STATEMENT

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Assessment of new sequencing information for genetically modified cotton DAS-24236-5 × DAS-21Ø23-5

EFSA Panel on Genetically Modified Organisms (GMO),
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Abstract

The GMO Panel has previously assessed genetically modified (GM) cotton DAS-24236-5 × DAS-21Ø23-5 and concluded that it is as safe as its conventional counterpart and other appropriate comparators with respect to potential effects on human and animal health and the environment in the context of its intended uses. On 17 November 2020, the European Commission requested EFSA to evaluate new DNA sequence information and updated bioinformatics data for cotton DAS-24236-5 × DAS-21Ø23-5 and to indicate whether the conclusions of the GMO Panel on the previously assessed cotton DAS-24236-5 × DAS-21Ø23-5 remain valid. The new sequence data of DAS-24236-5 showed the change of one nucleotide that results in one amino acid substitution, in the newly expressed Cry1F (synpro_L620Q) compared to the sequence originally reported. The GMO Panel concludes that this amino acid substitution in the protein is a mutation. Nonetheless with the exception of the bioinformatics analysis, the studies performed for the risk assessment of Cry1F in cotton DAS-24236-5 × DAS-21Ø23-5 remain valid. In addition, the new sequencing data showed a change in one nucleotide in the 5′ flanking region of DAS-21Ø23-5 compared to the original sequence reported on the stack cotton DAS-24236-5 × DAS-21Ø23-5. The bioinformatic analyses of the newly sequenced DAS-21Ø23-5 event in the stack DAS-24236-5 × DAS-21Ø23-5 shows that the nucleotide difference is in the 5′ flanking region outside the ORFs that span the 5′ junction and is therefore not considered further in the safety assessment. Based on the information provided, the GMO Panel concludes that the corrected sequence does not give rise to any safety concerns, and therefore, the original risk assessment of cotton DAS-24236-5 × DAS-21Ø23-5 remains valid.

Keywords: GMO, cotton, Cry1F, pat, DAS-24236-5, DAS-21Ø23-5

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Table of Contents

Abstract 1
1. Introduction 4
1.1. Background and Terms of Reference as provided by the requestor 4
2. Data and methodologies 4
2.1. Data 4
2.2. Methodologies 4
3. Information previously submitted to EFSA for the GM cotton event DAS-24236-5 × DAS-21Ø23-5 (281–24-236 × 3006–210-23) 4
3.1. Information on DAS-24236-5 (281–24-236) provided in application EFSA-GMO-NL-2005-16 (EFSA GMO Panel, 20102010) 5
3.2. Information on DAS-21Ø23-5 (3006–210-23) provided in application EFSA-GMO-NL-2005-16 (EFSA GMO Panel, 20102010) 5
4. New information submitted to EFSA for the GM cotton event DAS-24236-5 × DAS-21Ø23-5 (281–24-236 × 3006–210-23) as part of the current mandate 5
4.1. DAS-24236-5 (281–24-236) 6
4.2. DAS-21Ø23-5 (3006–210-23) 8
5. Assessment 8
5.1. DAS-24236-5 8
5.1.1. DAS-24236-5 sequencing information 8
5.1.2. DAS-24236-5 bioinformatics analyses 9
5.1.3. Cry1F(synpro_L620Q) protein in DAS-24236-5 9
5.2. DAS-21Ø23-5 9
5.2.1. DAS-21Ø23-5 sequencing information 9
5.2.2. DAS-21Ø23-5 Bioinformatics analyses 9
6. Conclusions 9
6.1. Conclusions on event DAS-24236-5 9
6.2. Conclusions on event DAS-21Ø23-5 10
7. Documentation as provided to EFSA 10
References 10
Abbreviations 11

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The GM stacked cotton DAS-24236-5 × DAS-21Ø23-5[1] has been produced by crossing the DAS-24236-5 and DAS-21Ø23-5 single lines. The applicant restricted the scope of this application to the stack DAS-24236-5 × DAS-21Ø23-5 excluding the single events DAS-24236-5 and DAS-21Ø23-5. Although the applicant claimed that the single lines would not be commercialised anywhere, information on the single events has been assessed by the EFSA GMO Panel to support the evaluation of the stacked cotton DAS-24236-5 × DAS-21Ø23-5 (EFSA GMO Panel, 2010).

