or II in the equivalence test, except for total fat, dihydrosterculic acid and α-linolenic acid, that fell under category III/IV.
- For both treated and not treated GHB614 × T304-40 × GHB119 cottonseed, palmitic acid fell under equivalence category III, although no statistically significant differences were identified with the comparator.

Table 6: Outcome of the comparative compositional analysis in GHB614 × T304-40 × GHB119 cottonseed. The table shows the number of endpoints in each category

		Test of difference[(a)]			
		Not Treated[(c)]		Treated[(c)]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[(b)]	Category I/II	18	30[(d)]	16	32[(d)]
	Category III/IV	1[(e)]	3[(f)]	1[(e)]	3[(f)]
	Not categorised	1[(g)]	–	1[(g)]	–
	Total endpoints	53		53	

(a): Comparison between GHB614 × T304-40 × GHB119 cottonseed and its comparator.
(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.

[19] Additional studies M-398053-01-1 (carried out in Spain in 2009) and M-372931-01-1 (carried out in US in 2009).
[20] Sodium, β and δ tocopherol and the fatty acids caprylic (C8:0), capric (C10:0), lauric (C12:0), myristoleic (C14:1), pentadecanoic (C15:0), pentadecenoic (C15:1), γ-Linolenic (C18:3), octadecatetraenoic (C18:4), Eicosenoic (C20:1), eicosadienoic (C20:2), eicosatrienoic (C20:3), arachidonic (C20:4), eicosapentaenoic (C20:5), erucic (22:1), n3-docosapentaenoic (C22:5), n6-docosapentaenoic (C22:5) and docosahexaenoic (C22:6).
[21] Moisture, ash, fat, protein, magnesium, carbohydrate, ADF, NDF, zinc, potassium, calcium, phosphorus, manganese, iron, copper, alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, tryptophan, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, α tocopherol, γ tocopherol, total tocopherols, free gossypol, total gossypol, 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 (C18:3), arachidic acid (C20:0), behenic acid (C22:0), lignoceric acid (C24:0), malvalic acid, phytic acid, sterculic acid, dihydrosterculic acid.

(c): Treated/not treated with intended herbicide: glyphosate- and glufosinate ammonium-containing herbicides (see Section 3.5.1).
(d): Endpoints with significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator falling in equivalence category I-II. For both treated and not treated GM: protein, carbohydrates, NDF, calcium, γ tocopherol, total tocopherols, free gossypol, total gossypol, sterculic acid, alanine, arginine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2); for the not treated GM only: magnesium, aspartic acid; for the treated GM only: ash, potassium, phosphorus, manganese. The EFSA GMO Panel noted that the free gossypol content in raw cottonseeds of both cotton GHB614 × T304-40 × GHB119 and its comparator was higher in certain sites than the limits set in Directive 2002/32 EC (5,000 mg/kg as fed) on undesirable substances in feed materials.
(e): Palmitic acid (16:0) fell under equivalence category III, although no statistically significant differences were identified with respect to the comparator.
(f): Endpoints with significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator and falling in equivalence category III-IV. Estimated means are reported for these endpoints in Table 7.
(g): Endpoints not categorised for equivalence and with no significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator: moisture.

The GMO Panel assessed all significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator, taking into account potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. Mean estimates for the endpoints showing significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator, falling under category III/IV are given in Table 7.

Table 7: Quantitative results (estimated means and equivalence limits) for endpoints with significant differences between GHB614 × T304-40 × GHB119 cottonseed and its comparator, falling under category III and category IV in the test of equivalence (see **Table 6**)

Endpoint	GHB614 × T304-40 × GHB119 cottonseed		Non-GM comparator	Equivalence limits from non-GM reference varieties	
	Not treated	Treated[(a)]		Mean	Equivalence limits
Fat (% DM)	16.4*	16.3*	17.6	18.34	(16.51, 20.39)
α-Linolenic acid (% FA)	0.215*	0.210*	0.187	0.18	(0.156, 0.202)
Dihydrosterculic acid (% FA)	0.137*	0.139*	0.157	0.21	(0.170, 0.251)

(a): Treated with herbicide/s glyphosate- and glufosinate ammonium-containing herbicides as described in Section 3.5.1. For GHB614 × T304-40 × GHB119 cottonseed, significantly different values are marked with an asterisk, while the outcomes of the test of equivalence are differentiated by greyscale backgrounds: light grey (equivalence category III) and dark grey (equivalence category IV). dm = dry matter; FA = total fatty acids.

3.5.4. Conclusion of the comparative analysis

Given the magnitude of the observed differences, the nature of the endpoints and the outcome of the equivalence test, the GMO Panel considers that none of the agronomic and phenotypic differences between GM cotton GHB614 × T304-40 × GHB119 and the comparator needs further assessment for its potential environmental impact except the percentage of lint, which is further discussed in Section 3.7.1.

The GMO Panel assessed all the compositional changes identified in the three-event stack cotton with respect to its comparator and the non-GM reference varieties. Based on the outcome of both the difference and the equivalence test, the GMO Panel does not identify any need for further food/feed safety assessment except for total fat, dihydrosterculic acid and α-linolenic acid, which are further discussed in Sections 3.6.3 and 3.6.6.

3.6. Food and feed safety assessment

3.6.1. Effects of processing

Cotton GHB614 × T304-40 × GHB119 will undergo existing production processes used for conventional cotton. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing cotton GHB614 × T304-40 × GHB119 into food and feed

products is not expected to result in products being different from those of conventional non-GM cotton varieties.

3.6.2. Influence of temperature and pH on newly expressed proteins

Effects of temperature and pH on 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins have been previously evaluated by the GMO Panel (Table 1). Additional studies[22] addressing heat stability of these newly expressed proteins were provided by the applicant. The outcome of these studies is consistent with previous analogous studies assessed by the GMO Panel.

3.6.3. Toxicology

Testing of newly expressed proteins[23]

Four proteins (2mEPSPS, PAT, Cry1Ab and Cry2Ae) are newly expressed in cotton GHB614 × T304-40 × GHB119 (Section 3.4.1). The GMO Panel has previously assessed these proteins in the context of the single cotton events (Table 1), and no safety concerns were identified for humans and animals. The GMO Panel is not aware of any new information that would change these conclusions.

The potential for a functional interaction between the proteins newly expressed in cotton GHB614 × T304-40 × GHB119 has been assessed with regard to human and animal health. The 2mEPSPS and PAT proteins are enzymes that catalyse distinct biochemical reactions and act on unrelated substrates with high substrate specificity. The Cry1Ab and Cry2Ae proteins are delta endotoxins with high-specific insecticidal properties acting through cellular receptors found in target insect 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; Koch et al., 2015). On the basis of the known biological functions of the individual newly expressed proteins (Table 3), there is currently no expectation for possible interactions relevant for the food and feed safety of the three-event stack cotton.

In vitro protein degradation studies on 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins have been previously evaluated by the EFSA GMO Panel (Table 1). Additional studies[24] addressing *in vitro* protein degradation of these newly expressed proteins were provided by the applicant. No indications of safety concerns are identified by the GMO Panel. The outcome of these studies is consistent with previous analogous studies assessed by the GMO Panel.

The applicant provided two acute studies in mice with the PAT protein, which were evaluated by the GMO Panel.[25] An *Escherichia coli* -derived PAT protein was administered by gavage in two separate studies at the doses of ~ 1,800 mg/kg body weight (bw) to male and female C57BL/6J mice and ~ 1,880 mg/kg bw to female C57BL/6J mice. No adverse effects related to the PAT protein were observed in these studies.

