STATEMENT

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Risk assessment of a new bioinformatics evaluation of the insertion sites of genetically modified soybean event 40-3-2

EFSA Panel on Genetically Modified Organisms (GMO),
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Abstract

Genetically modified (GM) soybean 40-3-2 expresses a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS), which confers tolerance to glyphosate. This event was previously assessed by the GMO Panel as a single event and as part of a two-event stack and was found to be as safe as its conventional counterparts and other appropriate comparators with respect to potential effects on human and animal health and the environment. On September 2021, the European Commission requested EFSA to evaluate a new bioinformatics study which revealed predicted genomic deletions at the insertion sites using the available soybean reference genome. Considering the variability of the soybean genome, with a number of structural variants such as presence/absence variants and copy number variants including genic regions, as well as the fact that a number of genes are present only in particular varieties, the GMO Panel concludes that comparing only to the reference genome does not allow to conclude that the transformation event resulted in gene loss. In support of this, the transcriptomic analysis did not show major differences in gene expression when comparing the soybean 40-3-2 with the most closely related conventional variety, indicating that the genetic redundancy may compensate for the potential gene loss. Moreover, the composition, phenotypic and agronomic analyses already assessed by the GMO Panel in previous opinions did not show differences between soybean 40-3-2 and its comparators suggesting that the potential gene loss may not have a significant phenotypic effect in soybean 40-3-2. For these reasons, the EFSA GMO Panel concludes that the new information provided by the applicant on soybean 40-3-2 does not alter EFSA's previous conclusions.

Keywords: GMO, soybean (*Glycine max*), 40-3-2, gene deletion, Regulation (EC) No 1829/2003

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Table of contents

Abstract 1
1. Introduction 4
2. Background and Terms of Reference as provided by the requestor 4
3. Data and methodologies 4
3.1. Data 4
3.2. Methodologies 4
4. EFSA safety assessment previously provided for GM soybean 40-3-2 4
4.1. EFSA safety assessment of GM soybean 40-3-2 as a single event for food and feed 4
4.2. EFSA safety assessment of GM soybean 40-3-2 as a single event for cultivation 5
4.3. EFSA safety assessment of GM soybean 40-3-2 in stack 305423 × 40-3-2 5
4.4. Sequencing information of soybean event 40-3-2 5
5. GM soybean 40-3-2 new information provided to EFSA 6
5.1. Additional information provided by the applicant in response to EFSA request 6
6. Assessment 7
7. Conclusions 7
Documentation as provided to EFSA 8
References 8
Abbreviations 9
Appendix A – List of studies submitted by the applicant 10

1. Introduction

Genetically modified (GM) soybean 40-3-2 has been developed to confer tolerance to glyphosate herbicides by the introduction, via particle gun acceleration technology, of a gene coding for 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* (renamed *Rhizobium radiobacter*) strain CP4 (CP4 EPSPS). Soybean 40-3-2 event contains two T-DNA inserts: (1) a primary functional T-DNA insert which is comprised of the full CP4 EPSPS sequence; and (2) a secondary non-functional insert which is comprised of a 72-bp segment of the CP4 EPSPS.

The GMO Panel has previously assessed soybean 40-3-2 as a single event in the frame of applications EFSA-GMO-RX-40-3-2[8-1a/20-1a], [8-1b/20-1b] (EFSA GMO Panel, 2010) and EFSA-GMO-NL-2005-24 (EFSA GMO Panel, 2012), and as part of a two-event stack (soybean 305423 × 40-3-2) in the frame of application EFSA-GMO-NL-2007-47 (EFSA GMO Panel, 2016) and was found to be as safe as their conventional counterparts and other appropriate comparators with respect to potential effects on human and animal health and the environment. Moreover, EFSA was requested by European Commission to analyse new nucleic acid sequencing and updated bioinformatics data for soybean 40-3-2. EFSA concluded that the original risk assessment of the soybean event as a single and the stacked soybean 305423 × 40-3-2 remained valid (EFSA, 2017).

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

On 6 September 2021, Bayer CropScience LP informed the EC in accordance with Articles 9(3) and 21(3) of Regulation (EC) 1829/2003 of a new updated bioinformatic analysis to assess any interruption of plant genes by the inserts in soybean 40-3-2. On 28 September 2021, the EC requested EFSA to evaluate the new data and the performed analysis provided by Bayer CropScience LP and indicate whether the conclusions of the adopted opinions on soybean 40-3-2 and 305423 × 40-3-2 remain valid. On 18 November 2021, EFSA accepted the EC mandate. EFSA requested deadline extension on 18 March 2022.

3. Data and methodologies

3.1. Data

In delivering this statement, the EFSA GMO Panel took into account 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 GM plants (EFSA GMO Panel, 2011; Regulation (EU) No 503/2013[1]).