The GMO Panel has previously assessed GM cotton DAS-24236-5 × DAS-21Ø23-5 in the frame of application EFSA-GMO-NL-2005-16 (EFSA GMO Panel, 2010). This GMO Panel Statement assesses the new sequencing information received for the GM cotton event DAS-24236-5 × DAS-21Ø23-5 and their potential impact on the previous risk assessment and conclusions of the GMO Panel (EFSA GMO Panel, 2010).

DAS-24236-5 cotton event expresses the *cry*1F (synpro) gene encoding a synthetic insecticidal crystal protein, and the *pat* gene encoding a phosphinothricin acetyltransferase (PAT) which provides tolerance to glufosinate-based herbicides (EFSA GMO Panel, 2010).

DAS-21Ø23-5 cotton event expresses the *cry*1Ac (synpro) gene encoding a synthetic insecticidal crystal protein and the *pat* gene encoding a phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides (EFSA GMO Panel, 2010).

On 13 July 2018, the European Commission (EC) received from Dow Agrosciences new sequencing information related to cotton event DAS-24236-5 × DAS-21Ø23-5, on the basis of Articles 9 and 21 of Regulation (EC) 1829/2003. On 17 November 2020, the EC requested EFSA to evaluate the data and analyses provided by Dow Agrosciences and to indicate whether, on the basis of these elements, the conclusions of the adopted opinion for GM stacked cotton DAS-24236-5 × DAS-21Ø23-5 remain valid. Following the receipt of the mandate, EFSA has evaluated the data and methodology provided for GM cotton DAS-24236-5 × DAS-21Ø23-5 and considered these elements in the context of previous conclusions.

2. Data and methodologies

2.1. Data

In delivering this statement, the EFSA GMO Panel considered information provided by the applicant and relevant scientific publications.

In delivering this statement, EFSA took into account the appropriate principles described in the GMO Panel guidelines for the risk assessment of genetically modified (GM) plants (EFSA GMO Panel, 2011) and Regulation (EU) No 503/2013[2].

2.2. Methodologies

The applicant followed the relevant parts of the GMO Panel guidelines for the risk assessment of genetically modified (GM) plants (EFSA GMO Panel, 2011) to investigate the insert sequence and to perform the bioinformatics analyses.

3. Information previously submitted to EFSA for the GM cotton event DAS-24236-5 × DAS-21Ø23-5 (281–24-236 × 3006–210-23)

The GM stacked cotton DAS-24236-5 × DAS-21Ø23-5 was produced by crossing between lines carrying the GM cotton single events DAS-24236-5 and DAS-21Ø23-5 to combine different resistances to certain lepidopteran insect pests. Cotton DAS-24236-5 × DAS-21Ø23-5 contains the *cry*1F from event DAS-24236-5, the *cry*1Ac from event DAS-21Ø23-5 and the *pat* genes from both events (EFSA GMO Panel, 2010). In application EFSA-GMO-NL-2005-16 information on the sequences of the single GM cotton events DAS-24236-5 and DAS-21Ø23-5 was submitted and assessed by the EFSA GMO

[1] Also known as WideStrike cotton (281 × 3006, or 281–24-236 × 3006–210-23).

[2] Commission 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.

Panel (EFSA GMO Panel, 2010). At that time, no sequencing data on the two-event stacked cotton DAS-24236-5 × DAS-21Ø23-5 was provided by the applicant.

3.1. Information on DAS-24236-5 (281–24-236) provided in application EFSA-GMO-NL-2005-16 (EFSA GMO Panel, 2010)

The vector pAGM281 used to generate cotton DAS-24236-5 contains two expression cassettes on the T-DNA, one for the *cry*1F and one for the *pat*. The *cry*1F is a synthetic chimeric gene with the N-terminal core toxin *cry*1Fa and the C-terminal part from *cry*1Ca and *cry*1Ab. The coding sequence was modified in order to introduce two restriction sites and this resulted in two amino acid substitutions (F604L and Q640R). The *pat* expression cassette contains the synthetic *pat* gene based on the sequence from *Streptomyces viridochromogenes* Tu494 codon optimised for plants, which results in tolerance to glufosinate-ammonium herbicides.