The GMO Panel concludes that there are no safety concerns for human and animal health related to the newly expressed proteins 2mEPSPS, PAT, Cry1Ab and Cry2Ae in cotton GHB614 × T304-40 × GHB119.

Testing of new constituents other than proteins[24]

No new constituents other than newly expressed proteins have been identified in cotton GHB614 × T304-40 × GHB119. Therefore, no further food and feed safety assessment of components other than the newly expressed proteins is required.

Information on altered levels of food and feed constituents[25]

Fat, α-linolenic acid and dihydrosterculic acid were significantly different in cotton GHB614 × T304-40 × GHB119 when compared to its comparator and showed lack of equivalence with the set of non-GM reference varieties (see Section 3.5.3).

Changes observed in fat and in α-linolenic acid are considered not to pose a toxicological concern in cotton GHB614 × T304-40 × GHB119 considering their biological characteristics and functions and the magnitude of the observed changes.

[22] Dossier: Part II–Section 7 and additional information: 13/12/2016.
[23] Dossier: Part II – Section 1.4.1.
[24] Dossier: Part II – Section 1.4.2.
[25] Dossier: Part II – Section 1.4.3.

Dihydrosterculic acid belongs to the category of cyclopropenoid fatty acids (CPFA) present in cotton oil described as antinutrients (OECD, 2009). CPFA are considered to inhibit the desaturation of saturated fatty acids and have been associated with detrimental effects in animals (OECD, 2009). Since the level of dihydrosterculic acid was lower in cotton GHB614 × T304-40 × GHB119 as compared to its comparator, the GMO Panel considers that there are no toxicological concerns related to this change.

The GMO Panel concludes that there are no toxicological concerns as regards the changes in fat, α-linolenic acid and dihydrosterculic acid observed in cotton GHB614 × T304-40 × GHB119 as compared to its comparator and the non-GM reference varieties.

Testing of the whole genetically modified food and feed[26]

No indication of potential adverse effects relevant for food/feed safety were identified for cotton GHB614 × T304-40 × GHB119 regarding the stability of the inserts, the expression of the inserts and the potential synergistic or antagonistic effects resulting from the combination of the transformation events (see Sections 3.4.1, 3.5.3 and 3.6.3). Therefore, animal studies on food/feed derived from cotton GHB614 × T304-40 × GHB119 are not necessary (EFSA GMO Panel, 2011a). In accordance to Regulation (EU) No 503/2013, the applicant provided 90-day oral repeated-dose toxicity studies on whole food and feed from cottons GHB614, T304-40 and GHB119. The GMO Panel previously concluded that the 90-day studies on cotton GHB119 and T304-40 were not suitable for the assessment, due to the low number of experimental units used (cage) (EFSA GMO Panel, 2013, 2016). The 90-day study on cotton GHB614 had the same study design limitations. In the frame of the present application, the applicant performed complementary statistical analyses allowing the use of individual animals instead of cage as the experimental unit following the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011).

90-day feeding study in rats – GHB119 cotton

Wistar Rj:WI (IOPS HAN) rats (10/sex per group) were allocated to four groups and to different cages within each group (2 cages/sex per group, 5 animals/cage), using a computerised, stratified randomisation based on individual body weights within each sex. Groups were fed diets containing approximately 5% or 10%(w/w) cottonseed toasted meal from GHB119 cotton sprayed with glufosinate[27] (test diets); 10%(w/w) cottonseed toasted meal from the conventional counterpart (Coker 312, control diet). In addition; 10 rats/sex per group were given a diet containing 10%(w/w) cottonseed toasted meal from a commercial non-transgenic cotton (FM958, reference diet). The study provided was adapted from OECD TG 408 and complying with the principles of Good Laboratory Practice (GLP).

The GMO Panel previously concluded that this study was not suitable for the assessment, due to inappropriate statistical analysis and low number of experimental units (cage) (EFSA GMO Panel, 2016). Following the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011), the applicant performed complementary statistical analyses allowing the use of individual animals instead of cage as the experimental unit.[28] The analysis showed that there was overall no relevant cage effect, i.e. no relevant variation of the parameters between different cages. Based on those results, the GMO Panel accepts the statistical analysis of data based on individual animals.

Balanced diets were prepared according to the specifications for SAFE standard rodent maintenance diet. Comprehensive compositional analysis of the cottonseed meal from GHB119 cotton, from the conventional counterpart and from the reference variety was performed.[29] Test, control and reference diets were analysed for key nutrients, fibres, minerals, gross energy, cotton antinutrients, mycotoxins, heavy metals and pesticides. Event-specific polymerase chain reaction (PCR) analysis on the test diets confirmed their molecular identity.

The diets were prepared and analysed in a non-GLP facility; however, standardised procedures and quality measures were followed. Therefore, the GMO Panel considers that this is not a major deviation impacting the study.

Stability of the test and control materials was not tested; however, in accordance to product expiration standards declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. There are currently no practical analytical methods available

[26] Dossier: Part II – Section 1.4.4.
[27] Glufosinate was applied once during the crop growth stage 1st flowering, at a rate of 500 g a.i./ha. Additional information: 20/2/2018.
[28] Study: M-513407-01-1 and additional information 19/2/2018 and 15/5/2018.
[29] Dossier: Part II – Section 1.4.1.1.

to determine homogeneity and concentration of cottonseed toasted meal in the formulated diets. Diet preparation procedures guaranteed their homogeneity and the proper concentration of the test, control or reference substances in them.

Feed and water were provided ad libitum. Animals were checked twice daily for mortality and at least once daily for clinical signs. Detailed physical examinations were conducted on all animals pretreatment and then weekly during the dosing period. Individual body weights were recorded pretreatment, the first day of dosing, weekly during the dosing period and on the day prior to the scheduled necropsy. Feed consumption (per cage) was determined weekly during the study (twice weekly the first 6 weeks). Ophthalmoscopy and functional observation battery (FOB) and motor activity parameters were recorded on all animals pretreatment and at the end of the study. Clinical pathology (i.e. haematology, clinical chemistry and coagulation), urine analyses and necropsy examination with selected organs weighing were conducted at the end of the treatment period on all animals. The animals were fasted overnight prior to blood collection and while in metabolism cages for urine collection. Organs and tissues from 10% test diet, control and reference diet as well as gross lesions from all groups were subjected to a detailed histopathological examination. Upon completion of the histopathologic assessment of all tissues, histopathology was reviewed by a peer review pathologist.

The main statistical analysis compared rats consuming each of the two test diets (5% and 10%) with those consuming the control diet. The comparison was done using two analysis of variance (ANOVA) models: combined-gender ANOVA[30] and gender-specific ANOVA. Additional comparisons were performed between the two test groups and the reference group (both combined-gender and gender-specific ANOVA), and between the control group and the reference group (only gender-specific ANOVA).[31]

The few statistically significant differences between the control and reference diet groups in the examined parameters are considered by the GMO Panel to be within normal biological variability.

No mortalities were observed during the study. No test diet-related clinical signs and ophthalmoscopic findings were seen.

Feed consumption was similar across groups.

A statistically significant increase in mean body weight gain/day observed in females given 10% test diet (on day 57 only) as compared to controls is not considered to be test diet-related.

A statistically significantly lower hind limb grip strength was observed in the neuromuscular observations in test diet-fed female rats (15% and 17% decrease in 5% and 10% test diet groups, respectively, as compared to controls). This was the only change among the parameters examined in the FOB and therefore, not considered to be test diet-related.