3.2. Methodologies

The applicant followed the relevant parts of the GMO Panel guidelines for the risk assessment of GM plants to perform the bioinformatics analyses (EFSA GMO Panel, 2011; Regulation (EU) No 503/2013[1]). EFSA has evaluated the data and methodology provided for GM soybean 40-3-2 and considered these elements in the context of previous conclusions.

4. EFSA safety assessment previously provided for GM soybean 40-3-2

4.1. EFSA safety assessment of GM soybean 40-3-2 as a single event for food and feed

The original food safety assessment of soybean 40-3-2 within the European Union was performed by the Advisory Committee on Novel Foods and Processes in the UK (UK-ACNFP, 1995) according to Directive 90/220/EEC (European Commission, 1996). Similarly, the Advisory Committee on Release to the Environment (ACRE) to the Secretary of State for the Environment, Transport and the Regions and Minister of Agriculture, Fisheries and Food of the UK advised on the importation storage and use of soybean 40-3-2 for processing to non-viable soybean fractions suitable for use in animal feeds, foods and any other products in which soybean fractions are used. On that occasion, ACRE concluded that

[1] 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.

the risk of marketing this product would be no different from that of other soybeans marketed for the same purposes. Other national approvals for the food and feed use of soybean 40-3-2 and its derivatives were received from the UK, the Netherlands and Denmark prior to the entry into force of Regulation (EC) No 258/97.

The EFSA GMO Panel has assessed soybean 40-3-2 as a single event in the frame of applications received from European Commission on 29 June 2007: EFSA-GMO-RX-40-3-2[8-1a/20-1a], [8-1b/20-1b] for the continued marketing of (1) food containing, consisting of, or produced from genetically modified soybean 40-3-2; (2) feed containing, consisting of, or produced from soybean 40-3-2; (3) other products containing or consisting of soybean 40-3-2 with the exception of cultivation, all under Regulation (EC) No 1829/2003 (EFSA GMO Panel, 2010). In this scientific opinion, molecular analyses indicated that soybean 40-3-2 contains one functional insert (i.e. primary insert) expressing CP4 EPSPS and a non-functional insert (i.e. secondary insert) consisting of a non-functional fragment of the CP4 EPSPS coding sequence. The updated bioinformatic analyses of the inserts' flanking sequences and the open reading frames (ORFs) spanning the junctions between the insert and the flanking plant genomic DNA, and the quantification of the levels of the newly expressed protein in soybean 40-3-2 did not raise any safety concern. Moreover, the available compositional and agronomic data showed that soybean 40-3-2 was compositionally and agronomically equivalent to its conventional counterpart and to other commercial soybean varieties, except for the expression of CP4 EPSPS protein. The Panel concluded that the soybean 40-3-2 event was unlikely to have any adverse effects on human and animal health and the environment, in the context of its intended uses.

4.2. EFSA safety assessment of GM soybean 40-3-2 as a single event for cultivation

The EFSA GMO Panel has assessed application EFSA-GMO-NL-2005-24 for the placing on the market of the herbicide tolerant genetically modified soybean 40-3-2 for cultivation under Regulation (EC) No 1829/2003 (EFSA GMO Panel, 2012). In this opinion, the bioinformatic analyses of the ORFs spanning the junction site within the functional insert or between the inserts and the plant genomic DNA did not raise safety concerns. The stability of the inserted DNA and the herbicide tolerance trait were confirmed over several generations. In addition, following the comparison of the composition of agronomic and phenotypic characteristics for transgenic and its conventional counterpart, the EFSA GMO Panel reported that soybean 40-3-2 was compositionally equivalent to commercial non-GM soybean varieties, except for the newly expressed protein. The EFSA GMO Panel concluded that soybean 40-3-2 was unlikely to raise additional environmental safety concerns compared with conventional soybean (EFSA GMO Panel, 2012).

4.3. EFSA safety assessment of GM soybean 40-3-2 in stack 305423 × 40-3-2

The EFSA GMO Panel has assessed application EFSA-GMO-NL-2007-47 for the placing on the market of the herbicide-tolerant, high-oleic acid, genetically modified soybean 305423 × 40-3-2 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (EFSA GMO Panel, 2016). Based on the molecular, agronomic, phenotypic and compositional characteristics, the GMO Panel considered that the combination of soybean single events 305423 and 40-3-2 in the two-event stack soybean 305423 × 40-3-2 did not raise food and feed or nutritional safety concerns. Therefore, the EFSA GMO Panel concluded that the combination of the newly expressed proteins in the two-event stack soybean did not raise concerns for human and animal health.