The DNA sequences obtained from the plant confirmed the presence of a 231-bp partial *pat* coding sequence as well as the entire ZmUbi1 promoter. The applicant reported only 2 bp differences within the ZmUbi1 promoter region compared to the plasmid sequence in EFSA-GMO-NL-2005-16. The cotton DAS-24236-5 event contains one intact copy of the pAGM281 T-DNA, with the *cry*1F and *pat* genes, and in addition one partial *pat* gene. The partial *pat* cassette is located downstream of the T-DNA border at the 3′ end of the intact T-DNA in the opposite orientation. Sequence analysis indicated that the pre-insertion locus was preserved except for the deletion of 53 bp from the original locus.

The transcript expression level of the partial *pat* gene was at least 16 times lower than the transcript level of the full-length *pat* gene. Moreover, the peptide produced by the partial *pat* gene was undetectable. The absence of vector backbone sequences in event DAS-24236-5 was confirmed. Bioinformatic analyses of the regions flanking the insert indicated that the DAS-24236-5 insertion occurred into the 3′ untranslated region of the GA 20-oxidase gene. However, compositional and agronomic analyses showed that event DAS-24236-5 is equivalent to its conventional counterpart except for the newly introduced traits. No biologically relevant similarity to allergens, toxins or bioactive proteins was observed for any of the ORFs spanning the junctions.

3.2. Information on DAS-21Ø23-5 (3006–210-23) provided in application EFSA-GMO-NL-2005-16 (EFSA GMO Panel, 2010)

The vector pMYC3006 used to generate cotton DAS-21Ø23-5 contains the *cry*1Ac gene on the T-DNA driven by the ZmUbi1 promoter and the *pat* coding sequence driven by the (4OCS) ΔMas2′ promoter. Termination of transcription of both genes is mediated by the bidirectional orf25 terminator. The *cry*1Ac is a synthetic chimeric gene with the N-terminal core toxin from *cry*1Ac and the C-terminal part from *cry*1Ca and *cry*1Ab. The *cry*1Ac and *pat* genes are codon optimised for plants (Gao et al., 2006; Shan et al., 2007). The *pat* coding sequence is identical to the one present in the pAGM281 vector.

Cotton DAS-21Ø23-5 contains one intact copy of the pMYC3006 T-DNA expressing the *cry*1Ac and *pat* genes. The absence of vector backbone sequences in event DAS-21Ø23-5 was confirmed. The analysis of the insert of cotton event DAS-21Ø23-5 confirmed the expected sequence of the insert. The analysis of the locus in the untransformed cotton genome showed that 16 bp from the original locus was deleted at the insertion site. Updated bioinformatic analyses of the sequences flanking the insertion site of event DAS-21Ø23-5 did not indicate the interruption of any endogenous coding or regulatory sequences. No biologically relevant similarity to allergens, toxins or bioactive proteins was observed for any of the putative peptides spanning the junctions.

4. New information submitted to EFSA for the GM cotton event DAS-24236-5 × DAS-21Ø23-5 (281–24-236 × 3006–210-23) as part of the current mandate

The EFSA GMO Panel assessed the new sequencing information, submitted by the applicant in July 2018, relating to cotton DAS-24236-5 × DAS-21Ø23-5 on the basis of Articles 9 and 21 of Regulation (EC) 1829/2003 and the additional information provided. This is the first time that the sequences of events DAS-24236-5 and DAS-21Ø23-5 from the GM stacked cotton material DAS-24236-5 × DAS-21Ø23-5 are reported. On 21 February 2020 and 27 October 2020, the JRC verified and concluded that the sequencing methodology of DAS-24236-5 and DAS-21Ø23-5 in the GM stacked

cotton DAS-24236-5 × DAS-21Ø23-5 complies with the requirements of the updated JRC Guideline for the submission of DNA sequences within the framework of Regulation (EC) No 1829/2003.