An increased mean exploratory locomotor activity (at one of six repeated measurements) in male rats given the 5% test diet as compared to controls was considered as an incidental finding.

No statistically significant haematological changes were noted in rats given the test diets as compared to controls.

Statistically significant increased prothrombin time was noted in females given the 10% test diet as compared to controls (~ 9%). Due to the small magnitude of the change and since this was not associated with changes in related endpoints (e.g. no difference in the platelet count across groups given the test diets and the controls or in the spleen and bone marrow histopathology), the GMO Panel considers that this change is not adverse.

No statistically significant differences in clinical chemistry parameters were noted between rats given the test diets and their controls, except for a decreased total bilirubin in males given the 10% test diet (~ 29%). This finding was not associated with changes in related endpoints (e.g. other liver clinical chemistry parameters and liver histopathology) and therefore, not considered as being adverse.

No statistically significant differences in urinalysis parameters were observed in animals given the test diets, as compared to controls.

Males given the 5% test diet showed statistically significant increased thymus weight (~ 30% for both absolute and relative to body weight and to brain weight) as compared to controls. Since this finding was not dose-related and no test diet-related changes were seen for thymus weight and in

[30] For the combined-gender analysis, following on a preliminary check for homogeneity of variance (Bartlett test), the applicant performed either a parametric ANOVA or a non-parametric (aligned rank transform) ANOVA, followed by a test of sex-by-dose interaction. If the interaction was not significant, differences between groups were investigated using ANOVA and either a parametric (Dunnet's) or non-parametric (Dunn's) test. If the interaction was significant, the combined-gender analysis was considered not appropriate and no further testing was done. The ANOVA models included sex and dose as main effects and (only for interaction testing) a sex-by-dose interaction term.

[31] Study: M-432216-01-1.

macroscopic and microscopic examination of the thymus from rats given the 10% test diet, the GMO Panel does not consider this finding to be toxicologically relevant.

Rats given the 5% test diet showed a statistically significant increase (in the combined analysis) in thyroid weight (~ 19% for both absolute and relative to body weight and to brain weight) as compared to controls. In the gender separated analysis, the thyroid weight (~ 24% relative to brain weight only) in the 5% test diet-fed males was significantly increased as compared to controls. Since this finding was not dose-related and no test diet-related changes were seen for thyroid weight and in macroscopic and microscopic examination of the thyroid from rats given the 10% test diet, the GMO Panel does not consider this finding to be toxicologically relevant.

A statistically significant decreased spleen weight (~ 8%, for both absolute and relative to body weight) was noted in rats given the 10% test diet (in the combined analysis) as compared to controls; since the magnitude of the change was small and not associated with macroscopic or microscopic findings in the spleen, this finding is not considered to be adverse.

No test diet-related gross lesions or microscopic findings were noted in the examined organs or tissues. Sporadic histopathological findings are considered compatible with the spontaneous background pathology of rats of this strain and age.

The GMO Panel concludes that no cotton GHB119-related adverse effects were observed in this study after a 90-day administration to rats of a diet formulated with 5% and 10% cottonseed toasted meal.

90-day feeding study in rats – GHB614 cotton

Wistar Rj:WI (IOPS HAN) rats (10/sex per group) were allocated to four groups and to different cages within each group (2 cages/sex per group, 5 animals/cage), using a computerised, stratified randomisation based on individual body weights within a sex. Groups were fed diets containing approximately 5% or 10%(w/w) cottonseed toasted meal from GHB614 cotton sprayed with the glyphosate[32] (test diets); 10%(w/w) cottonseed toasted meal from the comparator Fibermax 958 (control diet). In addition; 10 rats/sex per group were given a diet containing 10%(w/w) cottonseed toasted meal from a non-transgenic cotton (Acala GTO Maxxa, reference diet). The study provided was adapted from OECD TG 408 and complying with the principles of GLP.

Following the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011), the applicant performed complementary statistical analyses[33] allowing the use of individual animals instead of cage as the experimental unit. The analysis showed that there was overall no relevant cage effect, i.e. no relevant variation of the parameters between different cages. Based on those results, the GMO Panel accepts the statistical analysis of data based on individual animals.

Balanced diets were prepared according to the specifications for SAFE standard rodent maintenance diet. Comprehensive compositional analysis of the cottonseed meal from GHB614 cotton, from the comparator and from the reference variety was performed.[34] Test, control and reference diets were analysed for key nutrients, fibres, minerals, gross energy, cotton antinutrients, mycotoxins, heavy metals and pesticides. Event-specific PCR analysis on the test diets confirmed their molecular identity.

The diets were prepared and analysed in a non-GLP facility; however, standardised procedures and quality measures were followed. Therefore, the GMO Panel considers this not a major deviation impacting the study.

Stability of the test and control materials was not tested; however, in accordance to product expiration standards declared by the diet manufacturer, the constituents of the diets are considered stable for duration of the treatment. There are currently no practical analytical methods available to determine homogeneity and concentration of cottonseed toasted meal in the formulated diets. Diet preparation procedures guaranteed their homogeneity and the proper concentration of the test, control or reference substances in them.

Feed and water were provided ad libitum. Animals were checked twice daily for mortality and at least once daily for clinical signs. Detailed physical examinations were conducted on all animals pretreatment and then weekly during the dosing period. Individual body weights were recorded pretreatment, the first day of dosing, weekly during the dosing period and on the day prior to the scheduled necropsy. Feed consumption (per cage) was determined weekly during the study (twice weekly the first 6 weeks). Ophthalmoscopy and FOB and motor activity parameters were recorded on

[32] Glyphosate was applied once during the crop growth stage 4 leaves, at a rate of 1260 g a.i./ha. Dossier: Part II – 1.4.1.1 and additional information: 20/2/2018.
[33] Study: M-513410-01-1 and additional information 19/2/2018 and 15/5/2018.
[34] Technical dossier Part II – 1.4.1.1.

all animals pretreatment and at the end of the study. Clinical pathology (i.e. haematology, clinical chemistry and coagulation), urinalyses and necropsy examination with selected organs weighing were conducted at the end of the treatment period on all animals. The animals were fasted overnight prior to blood collection and while in metabolism cages for urine collection. Organs and tissues from 10% test diet, control and reference diet as well as gross lesions from all groups were subjected to a detailed histopathological examination. Upon completion of the histopathologic assessment of all tissues, histopathology was reviewed by a peer review pathologist.

The main statistical analysis compared rats consuming each of the two test diets (5% and 10%) with those consuming the control diet. Additional comparisons were performed between the two test groups and the reference group and between the control group and the reference group.[35] All comparisons were done with an ANOVA applied separately to males and females.

The few statistically significant differences between the control and reference diet groups in the examined parameters are considered by the GMO Panel to be within normal biological variability.

No treatment-related mortalities occurred. One control male was killed for humane reasons; this was attributed to an accidental trauma. No test diet-related clinical signs and ophthalmoscopic findings were seen.

Feed consumption was similar across groups.

No statistically significant differences in body weight parameters were noted in rats given the test diets as compared to controls.

No differences in FOB and exploratory locomotor activity parameters were seen between rats given the test diets and controls.

No test diet-related haematological and coagulation changes were seen.

Females given 10% test diet showed statistically significant increased mean alkaline phosphatase activity (~ 29%) and increased mean inorganic phosphorus concentration (~ 11%) as compared to controls. These findings were not associated with changes in related endpoints (e.g. other clinical chemistry parameters and histopathological findings indicating liver or kidney toxicity) and therefore not considered to be adverse.