4.4. Sequencing information of soybean event 40-3-2

In April 2017, European Commission requested EFSA to assess new sequencing information for soybean 40-3-2 and to indicate whether the conclusions of the EFSA GMO Panel on previously assessed GM soybean remain valid. The new sequencing data indicated that soybean 40-3-2 event present in the stacked soybean 305423 × 40-3-2 contained an additional nucleotide in the 5′ flanking region of the 72-bp non-functional insert (see Section 4.1). Following the re-examination of the original sequencing data of the single soybean event 40-3-2 provided by the applicant, the EFSA GMO Panel indicated that this additional nucleotide was already present in the original plant material used for the risk assessment of soybean event 40-3-2. In addition, the assessment of the updated bioinformatic analyses performed on the corrected sequence did not give rise to safety concerns. Thus,

the EFSA GMO Panel previous conclusions of the single event soybean 40-3-2 and the two-event stack soybean 305423 × 40-3-2 remained valid (EFSA, 2017).

5. GM soybean 40-3-2 new information provided to EFSA

In August 2021, the applicant generated a new bioinformatic study to assess whether the insertion of the primary and secondary inserts interrupted any endogenous gene in soybean 40-3-2 using the most recent GenBank soybean reference genome database (*Glycine max* reference genome GCF_000004515.5_Glycine_max_v2.1) (Appendix A).

The search against the *Glycine max* reference genome GCF_000004515.5_Glycine_max_v2.1[2] using the genomic flanking sequences of the primary and secondary inserts revealed that the top hit was the accession NC_016089.3 'Glycine max cultivar Williams 82 chromosome 2, Glycine_max_v2.1, whole genome shotgun sequence', indicating that the primary and secondary inserts were inserted into the chromosome 2 of the soybean genome.

The study reported that the 5′ and the 3′ flanking sequences were separated by approximately 135 Kb for the primary functional insert, and by approximately 332 Kb for the secondary non-functional insert. Overall, a total of 12 predicted endogenous genes were located between the genomic flanking regions of the primary insert, and a total of 30 predicted endogenous genes were located between the genomic flanking regions of the secondary insert. According to Salomon and Puchta (1998), the applicant clarified that these disruptions likely resulted from double stranded break (DSB) repair mechanisms in the plant during the transformation process and were already present in soybean 40-3-2 during all safety assessments and 25 years of commercial cultivation. The applicant stated that the ability to detect these disruptions is the result of the recent higher-resolution analysis allowed by the high-quality crop reference genomes. In addition, the study reported that an endogenous gene (LOC100791584) overlapped with the 5′ flanking sequence of the secondary insertion, indicating that the first exon of this gene has been interrupted.

The applicant provided a series of references already assessed by the GMO Panel showing that these predicted genomic deletions were not biologically relevant since there were no significant phenotypic, compositional and nutritional differences reported between soybean 40-3-2 and the non-transgenic control soybean varieties (Re et al., 1993; Delannay et al., 1995; Padgette et al., 1995; Hammond et al., 1996; Taylor et al., 1999; Nair et al., 2002; Harrigan et al., 2007).

The applicant concluded that the potential genomic deletions caused by the primary and secondary inserts do not pose any risk from a food, feed, or environmental perspective.

5.1. Additional information provided by the applicant in response to EFSA request[3]

On 21 December 2021, EFSA requested the applicant to discuss and/or provide additional evidence elucidating the implications of the predicted gene loss in soybean 40-3-2. On 8 March 2022, the applicant provided additional information in response to EFSA.

The applicant reported that the impact of the potential genomic deletions is minimised by the activity of redundant genes. The soybean genome is highly duplicated since it has undergone at least two large-scale duplication events leading to significant amounts of duplicated regions (Schlueter et al., 2007). This resulted in nearly three-quarters of soybean genes having multiple copies (Schmutz et al., 2010). The applicant indicated the presence of a high number of structural variants (SVs) such as presence/absence variants (PAVs) and copy number variants (CNVs) in different soybean accessions (Liu et al., 2020). Thus, considering this high level of duplication in soybean, one or a few reference genomes cannot represent the full range of genetic diversity.

Specifically, two synteny blocks in chromosomes 5 and 19 reveal potential genetic redundancy for the ~135-Kb segment in the soybean genome, and 6 of the predicted 12 genes in this segment share high protein sequence similarities with their paralogs (80–97%, with an average of 88.13%). Whereas 27 of the 30 predicted genes in the ~332-Kb segment displayed highly similar protein sequences to their paralogs (81–99.7%, with an average of 93.45%) (Schmutz et al., 2010). The applicant stated that such high similarity of protein sequences implies functional conservation between the genes predicted in the two segments and the corresponding paralogs.