4.1. DAS-24236-5 (281–24-236)

Sanger resequencing of the single line DAS-24236-5 was performed in 2018 on the DNA material used in the original sequencing experiment of 2002. The new sequence of the single line DAS-24236-5 shows 38 nucleotide differences when compared to the original sequence of 2002 submitted in the context of application EFSA-GMO-NL-2005-16.

When the new sequence of the single line DAS-24236-5 was compared with the sequence of this event in the stacked cotton DAS-24236-5 × DAS-21Ø23-5, two differences were observed. These discrepancies (indicated as 'c' in Table 1) were identified in the polyC/G regions, which also contain a change in the original sequencing result (indicated as 'd' in Table 1), are: (i) one nucleotide difference in the number of cytosines (C) (13 C's to 12 C's) in the ZmUbi1 promoter adjacent to AtuORF25/26 3'UTR at positions 3,339–3,352 bp and (ii) one nucleotide difference in the number of guanines (G) (13 G's to 12 G's) in the ZmUbi1 promoter adjacent to the partial *pat* fragment at positions 11,275–11,290 bp (Table 1).

Finally, the applicant submitted resequencing information of the T-DNA from the plasmid pAGM281 used for the transformation. A comparison between the resequenced cotton single line, the newly sequenced event in the stacked cotton DAS-24236-5 × DAS-21Ø23-5 and the T-DNA confirmed the existence of the sequencing uncertainty of the two aforementioned polyC/G region (indicated as 'e' in Table 1). An additional difference was found in the resequenced T-DNA at position 7,240 bp which is in the *cry1F(synpro)* protein (indicated as 'f' in Table 1).

Among the nucleotide differences identified, compared to the originally submitted sequence, and as summarised in Table 1, two of these are present in the *cry*1F(synpro) coding sequence: one G to A transition at position 8,972 bp of the *cry*1F(synpro) coding sequence that would lead to a silent mutation and one A to T transversion at position 7,240 bp, the latter one resulting in an amino acid substitution at location 620 of the Cry1F(synpro) amino acid sequence generating the Cry1F (synpro_L620Q) version. According to the applicant, the first change corrects a previous PCR error and therefore confirms that the sequence at position 8,972 bp is identical to the sequence in the plasmid pAGM281 used during transformation to obtain the breeding line DAS-24236-5 cotton used in the production of the stacked cotton DAS-24236-5 × DAS-21Ø23-5. The second A-to-T change in cry1F (synpro) is present in the resequenced DAS-24236-5 single line and the DAS-24236-5 event sequence in the stack, both performed in 2018. In regard to this second change, which is outside of the active domain of Cry1F, the applicant claims that it does not alter the protein function, the biochemical properties or the allergenicity and toxicity potential of the protein (see Section 5.1.2). The applicant compared the functional properties, biochemical properties and digestibility of the Cry1F (synpro_L620Q) with Cry1F(synpro), which was the intended protein and concluded that no differences were reported.

The applicant provided new bioinformatic analyses to assess whether the open reading frames (ORFs) newly predicted in the event due to the reported nucleotide changes show similarities to toxins or allergens according to EFSA guidance (EFSA GMO Panel, 2011).

Table 1: Identified differences in the sequence of the insert and flanking regions for the DAS-24236-5 single line, the DAS-24236-5 × DAS-21Ø23-5 cotton breeding stack and the T-DNA from plasmid pAGM281 performed in 2018, compared to the original sequence reported in EFSA-GMO-NL-2005-16