No test diet-related changes in the urinalysis were seen.

A statistically significant increased heart weight (~ 11%, relative to brain weight only) was noted in females given the 10% test diet as compared to controls; this finding is not considered to be adverse since the magnitude of the change was small, it was not accompanied by statistical significant difference in both the heart absolute and relative to body weight, and it was not associated with macroscopic or microscopic findings in the heart.

No test diet-related gross lesions or microscopic findings were noted in the examined organs or tissues. Sporadic histopathological findings are considered compatible with the spontaneous background pathology of rats of this strain and age.

The GMO Panel concludes that no cotton GHB614-related adverse effects were observed in this study after a 90-day administration to rats of a diet formulated with 5% and 10% cottonseed toasted meal.

90-day feeding study in rats – T304-40 cotton

Wistar Rj:WI (IOPS HAN) rats (10/sex per group) were allocated to four groups, and to different cages within each group (2 cages/sex per group, 5 animals/cage), using a computerised, stratified randomisation based on individual body weights within a sex. Groups were fed diets containing approximately 5% or 10%(w/w) cottonseed toasted meal from T304-40 cotton (test diets); 10%(w/w) cottonseed toasted meal from the conventional counterpart (Coker 315, control diet). In addition; 10 rats/sex per group were given a diet containing 10%(w/w) cottonseed toasted meal from a commercial non-transgenic cotton (FM958, reference diet). The study provided was adapted from OECD TG 408 and complying with the principles of GLP.

The GMO Panel previously concluded that this study was not suitable for the assessment, due to inappropriate statistical analysis and low number of experimental units (cage) (EFSA GMO Panel, 2013). Following the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011), the applicant performed complementary statistical analyses allowing the use of individual animals instead of cage as the experimental unit.[36] The analysis showed that there was overall no relevant cage effect, i.e. no relevant variation of the parameters between different cages. Based on those results, the GMO Panel accepts the statistical analysis of data based on individual animals.

[35] Study: M-430107-01-1.
[36] Study: M-513420-01-1 and additional information 19/2/2018 and 15/5/2018.

Balanced diets were prepared according to the specifications for SAFE standard rodent maintenance diet. Comprehensive compositional analysis of the cottonseed meal from T304-40 cotton, from the conventional counterpart and from the reference variety was performed.[37] Test, control and reference diets were analysed for key nutrients, fibres, minerals, gross energy, cotton antinutrients, mycotoxins, heavy metals and pesticides. Event-specific PCR analysis on the test diets confirmed their molecular identity.

The diets were prepared and analysed in a non-GLP facility; however, standardised procedures and quality measures were followed. Therefore, the GMO Panel considers this not a major deviation impacting the study.

Stability of the test and control materials was not tested; however, in accordance to product expiration standards declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. There are currently no practical analytical methods available to determine homogeneity, and concentration of cottonseed toasted meal in the formulated diets. Diet preparation procedures guaranteed their homogeneity and the proper concentration of the test, control or reference substances in them.

Feed and water were provided ad libitum. Animals were checked twice daily for mortality and at least once daily for clinical signs. Detailed physical examinations were conducted on all animals pretreatment and then weekly during the dosing period. Individual body weights were recorded pretreatment, the first day of dosing, weekly during the dosing period and on the day prior to the scheduled necropsy. Feed consumption (per cage) was determined weekly during the study (twice weekly the first 6 weeks). Ophthalmoscopy and FOB and motor activity parameters were recorded on all animals pretreatment and at the end of the study. Clinical pathology (i.e. haematology, clinical chemistry and coagulation), urinalyses and necropsy examination with selected organs weighing were conducted at the end of the treatment period on all animals. The animals were fasted overnight prior to blood collection and while in metabolism cages for urine collection. Organs and tissues from 10% test diet, control and reference diet as well as gross lesions from all groups were subjected to a detailed histopathological examination. Upon completion of the histopathologic assessment of all tissues, histopathology was reviewed by a peer review pathologist.

The main statistical analysis compared rats consuming each of the two test diets (5% and 10%) with those consuming the control diet. An additional comparison was performed between the control group and the reference group. All comparisons were done with an ANOVA applied separately to males and females.

The few statistically significant differences between the control and reference diet groups in the examined parameters are considered by the GMO Panel to be within normal biological variability.

No mortalities were observed during the study. No treatment-related clinical signs and ophthalmoscopic findings were observed.

Feed consumption was similar across groups.

No statistically significant differences in body weight parameters were noted in rats given the test diets as compared to controls.

A statistically significantly higher mean forelimb grip strength in females given the 10% test diet and increased mean landing foot splay value in females given the 5% test diet were noted as compared to controls. These were the only changes among the parameters examined in the FOB and therefore, not considered to be test diet-related. No test diet-related changes in exploratory locomotor activity parameters were seen.

No test diet-related haematological, coagulation, clinical chemistry and urinalysis findings were seen.

An increased spleen weight (~ 19%, relative to body weight) was seen in females given the 5% test diet as compared to controls. Since this finding was not dose-related and no test diet-related changes were noted for spleen weight and in macroscopic and microscopic examination of the spleen from rats given the 10% test diet, the GMO Panel does not consider this finding to be toxicologically relevant.

No test diet-related gross lesions or microscopic findings were noted in the examined organs or tissues. Sporadic histopathological findings are considered compatible with the spontaneous background pathology of rats of this strain and age.

The GMO Panel concludes that no cotton T304-40-related adverse effects were observed in this study after a 90-day administration to rats of a diet formulated with 5% and 10% cottonseed toasted meal.

[37] Dossier: Part II – 1.4.1.1

3.6.4. Allergenicity

For the allergenicity assessment, a weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed proteins, as no single piece of information or experimental method yields sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a; Regulation (EU) 503/2013). In addition, when known functional aspects of the newly expressed protein or structural similarity to known adjuvants may indicate an adjuvant activity, the possible role of these proteins as adjuvants is considered. When newly expressed proteins with a potential adjuvant activity are expressed together, possible interactions increasing adjuvanticity and impacting the allergenicity of the GM crop are assessed.

Assessment of allergenicity of newly expressed proteins[38]

For allergenicity, the GMO Panel has previously evaluated the safety of the proteins 2mEPSPS, PAT, Cry1Ab and Cry2Ae individually, and no concerns on allergenicity were identified in the context of the applications assessed (Table 1). No new information on allergenicity of these proteins that might change the previous conclusions of the GMO Panel in the context of the GM events assessed has become available. Based on the current knowledge, and as none of the newly expressed proteins showed allergenicity, no reasons for concerns regarding the simultaneous presence of these newly expressed proteins in this three-event stack cotton affecting their allergenicity were identified.

For adjuvanticity, proteins derived from *Bacillus thuringiensis* (Bt proteins) have been suggested to possess adjuvant activity based on animal studies on Cry1Ac when applied at relatively high doses (e.g. Vazquez et al., 1999). The GMO Panel has previously evaluated the safety of the Cry1Ab and Cry2Ae proteins and no concerns on adjuvanticity in the context of the applications assessed were identified (Table 1). The levels of Bt proteins in this three-event stack cotton are similar to those in the respective single cotton events (Appendix A). From the limited experimental evidence available, the GMO Panel does not find indications that the presence of the Bt proteins at the levels expressed in this three-event stack cotton might act as adjuvants with the potential to enhance a specific immunoglobulin E (IgE) response and to favour the development of an allergic reaction.