[2] A new version of the *Glycine max* reference genome is available (V 4.0). The analysis of the inserts' flanking regions using the reference genome version 4.0 confirms the results obtained using the version 2.1.
[3] Additional information: 3 August 2022.

The applicant highlighted the plasticity of the soybean genome. Liu et al. (2020) indicated that an important fraction of gene families can be defined as dispensable since they were present only in 24 or fewer of the 27 analysed soybean accessions. Similarly, Bayer et al. (2021) analysed a large set of soybean accessions (> 1,000) showing that 13% of soybean genes were considered dispensable, as they were absent from at least one accession. Therefore, the applicant stated that the potential gene loss may not impact the phenotype of commercial soybean. Therefore, it is not surprising that the disruptions identified in soybean 40-3-2 did not result in any observed phenotypes when compared to a conventional comparator.

In addition, the applicant cited the study by Cheng et al. (2008) which reported that gene expression in soybean 40-3-2 was practically unchanged. They found that while only eight genes were differentially expressed in soybean 40-3-2 varieties compared to the most closely related conventional variety, over 1,000 genes were differentially expressed when comparing specific conventional varieties to each other. Despite the genomic disruptions caused by the two insertions and the expression of the CP4 EPSPS protein, the applicant demonstrated the minimal impact in soybean 40-3-2 of the potential loss of the 42 annotated genes of the disrupted regions, and also the level of genetic variability that already exists between conventional varieties of soybean.

Based on this information, the applicant suggested that any genomic disruption caused by the two inserts in soybean 40-3-2 has virtually no impact on the characteristics of the plant. Therefore, the applicant concluded that the original assessment on the safety of soybean 40-3-2 for food, feed and the environment remains valid.

6. Assessment

The alignment of the flanks of the event with the soybean reference genome revealed that the regions showing sequence similarity to the 5′ and the 3′ flanks are separated by ~ 135 Kb and ~ 332 Kb for the primary and secondary inserts, respectively. Moreover, the analysis identified the disruption of the first exon of an endogenous gene (LOC100791584) in the 5′ flanking sequence of the secondary insert.

The analysis of the flanking regions of the primary and secondary inserts using the available soybean reference genome may suggest genomic disruptions and consequent gene loss. Considering the variability of the soybean genome, with a number of SVs such as PAVs and CNVs including genic regions, does not allow to conclude that the insertions did result in potential gene loss.

Recent studies (Liu et al., 2020; Bayer et al., 2021) described the plasticity of the soybean genome, also highlighting that a number of genes are present only in particular varieties. Moreover, a transcriptomic study (Cheng et al., 2008) showing that few genes were differentially expressed when comparing soybean 40-3-2 varieties to the most closely related conventional soybean cultivars may also suggest that genetic redundancy could compensate the potential gene loss.

Finally, the soybean event 40-3-2 has been previously assessed by the EFSA GMO Panel (2010, 2012, 2016) and EFSA concluded that soybean 40-3-2 is unlikely to pose any risk to human and animal health.

7. Conclusions

The assessment of the new bioinformatics analysis provided by the applicant reveals predicted genomic deletions at the insertion sites in genetically modified soybean 40-3-2. However, the variability of the soybean genome as well as the fact that a number of genes are present only in particular varieties implies that comparing only to the reference genome does not allow to conclude that the insertions result in gene loss.

Composition, phenotypic and agronomic analyses already assessed by the GMO Panel in previous opinions did not show relevant differences between soybean 40-3-2 and its comparators suggesting that the potential gene loss may not have significant phenotypic effect in soybean. Moreover, the transcriptomic analysis of the GM soybean 40-3-2 did not show major differences in gene expression with the conventional soybean cultivars.

Therefore, the EFSA GMO Panel concludes that the new information provided by the applicant on soybean 40-3-2 does not alter EFSA's previous conclusions (EFSA GMO Panel, 2010, 2012, 2016).

Documentation as provided to EFSA

- Mandate from the European Commission (EC) received on 28 September 2021 concerning a request to evaluate the new data and the performed analysis provided by Bayer CropScience LP.
- Mandate accepted on 18 November 2021.
- Additional information (Clock 1) was requested on 21 December 2021.
- Additional information (Clock 1) was received on 8 March 2022.

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Abbreviations

CNV	copy number variant
CP4 EPSPS	*Agrobacterium* sp. strain CP4 5-enolpyruvylshikimate-3-phosphate synthase
DSB	double stranded break
GM	genetically modified
GMO	EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms)
Kb	kilobase
ORF	open reading frame
PAV	presence/absence variant
SV	structural variant

Appendix A – List of studies submitted by the applicant

Study identification	Study title
REG-2021-0077 TRR0000841	Updated Bioinformatics Evaluation of the MON 40-3-2 Insertion Site Utilizing the GMA_2021 Database