Identified difference	Position (bp)[a]	Reported in application EFSA-GMO-NL-2005-16	Resequenced DAS-24236-5	DAS-24236-5 sequence in DAS-24236-5 × DAS-21Ø23-5	Resequenced T-DNA from plasmid pAGM281
5′ border	570	TAAG**G**ATTG	TAAG**A**ATTG	TAAG**A**ATTG	NA[g]
5′ border	882	GTTT**A**CCAT	GTTT**G**CCAT	GTTT**G**CCAT	NA
5′ border	980	AAAA**C**GAAT	AAAA**T**GAAT	AAAA**T**GAAT	NA
5′ border	1,395	ATTC**C**CTTA	ATTC**T**CTTA	ATTC**T**CTTA	NA
5′ border	1,503	TGAA**G**GTAG	TGAA**A**GTAG	TGAA**A**GTAG	NA
ZmUbi1 promoter	2,636–2,643/ **2,644**[b]	8 T	9 T	9 T	9 T
ZmUbi1 promoter	3,339–**3,351**/ 3,352[b]	14C[d]	13C[d]	12C[c]	13C[e]
Cry1F (synpro) CDS	7,240	GAAC**A**GAGC	GAAC**T**GAGC*	GAAC**T**GAGC	GAAC**A**GAGC[f]
Cry1F (synpro) CDS	8,972	AGGA**G**ACGT	AGGA**A**ACGT	AGGA**A**ACGT	AGGA**A**ACGT
AtuMas promoter	9,718	CACC**C**TCGA	CACC-TCGA	CACC-TCGA	CACC-TCGA
Intervening sequence	9,815	ATCT**G**CAAA	ATCT**A**CAAA	ATCT**A**CAAA	ATCT**A**CAAA
ZmUbi1 promoter	11,275–**11,286**/ 11290[b]	16G[d]	13G[d]	12G[c]	13G[e]
ZmUbi1 promoter	11,303	CGGC**T**GTAC	CGGC-GTAC	CGGC-GTAC	CGGC-GTAC
ZmUbi1 promoter	11,776	TTTG**C**TTAA	TTTG**T**TTAA	TTTG**T**TTAA	TTTG**T**TTAA
ZmUbi1 promoter	12,235	TAGA**G**ATGC	TAGA**C**ATGC	TAGA**C**ATGC	TAGA**C**ATGC
Intervening sequence	12,360	TTTG**C**TTAT	TTTG**T**TTAT	TTTG**T**TTAT	TTTG**T**TTAT
Intervening sequence	12,403	GTCG**C**TTTA	GTCG**T**TTTA	GTCG**T**TTTA	GTCG**T**TTTA
Intervening sequence	12,411	ATCA**G**AATG	ATCA**A**AATG	ATCA**A**AATG	ATCA**A**AATG
3′ border	12,781	AAGC**T**AGCT	AAGC**C**AGCT	AAGC**C**AGCT	NA
3′ border	12,811	AGCT**C**GGGA	AGCT**T**GGGA	AGCT**T**GGGA	NA
3′ border	12,866	CTGC**C**CAAG	CTGC**T**CAAG	CTGC**T**CAAG	NA
3′ border	12,882	TGTA**G**ATAC	TGTA**A**ATAC	TGTA**A**ATAC	NA
3′ border	12,918	CACA**A**CCTC	CACA**G**CCTC	CACA**G**CCTC	NA
3′ border	13,129	CCAG**G**GTCA	CCAG**A**GTCA	CCAG**A**GTCA	NA
3′ border	13,222	CAAG**C**CCTA	CAAG**T**CCTA	CAAG**T**CCTA	NA
3′ border	13,436–**13,440**/ 13,441[b]	6 T	5 T (13436-**13440**)[a],[b]	5 T (13436-**13440**)[a],[b]	NA
3′ border	13,982	CGCC**C**CTGC	CGCC**T**CTGC	CGCC**T**CTGC	NA
3′ border	14,009	AGAG**C**CCCC	AGAG**T**CCCC	AGAG**T**CCCC	NA
3′ border	14,185	TTGG**C**GGAA	TTGG**T**GGAA	TTGG**T**GGAA	NA
3′ border	14,218	AAAC**T**ATTG	AAAC**C**ATTG	AAAC**C**ATTG	NA
3′ border	14,429	GTTC**G**ATTG	GTTC**A**ATTG	GTTC**A**ATTG	NA
3′ border	14,613	TTGC**C**TGGT	TTGC**T**TGGT	TTGC**T**TGGT	NA
3′ border	15,297–15,298	TTTC**CA**TTGA	TTTC**TT**TTGA	TTTC**TT**TTGA	NA
3′ border	15,307	ATAG**C**CTGG	ATAG**T**CTGG	ATAG**T**CTGG	NA
3′ border	15,397	ACAC**T**CTAT	ACAC**A**CTAT	ACAC**A**CTAT	NA

(a): Reported position refers to the nucleotide location in the 15,490 bp of DAS-24236-5 original sequence of 2002.
(b): Bolded position in the homopolymer region refers to the nucleotide location at the 3′ end of the stretch. Other software putting the misalignment/gap towards the 5′ end of the homopolymer stretch results in the second position (not in bold).
(c): Additional differences between resequenced single line and stacked cotton DAS-24236-5 × DAS-21Ø23-5 sequence performed in 2018.
(d): Regions that contain a change also in the original sequencing result.