Assessment of allergenicity of the whole GM plant[39]

The GMO Panel regularly reviews the available publications on food allergy to cottonseed-derived products. However, to date, cotton has not been considered to be a common allergenic food[40] (OECD, 2009). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM cotton.

Conclusion

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, 3.5.3 and 3.6.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from this three-event stack cotton with respect to that derived from the comparator.

3.6.5. Dietary exposure assessment to endogenous and new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure to 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins present in cotton GHB614 × T304-40 × GHB119.

3.6.5.1. Human dietary exposure

The applicant estimated the dietary exposure to 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins as negligible based on the fact that all protein compounds from cottonseed are removed or destroyed during the production of refined cottonseed oil of food grade quality, and that no consumption data of cottonseed and derived products is available in the European Comprehensive Food Consumption Database.

[38] Dossier: Part II – 1.5.1 and 1.5.3, 7 and additional information: 13/12/2016.
[39] Dossier: Part II – 1.5.2.
[40] 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.

The GMO Panel identified different food products for human consumption such as flour and oil that can be derived from cottonseed, with the latter being the most important. Availability of consumption data on this type of products was searched in the EFSA Comprehensive European Food Consumption Database; this database contains information on food consumption data at individual level from the most recent national dietary surveys in different EU Member States (EFSA, 2011).[41] The GMO Panel confirms that no data on consumption of cottonseed oil as such or cottonseed flour are available in the EFSA consumption database. However, cottonseed oil is consumed as this oil is used as an ingredient in the production of a wide variety of food products such as dressings, mayonnaise, fine bakery wares, chocolate spreads and chips.[42] Considering that refined bleached deodorised cottonseed oil is free from proteins, no dietary exposure to 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins is expected from the consumption of oil derived from cotton GHB614 × T304-40 × GHB119. On the other hand, dietary exposure to these proteins cannot be excluded through the consumption of cottonseed flour. However, the presence of these products is currently minor in the European market, and therefore, the GMO Panel concludes that, under the current situation, the dietary exposure to 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins is negligible.

3.6.5.2. Animal dietary exposure

Daily dietary exposure (DDE) to the 2mEPSPS, PAT, Cry1Ab, and Cry2Ae proteins newly expressed in cotton GHB614 × T304-40 × GHB119 was provided by the applicant across different livestock animal species (poultry, swine, cattle and sheep)[43] based on EU estimates issued by OECD (OECD, 2013) for animal body weight, daily feed intake and the inclusion rates (percentage) of undelinted fuzzy cottonseed (i.e. dairy cows only) and cottonseed meal in animal diets. A conservative scenario with 100% replacement of the conventional cotton (e.g. undelinted fuzzy cottonseed and cottonseed meal) was considered. The mean levels of 2mEPSPS, PAT, Cry1Ab and Cry2Ae proteins in undelinted fuzzy cottonseeds were used by the applicant as occurrence data for both cottonseed and meal.[44]

Estimated DDEs to the 2mEPSPS, based on mean levels in GM cotton undelinted seed was 617.34 μg/kg bw per day in dairy cows. Estimated DDEs to the 2mEPSPS protein based on mean levels in GM cotton meal ranged from 192.42 μg/kg bw per day in beef to 1144.89 μg/kg bw per day in turkey.[45]

Estimated DDEs to the PAT, based on mean levels in GM cotton undelinted seed was 844.92 μg/kg bw per day in dairy cows. Estimated DDEs to the PAT protein based on mean levels in GM cotton meal ranged from 263.35 μg/kg bw per day in beef to 1566.94 μg/kg bw per day in turkey.[46]

Estimated DDEs to the Cry1Ab, based on mean levels in GM cotton undelinted seed was 24.52 μg/kg bw per day in dairy cows. Estimated DDEs to the Cry1Ab protein based on mean levels in GM cotton meal ranged from 7.64 μg/kg bw per day in beef to 45.48 μg/kg bw per day in turkey.[47]

Estimated DDEs to the Cry2Ae, based on mean levels in GM cotton undelinted seed was 112.22 μg/kg bw per day in dairy cows. Estimated DDEs to the Cry2Ae protein based on mean levels in GM cotton meal ranged from 34.98 μg/kg bw per day in beef to 208.13 μg/kg bw per day in turkey.[48]

The GMO Panel considers that after extraction of the oil, crude protein in cottonseed meals/cakes increases by a factor of 1.6–2, based on the protein content of cotton meal relative to cottonseed (OECD, 2009), assuming that no protein is lost during the processing. Therefore, the above-reported values for the newly expressed proteins present in meal should be adjusted accordingly.

3.6.6. Nutritional assessment of GM food/feed

The intended trait of cottonseed GHB614 × T304-40 × GHB119 is insecticide resistance and herbicide tolerant, with no intention to alter the nutritional parameters. However, total fat, α-linolenic

[41] https://www.efsa.europa.eu/en/applications/gmo/tools

[42] The Mintel's Global New Products Database (GNPD) is an online database which monitors product introduction in consumer packaged goods markets worldwide (http://www.mintel.com/global-new-products-database).

[43] Poultry (broiler, layer and turkey); swine (breading and finishing); cattle (beef and dairy cows); sheep (ram/ewe and lamb).

[44] Following the submission of additional information upon EFSA request, the GMO Panel referred to mean level data (Appendix A).

[45] Estimated DDEs: 192.42, 307.87, 801.75, 681.48, 370.40, 240.52, 566.03, 548.39, 1144.89 μg/kg per bw, respectively, in beef and dairy cows, ram/ewe and lamb, breeding and finishing swine, broiler chicken, layer hen and turkey.

[46] Estimated DDEs: 263.35, 421.36, 1097.3, 932.7, 506.95, 329.19, 774.69, 750.55, 1566.94, μg/kg per bw, respectively, in beef and dairy cows, ram/ewe and lamb, breeding and finishing swine, broiler chicken, layer hen and turkey.

[47] Estimated DDEs: 7.64, 12.23, 31.85, 27.07, 14.71, 9.55, 22.48, 21.78, 45.48 μg/kg per bw, respectively, in beef and dairy cows, ram/ewe and lamb, breeding and finishing swine, broiler chicken, layer hen and turkey.

[48] Estimated DDEs: 34.98, 55.96, 145.75, 123.88, 67.33, 43.72, 102.89, 99.69, 208.13 μg/kg per bw, respectively, in beef and dairy cows, ram/ewe and lamb, breeding and finishing swine, broiler chicken, layer hen and turkey.

acid and dihydrosterculic acid in both treated and not treated GHB614 × T304-40 × GHB116 cottonseeds were significantly different from its comparator and showed a lack of equivalence with the set of non-GM reference varieties (Section 3.5). The biological role of these compounds, their levels in cottonseed and the magnitude and direction of the observed changes were considered during the nutritional assessment.

3.6.6.1. Human nutrition[49]

Oil is the most important cottonseed-derived product used for human consumption. In Europe, the consumption of cottonseed oil as such seems to be minor as reflected by the lack of consumption data reported in the EFSA Comprehensive Food Consumption Database. However, cottonseed oil is used as an ingredient in the production of a wide variety of food products such as dressings, mayonnaise, fine bakery wares, chocolate spreads and chips. Cottonseed oil is rich in linoleic acid (about 50% of total FA), poor in α-linolenic acid (~ 0.2% of total FA) and contains approximately 25% saturated fatty acids, among which palmitic acid is the most prominent (about 20% of total FA) (Dowd et al., 2010). Because it has no flavour, it is suitable for cooking.

The 12–15% increase in content of α-linolenic acid (% of total FA) in GHB614 × T304-40 × GHB119 cottonseed as compared to the comparator will not substantially change the daily intake of α-linolenic acid when GHB614 × T304-40 × GHB116 cottonseed oil replaces conventional cottonseed oil.

The dihydrosterculic acid content in GHB614 × T304-40 × GHB119 cottonseed was lower than that in the comparator (11–13% of total FA) and the six non-GM reference varieties (19–45%). Dihydrosterculic acid (cyclopropaneoctanoic acid, 8-(2-octylcyclopropyl)octanoic acid) is one of a group of cyclopropane fatty acids (CPA) that together with cyclopropene fatty acids (CPE) occur infrequently in most plants. Dihydrosterculic acid makes up 0.2–0.4% of total fatty acids in cottonseed (Xiao-Hong et al., 2011). CPAs are derived from unsaturated fatty acids, e.g. oleic acid, by cyclopropanation. No information is available about effects in humans. CPAs will be destroyed during refining and hydrogenation of vegetable oils; therefore, changes in levels of dihydrosterculic acid in cottonseed should not raise health concerns.

3.6.6.2. Animal nutrition[50]

Cotton can be fed to animals, mainly as cottonseed cake/meal or full fat seeds, especially in ruminants. In complete feeds, its use is limited to 5–10% because of the content of antinutritional factors.

A decrease in total fat content does not affect animal nutrition since complete diets are balanced to energy content.

Cyclopropane fatty acids (CPA) are inhibitors of several fatty acid desaturases in animals, and this implies an increase of saturated fatty acids in animal fat (Phelps et al., 1965). Page et al. (1997) observed that feeding whole cottonseeds containing CPA, affect lipogenesis, but does not influence the activity of stearoyl coenzyme desaturase in liver and adipose tissue. Therefore, a reduction in dihydrosterculic acid content in GHB614 × T304-40 × GHB119 cottonseed is not expected to affect animal health.

Linolenic acids are commonly present in most commercially edible plant oils fed to animals; α-linolenic acid cannot be synthetised in mammals and is commonly supplied to animals in diets as an essential fatty acid (Mc Donalds, 2011). The GMO Panel considers the increase in content of α-linolenic acid in GHB614 × T304-40 × GHB119 of no safety concern, since animal complete feeds are regularly balanced for linolenic acid.

3.6.7. Conclusion of the food and feed safety assessment

The proteins 2mEPSPS, PAT, Cry1Ab and Cry2Ae newly expressed in cotton GHB614 × T304-40 × GHB119 do not raise safety concerns for human and animal health in light of the scope of this application. No interactions between these newly expressed proteins relevant for food and feed safety were identified. Similarly, the GMO Panel does not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in cotton GHB614 × T304-40 × GHB119, or regarding the overall allergenicity of the three-event stack cotton. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the nutritional impact of cotton GHB614 × T304-40 × GHB119-derived food and

[49] Dossier: Part II – Section 1.6.1.
[50] Dossier: Part II – Section 1.6.2

feed is expected to be the same as those derived from the comparator and non-GM reference varieties. The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is nutritionally equivalent to and as safe as the comparator and the non-GM reference varieties tested.

3.7. Environmental risk assessment[51]

Considering the scope of application EFSA-GMO-NL-2014-122, which excludes cultivation, the ERA of cotton GHB614 × T304-40 × GHB119 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable GHB614 × T304-40 × GHB119 cottonseeds during transportation and processing (EFSA GMO Panel, 2010).

3.7.1. Persistence and invasiveness of the GM plant[52]

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

It is unlikely that the intended traits of cotton GHB614 × T304–40 × GHB119 will provide a selective advantage to cotton plants, except when they are exposed to glyphosate- and/or glufosinate ammonium-containing herbicides or infested by insect pests that are susceptible to the Cry1Ab and/or Cry2Ae proteins.

The GMO Panel considers that the fitness advantage provided by the intended traits, and the observed difference in percentage of lint (see Section 3.5.2.1) 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 and other observed differences will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers it very unlikely that cotton GHB614 × T304–40 × GHB119 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 GHB614 × T304-40 × GHB119 cottonseeds.

3.7.2. Potential for gene transfer[53]

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

Plant-to-microorganism gene transfer

The probability and potential adverse effects of HGT of the recombinant DNA have been assessed previously for the single events (see Table 1). No concern as a result of an unlikely, but theoretically possible, HGT of the recombinant genes to bacteria in the gut of domesticated animal and humans fed GM material or other receiving environments was identified. The applicant submitted an updated bioinformatic analysis for each of the single events in order to assess possibility for HGT by HR.

[51] Dossier: Part II – Section 5.
[52] Dossier: Part II – Section 5.3.1.
[53] Dossier: Part II – Section 5.3.1 and 5.3.2.

Bioinformatic analysis[54] for event GHB614 revealed no sequence identity with bacterial DNA; thus, there is no indication of facilitated gene transfer of recombinant DNA of event GHB614 to bacteria.

The bioinformatic analysis[54] for events T304-40 and GHB119 confirmed previous assessments, in which for both events, two elements with sufficient sequence identity and length with bacterial DNA were observed. These were the *bar*-coding sequence from *Streptomyces hygroscopicus* and the 3' nos terminator from the *Agrobacterium tumefaciens* Ti plasmid. Considering that these sequences occur in two relatively unrelated bacterial genomes, there is no indication for a potential double HR.

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 three-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 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 cottonseeds 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 cottonseeds to establish, grow and produce pollen is extremely low and transient (see Section 3.7.1). The likelihood/frequency of cross-pollination between occasional feral GM cotton plants resulting from seed spillage and weedy or cultivated *Gossypium* plants is therefore also considered extremely low. Even if cross-pollination would occur, the GMO Panel is of the opinion that the likelihood of environmental effects as a consequence of the spread of genes from occasional feral GM cotton plants in Europe will not differ from that of conventional cotton varieties for the reasons given in Section 3.7.1.

3.7.3. Interactions of the GM plant with target organisms[55]

Taking the scope of application EFSA-GMO-NL-2014-122 (no cultivation) and thus the absence of target organisms into account, potential interactions of occasional feral cotton GHB614 × T304–40 × GHB119 plants arising from seed import spills with the target organisms are not considered a relevant issue by the GMO Panel.

3.7.4. Interactions of the GM plant with non-target organisms[56]

Given that environmental exposure of non-target organisms to spilled GM seeds or occasional feral GM cotton plants arising from spilled GHB614 × T304-40 × GHB119 cottonseeds is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM cottonseed, potential interactions of the cotton GHB614 × T304–40 × GHB119 with non-target organisms are not considered by the GMO Panel to raise any environmental safety concern. Interactions that may occur between the Cry proteins (as mentioned in Section 3.4.1.1) will not alter this conclusion.

3.7.5. Interactions with abiotic environment and biogeochemical cycles[57]

Given that environmental exposure to spilled seeds or occasional feral cotton GHB614 × T304–40 × GHB119 plants arising from seed import spills is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM cotton, potential

[54] Additional information: 2/2/2018.
[55] Dossier: Part II – Section 5.3.3.
[56] Dossier: Part II – Section 5.3.4.
[57] Dossier: Part II – Section 5.3.6.

interactions with the abiotic environment and biogeochemical cycles are not considered by the GMO Panel to raise any environmental safety concern.