(e): Sequence uncertainty in the polyC/G region between the re-sequenced single line, the newly sequenced event in the stack DAS-24236-5 × DAS-21Ø23-5 and the re-sequenced T-DNA.
(f): Sequence difference found in the re-sequenced T-DNA (2018) compared with the re-sequenced DAS-24236-5 in the single and stack material done in 2018.
(g): NA: not applicable.
*: This change leads to Cry1F (synpro_L620Q).

4.2. DAS-21Ø23-5 (3006–210-23)

The applicant sequenced genomic DNA from 10 pooled seeds of the stacked cotton DAS-24236-5 × DAS-21Ø23-5 (commercialised product) in order to obtain a new sequence for the DAS-21Ø23-5 event in the stack. The resulting 9,043 bp sequence consisted of 358 bp of the 5′ flanking border, 317 bp of the 3′ flanking border and 8,368 bp of the transgenic insert derived from plasmid pMYC3006. When compared to the reference sequence of DAS-21Ø23-5 single line, the sequence of DAS-21Ø23-5 from the stacked cotton DAS-24236-5 × DAS-21Ø23-5 contains a C to A transversion in the 5′ flanking border at position 70 bp (position 244 bp in the reference sequence). No other nucleotide changes were reported by the applicant (Table 2). The applicant provided new bioinformatic analyses to assess whether the ORFs predicted within the insert and spanning the junction between the insert and flanking regions show similarities to toxins or allergens according to EFSA guidance (EFSA GMO Panel, 2011).

Table 2: Identified differences in the sequence of the insert and flanking regions for the DAS-21Ø23-5 single line and the DAS-24236-5 × DAS-21Ø23-5 cotton breeding stack

Identified difference	Position (bp)[(a)]	Reported in application EFSA-GMO-NL-2005-16	DAS-21Ø23-5 sequence in DAS-24236-5 × DAS-21Ø23-5
5′ border	244	AATC**C**AAAT	AATC**A**AAAT

(a): The reported base position refers to the nucleotide location in the 9,382 bp of the DAS-21Ø23-5 originally reported sequence of 2002, which is at base position 70 in the study for sequencing the DAS-21Ø23-5 insert from DAS-24236-5 × DAS-21Ø23-5 cotton.

5. Assessment[3]

5.1. DAS-24236-5

5.1.1. DAS-24236-5 sequencing information

This is the first-time event DAS-24236-5 from the stacked cotton material DAS-24236-5 × DAS-21Ø23-5 was sequenced. The Sanger sequencing of this event in the stack material was performed in 2018, was evaluated by the JRC and was found to be compliant with the JRC guideline (2016)[4] and was considered as the reference sequence.

The applicant used the sequence obtained in 2018 from the single line DAS-24236-5 as reference to identify nucleotide differences when compared to the one derived from the stacked cotton material DAS-24236-5 × DAS-21Ø23-5 for the same single event. EFSA assessed the compliance of the nucleotide sequence obtained from the resequenced single line DAS-24236-5 according to the EFSA Technical Note (EFSA GMO Panel, 2018). The sequence was found to be compliant at the 5′ flanking region, the entire length of the insert and > 1 kb of the 3′ flank. Based on the fact that the material used in the resequencing of the DAS-24236-5 was the same original DNA used in the sequencing experiment of 2002, the observed differences are most likely due to sequencing errors and/or sequencing uncertainties of the polyG/C regions (*vide infra*), rather than mutations, except for the one at the *Cry*1F (7,240 bp). This change is probably the result of a mutation as supported by the resequencing of the plasmid T-DNA that is different from the resequenced event in the single and stack materials. The variations observed in the polyC/polyG regions of the ZmUbi1 promoter are most probably a result of the sequencing difficulties caused by DNA polymerase-associated slippage during the PCR amplification of polyG/C region, as reported previously (Levinson and Gutman, 1987; Clarke