3.7.6. Conclusion of the environmental risk assessment

The GMO Panel concludes that it is unlikely that cotton GHB614 × T304-40 × GHB119 would differ from conventional cotton varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-NL-2014-122, interactions of occasional feral cotton GHB614 × T304-40 × GHB119 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from the three-event stack cotton to bacteria does not indicate a safety concern. Therefore, considering the combined traits and their interactions, the outcome of the comparative analysis, the routes and levels of exposure, the GMO Panel concludes that cotton GHB614 × T304-40 × GHB119 would not raise safety concerns in the event of accidental release of viable GM cottonseeds into the environment.

3.8. Post-market monitoring

3.8.1. Post-market monitoring of GM food/feed

The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is nutritionally equivalent to and as safe as the comparator and the non-GM reference varieties tested, and no post-market monitoring (EFSA GMO Panel, 2011a) of food/feed is considered necessary.

3.8.2. Post-market environmental monitoring[58]

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

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

As the ERA did not identify potential adverse environmental effects from cotton GHB614 × T304-40 × GHB119, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for cotton GHB614 × T304-40 × GHB119 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 EuropaBio 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 and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of cotton GHB614 × T304-40 × GHB119. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

3.8.3. Conclusion on post-market monitoring

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of cotton GHB614 × T304-40 × GHB119.

3.9. Overall conclusions

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

No new data on the single cotton events GHB614, T304-40 and GHB119 that would lead to a modification of the original conclusions on their safety were identified.

[58] Dossier: Part II – Section 6.

The molecular data establish that the events stacked in cotton GHB614 × T304-40 × GHB119 have retained their integrity. Protein expression analysis shows that the levels of the newly expressed proteins are similar in the three-event stack cotton and in the single events. PAT shows the expected higher level in the stack resulting from the combination of the single cotton events T304-40 and GHB119. Therefore, there is no indication of an interaction that may affect the integrity of the events and the levels of the newly expressed proteins in this stack.

Given the magnitude of the differences observed in the comparative assessment, the nature of the endpoints and the outcome of the equivalence test, the GMO Panel considers that none of the agronomic and phenotypic differences between cotton GHB614 × T304-40 × GHB119 and the comparator needs further assessment for its potential environmental impact except the percentage of lint. This difference was further assessed and found not to affect the ability of cotton GHB614 × T304-40 × GHB119 to survive until subsequent seasons or to establish occasional feral plants under European environmental conditions. The GMO Panel does not identify the need for further food/feed safety assessment on the composition characteristics except for total fat, dihydrosterculic acid and α-linolenic acid. These differences were assessed and do not raise concerns with regard either food and feed safety or nutritional relevance.

The proteins 2mEPSPS, PAT, Cry1Ab and Cry2Ae newly expressed in the three-event stack cotton do not raise concerns for human and animal health and no interactions between these proteins relevant for food and feed safety were identified. Similarly, the GMO Panel does not identify indications regarding the overall allergenicity of the three-event stack cotton. The nutritional impact of cotton GHB614 × T304-40 × GHB119-derived food and feed is expected to be the same as those derived from the comparator and non-GM reference varieties.

The GMO Panel concludes that cotton GHB614 × T304-40 × GHB119, as described in this application, is nutritionally equivalent to and as safe as the comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable seeds from cotton GHB614 × T304-40 × GHB119 into the environment.

Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the intended uses of cotton GHB614 × T304-40 × GHB119. In the context of PMEM, the applicant should improve the future literature searches according to the GMO Panel recommendations.

The PMEM plan and reporting intervals are in line with the intended uses of cotton GHB614 × T304-40 × GHB119.

In conclusion, the GMO Panel considers that cotton GHB614 × T304-40 × GHB119, as described in this application is as safe as its comparator and the tested non-GM cotton reference varieties with respect to potential effects on human and animal health and the environment.

Documentation requested and provided to EFSA

- Letter from the Netherlands to EFSA received on 8 October 2014 for placing on the market of genetically modified cotton GHB614 × T304-40 × GHB119 in accordance with articles 6 and 18 of Regulation (EC) No 1829/2003 by Bayer CropScience N.V. (EFSA-GMO-NL-2014-122).
- Acknowledgement letter dated 15 October 2014 from EFSA to the Netherlands.
- Letter from EFSA to applicant dated 4 December 2014 requesting additional information under completeness check.
- Letter from applicant to EFSA received on 23 December 2014 extending the timeline to provide additional information under completeness check.
- Letter from applicant to EFSA received on 23 February 2015 extending the timeline to provide additional information under completeness check.
- Letter from applicant to EFSA received on 16 March 2015 extending the timeline to provide additional information under completeness check.
- Letter from applicant to EFSA received on 23 March 2015 providing additional information under completeness check.
- Letter from applicant to EFSA received on 9 April 2015 providing additional information under completeness check.
- Letter from EFSA to applicant dated **30 April 2015** delivering the'Statement of Validity' for application EFSA-GMO-NL-2014-122.

- Letter from EFSA to applicant dated 30 April 2015 stopping the clock due to the pending risk assessment of single event cotton GHB119.
- Letter from EFSA to applicant dated 23 September 2016 restarting the clock due to finalisation of risk assessment of single event cotton GHB119.
- Letter from EFSA to applicant dated 27 September 2016 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 13 December 2016 providing additional and spontaneous information.
- Email from EFSA to applicant, dated 15 December 2016, re-starting the clock from 13 December 2016.
- Letter from EFSA to applicant dated 16 December 2016 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 14 February 2017 providing additional information.
- Email from EFSA to applicant, dated 14 February 2017, re-starting the clock.
- Letter from EFSA to applicant dated 23 February 2017 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 27 March 2017 providing additional information.
- Email from applicant to EFSA dated 30 March 2017 providing additional information.
- Email from EFSA to applicant, dated 30 March 2017, re-starting the clock.
- Letter from EFSA to applicant dated 12 April 2017 requesting additional information and stopping the clock.
- Letter from EFSA to applicant dated 11 May 2017 requesting additional information and maintaining the clock stopped.
- Letter from applicant to EFSA dated 12 May 2017 extending the timeline to provide additional information.
- Letter from applicant to EFSA dated 9 June 2017 extending the timeline to provide additional information.
- Letter from applicant to EFSA received on 28 July 2017 providing additional information.
- Email from EFSA to applicant, dated 31 July 2017, re-starting the clock from 28 July 2017.
- Letter from EFSA to applicant dated 1 August 2017 requesting additional information and stopping the clock.
- Letter from EFSA to applicant dated 1 September 2017 requesting additional information and maintaining the clock stopped.
- Letter from applicant to EFSA received on 15 September 2017 providing additional information.
- Email from EFSA to applicant, dated 18 September 2017, re-starting the clock from 15 September 2017.
- Letter from EFSA to applicant dated 7 December 2017 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 2 February 2018 providing additional information.
- Email from EFSA to applicant, dated 6 February 2018, re-starting the clock from 2 February 2018.
- Letter from EFSA to applicant dated 15 February 2018 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 20 February 2018 providing additional information.
- Email from EFSA to applicant, dated 21 February 2018, re-starting the clock from 20 February 2018.
- Letter from EFSA to applicant dated 28 March 2018 requesting additional information and stopping the clock.
- Letter from applicant to EFSA received on 15 May 2018 providing additional information.
- Email from EFSA to applicant, dated 25 May 2018, re-starting the clock from 15 May 2018.