[3] Information provided with the mandate on 17/11/2020 and supplementary information 3 October 2021, 21/7/2021, 10 August 2021, 17/2/2022 and 29/4/2022.
[4] https://gmo-crl.jrc.ec.europa.eu/doc/Guideline-Sequencing-Feb-2016-mod-April-2017.pdf

et al., 2001; Fazekas et al., 2010; Kieleczawa, 2006). Therefore, it is not possible to make clear conclusions on the number of Cs or Gs in the polyC/polyG regions; thus, the observed differences are most probably due to PCR/sequencing errors.

5.1.2. DAS-24236-5 bioinformatics analyses

The new bioinformatic analysis of the corrected sequence of event DAS-24236-5 identified 28 ORFs which are newly predicted due to the nucleotide changes compared to the original sequence obtained in 2002. The allergenicity assessment indicated that two of these ORFs predicted in the complementary strand of the ZmUbi promoters show significant similarity to a collagen α2(I) (GenBank Accession BAB55663.1) from rainbow trout (*Oncorhynchus mykiss*). However, the absence of any promoter upstream these two ORFs and the lack of any start codon make their expression highly unlikely. The toxicity analysis indicated that none of the 28 ORFs showed significant similarities to toxins.

5.1.3. Cry1F(synpro_L620Q) protein in DAS-24236-5

One of the nucleotide changes identified by the applicant is located at position 7,240 bp (Table 1) and results in a single amino acid substitution at location 620 of the Cry1F(synpro) amino acid sequence, generating the Cry1F(synpro_L620Q) version. The applicant was requested to clarify which version of Cry1F protein was expressed in cotton DAS-24236-5 × DAS-21Ø23-5 and which one was used for the acute toxicity and *in vitro* digestibility studies submitted in the original application (EFSA-GMO-NL-2005-16). The applicant clarified that the single line DAS-24236-5 and the stack DAS-24236-5 × DAS-21Ø23-5 express the Cry1F(synpro_L620Q), while the Cry1F(synpro) was the microbially produced protein used in studies for the acute toxicity and *in vitro* digestibility.

To assess potential impacts on safety linked to the amino acid substitution, the applicant provided (i) an *in silico* pepsin cleavage study to assess the impact of the amino acid change in the Cry1F protein, and (ii) homology 3-D modelling studies of the target sequences to elucidate the impact of the L620Q substitution on the tertiary structure of the Cry1F protein. The *in silico* pepsin cleavage study indicated no relevant impact of the amino acid change on the results of *in vitro* digestibility for both Cry1F protein versions. The 3-D modelling suggests that the Cry1F (synpro_L620Q) and the Cry1F (synpro) are structurally equivalent. In addition, the functional equivalence between Cry1F microbially produced and the Cry1F *in planta* was demonstrated (EFSA-GMO-NL-2005-16 and Section 4.1).

The GMO Panel concludes that the amino acid substitution does not impact the safety profile of the Cry1F protein and supports previous assessments on the safety of Cry1F in cotton DAS-24236-5 × DAS-21Ø23-5 to humans and animals.

5.2. DAS-21Ø23-5

5.2.1. DAS-21Ø23-5 sequencing information

This is the first-time single event DAS-21Ø23-5 from the stacked cotton material DAS-24236-5 × DAS-21Ø23-5 was sequenced. The Sanger sequencing of this event in the stack material was performed in 2018, was evaluated by the JRC and was found to be compliant with the JRC Guideline (2016)[4] and can be considered as the reference sequence of the stack material.

5.2.2. DAS-21Ø23-5 Bioinformatics analyses

The new bioinformatic analyses of the newly sequenced DAS-21Ø23-5 event in the stack DAS-24236-5 × DAS-21Ø23-5 show that the nucleotide difference is in the 5′ flanking region outside the ORFs that span the 5′ junction and is therefore not considered further in the safety assessment.

6. Conclusions

6.1. Conclusions on event DAS-24236-5

The sequence of the DAS-24236-5 event was obtained for the first time from the stacked cotton material DAS-24236-5 × DAS-21Ø23-5 subject of application EFSA-GMO-NL-2005-16. This sequence was assessed by the JRC and was found to be compliant.

The resequenced single line DAS-24236-5 of 2018 was used as the reference sequence. The comparison between the reference sequence and the sequence of the stack indicated two sequence

differences (13Cs/12Cs at positions 3,269–3,281/3,280 and 13Gs/12Gs at position 11,203–11,215/11,202–11,213) on which bioinformatics analyses were performed and did not raise any safety concerns. The difficulties in sequencing these regions have been reported previously and are caused by DNA polymerase-associated slippage during PCR amplification (Levinson and Gutman, 1987; Clarke et al., 2001; Fazekas et al., 2010; Kieleczawa, 2006). Therefore, it is not possible to make clear conclusions on the number of Cs or Gs in the polyC/polyG regions; thus, the observed differences are most probably due to PCR/sequencing errors.

However, as mentioned above, the bioinformatics analyses did not raise any safety concerns.

From the observed sequence differences, the only confirmed change leading to the single amino acid substitution in the Cry1F protein, namely Cry1F(synpro_L620Q), was extensively analysed for its impact on the past risk assessment conclusions. Based on the fact that the material used in the re-sequencing of the DAS-24236-5 was the same original DNA used in the sequencing experiment of 2002, the observed differences are most likely due to sequencing errors, rather than mutations, except for the one at the *Cry*1F (7,240 bp), which is most likely the result of a mutation as supported by the resequencing of the plasmid T-DNA.

The bioinformatic analyses indicated that the expression of any newly predicted ORF within the insert and spanning the junction between the insert and the flanking genomic regions showing similarity with known allergens or toxins is highly unlikely.

Therefore, the conclusions of the adopted opinion for GM cotton DAS-24236-5 × DAS-21Ø23-5 remain valid.

6.2. Conclusions on event DAS-21Ø23-5

The sequence of the DAS-21Ø23-5 was obtained for the first time using the stacked cotton material DAS-24236-5 × DAS-21Ø23-5, subject of application EFSA-GMO-NL-2005-16. This sequence was assessed by the JRC and was found to be compliant. The new bioinformatic analyses indicated that the expression of any newly predicted ORF within the insert and spanning the junction between the insert and the flanking genomic regions showing similarity with known allergens or toxins is highly unlikely. Therefore, the conclusions of the adopted opinion for GM cotton DAS-24236-5 × DAS-21Ø23-5 remain valid.

7. Documentation as provided to EFSA

Mandate from the European Commission (EC) received on 17 November 2020 concerning a request to assess new sequencing information relating to cotton DAS-24236-5 x DAS-21Ø23-5 provided by Dow Agrosciences.

Mandate accepted on 4 January 2021.

Request for supplementary information (1) from the applicant on 18 January 2021.

Receipt of supplementary information (1) by the applicant on 10 March 2021.

Request for supplementary information (2) from the applicant on 21 April 2021.

Receipt of supplementary information (2) by the applicant on 21 July 2021.

Request for supplementary information (3) from the applicant on 3 August 2021.

Receipt of supplementary information (3) by the applicant on 8 October 2021.

Request for supplementary information (4) from the applicant on 16 December 2021.

Receipt of supplementary information (4) by the applicant on 17 February 2022.

Request for supplementary information (5) from the applicant on 4 March 2022.

Receipt of supplementary information (5) by the applicant on 29 April 2022.

Request to EC to extend the deadline of the mandate, from 4 March 2021 to 30 January 2022, sent on 5 November 2021.

Receipt of acceptance of deadline extension from EC on 19 November 2021.

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Abbreviations

DNA	deoxyribonucleic acid
GA	gibberellin
GM	genetically modified
GMO	genetically modified organism
GMO	Panel EFSA Panel on Genetically Modified Organisms
JRC	Joint Research Centre
ORF	open reading frame
pat	phoshosphinothricin-acetyl-transferase
PCR	polymerase chain reaction
T-DNA	transfer deoxyribonucleic acid
ΔMas2′	promoter mannopine synthase promoter