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Abbreviations

ANOVA	analysis of variance
bw	body weight
CPA	cyclopropane fatty acids
CPE	cyclopropene fatty acids
CPFA	cyclopropenoid fatty acids
DDE	Daily dietary exposure
ERA	environmental risk assessment
ELISA	enzyme-linked immunosorbent assay
FW	fresh weight
FOB	functional observation battery
GLP	Good Laboratory Practice
GM	genetically modified
GMO	Genetically modified organisms
HGT	horizontal gene transfer
HR	homologous recombination
IgE	immunoglobulin E
ORFs	Open Reading Frames
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring plan

Appendix A – Protein expression data

Means, standard deviation and ranges of protein levels (μg/g dry weight) from cotton GHB614 × T304-40 × GHB119 (not treated), GHB614 (not treated), T304-40 (not treated) and GHB119 (not treated) from field trials performed in USA in 2013[a]

	GHB614 × T304-40 × GHB119	GHB614	T304-40	GHB119
PAT				
Leaf (4–6 leaf)	551.30[b] ± 82.75[c] (452.46–678.41)[d]		366.50 ± 55.85 (292.28–455.13)	232.65 ± 34.16 (178.30–293.36)
Leaf (square initiation)	1366.02 ± 481.35 (852.22–2164.34)		663.13 ± 56.75 (553.32–746.27)	380.83 ± 73.21 (294.73–512.23)
Leaf (2 weeks after first flower)	770.37 ± 183.41 (478.33–1083.54)		372.36 ± 101.55 (220.05–552.15)	263.23 ± 66.97 (174.27–399.16)
Root (4–6 leaf)	247.88 ± 64.45 (160.15–361.73)		146.11 ± 17.82 (115.87–167.46)	88.32 ± 19.56 (34.90–105.17)
Pollen[e] (flowering)	1.12 ± 0.68 (0.50–2.30)		0.75 ± 1.00 (0.03–3.31)	1.53 ± 1.00 (0.29–3.06)
Squares (2 weeks after first flower)	1036.73 ± 169.99 (803.85–1356.51)		536.98 ± 195.59 (172.65–852.53)	323.46 ± 34.84 (277.09–396.89)
Bolls (2 weeks after first flower)	364.71 ± 34.77 (313.85–420.69)		220.55 ± 18.50 (196.41–251.86)	161.64 ± 32.09 (131.84–246.23)
Whole plant (2 weeks after first flower)	489.07 ± 80.88 (345.18–650.41)		322.14 ± 115.35 (147.96–586.87)	165.98 ± 14.14 (142.94–190.56)
Fuzzy seed (maturity)	216.17 ± 23.28 (175.51–248.65)		110.95 ± 12.80 (85.41–130.41)	96.10 ± 12.37 (74.80–117.54)
Cry1Ab	**GHB614 × T304-40 × GHB119**	**GHB614**	**T304-40**	**GHB119**
Leaf (4–6 leaf)	16.74 ± 6.77 (7.42–24.52)		15.15 ± 6.58 (6.90–23.01)	
Leaf (square initiation)	27.41 ± 12.92 (9.80–47.12)		20.03 ± 8.90 (10.81–37.33)	
Leaf (2 weeks after first flower)	10.06 ± 4.16 (2.31–15.46)		7.87 ± 4.05 (1.93–13.92)	
Root (4–6 leaf)	12.13 ± 2.43 (8.03–16.39)		11.36 ± 2.13 (8.98–16.17)	
Pollen (flowering)	0.20 ± 0.11 (0.09–0.41)		0.35 ± 0.23 0.12–0.82	
Squares (2 weeks after first flower)	13.13 ± 3.48 (9.82–19.02)		9.93 ± 3.94 (4.87–16.78)	
Bolls (2 weeks after first flower)	10.81 ± 2.57 (7.25–14.36)		9.38 ± 1.82 (6.37–12.87)	
Whole plant (2 weeks after first flower)	9.71 ± 1.76 (7.05–11.92)		10.07 ± 4.63 (5.98–22.19)	
Fuzzy seed (maturity)	6.37 ± 1.80 (4.07–9.34)		6.56 ± 1.25 (5.24–9.05)	

2mEPSPS	GHB614 × T304-40 × GHB119	GHB614	T304-40	GHB119
Leaf (4–6 leaf)	410.37 ± 63.16 (297.54–497.53)	405.31 ± 50.37 (317.74–480.82)		
Leaf (square initiation)	1912.52 ± 676.00 (876.48–2572.18)	1540.20 ± 767.54 (495.63–2790.01)		
Leaf (2 weeks after first flower)	494.21 ± 128.44 (303.34–749.60)	382.68 ± 48.55 (287.74–475.37)		
Root (4–6 leaf)	86.27 ± 18.46 (54.05–109.59)	85.62 ± 29.03 (22.19–123.87)		
Pollen (flowering)	8.24 ± 3.19 (5.32–14.90)	8.95 ± 6.69 (2.43–23.14)		
Squares (2 weeks after first flower)	390.51 ± 78.33 (267.43–499.86)	393.71 ± 101.61 (251.09–538.67)		
Bolls (2 weeks after first flower)	143.35 ± 21.32 (110.39–177.56)	158.84 ± 23.38 (126.42–195.79)		
Whole plant (2 weeks after first flower)	216.86 ± 45.19 (155.57–311.10)	253.92 ± 161.81 (171.84–754.31)		
Fuzzy seed (maturity)	154.57 ± 20.89 (128.93–192.99)	159.36 ± 13.06 (131.33–180.65)		
Cry2Ae	**GHB614 × T304-40 × GHB119**	**GHB614**	**T304-40**	**GHB119**
Leaf (4–6 leaf)	172.41 ± 54.07 (102.09–263.04)			190.74 ± 51.99 (95.49–268.24)
Leaf (square initiation)	226.52 ± 44.18 (165.69–305.99)			218.94 ± 55.07 (157.80–324.63)
Leaf (2 weeks after first flower)	138.84 ± 27.59 (96.10–202.71)			119.66 ± 37.03 (78.62–207.88)
Root (4–6 leaf)	10.81 ± 5.91 (3.64–21.95)			12.19 ± 2.90 (4.62–16.44)
Pollen (flowering)	0.21 ± 0.14 (0.04–0.58)			0.28 ± 0.41 (0.06–1.56)
Squares (2 weeks after first flower)	42.75 ± 13.09 (30.96–63.72)			42.16 ± 14.95 (26.80–69.75)
Bolls (2 weeks after first flower)	19.54 ± 4.10 (13.58–24.75)			23.99 ± 6.52 (14.91–38.02)
Whole plant (2 weeks after first flower)	111.81 ± 31.51 (79.15–173.47)			135.11 ± 46.13 (78.92–235.72)
Fuzzy seed (maturity)	29.15 ± 6.14 (19.10–39.14)			26.79 ± 7.53 (15.00–35.10)

(a): Number of samples is n = 12 except for: PAT in leaf at square initiation and fuzzy seed (n = 11 for GHB614 × T304-40 × GHB119); 2mEPSPS in leaf at square initiation and fuzzy seed (n = 11 for GHB614 × T304-40 × GHB119) and squares (n = 11 for GHB614); Cry1Ab in fuzzy seed (n = 11 for GHB614 × T304-40 × GHB119); Cry2Ae in leaf/square initiation and fuzzy seed (n = 11 for GHB614 × T304-40 × GHB119).
(b): Mean.
(c): Standard deviation.
(d): Range.
(e): Reported values for pollen are derived from fresh weight (FW) tissue.