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Scientific Opinion on an application by Dow AgroSciences LLC (EFSA-GMO-NL-2011-91) for the placing on the market of genetically modified herbicide-tolerant soybean DAS-68416-4 for food and feed uses, import and processing under Regulation (EC) No 1829/2003

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Abstract

Soybean DAS-68416-4 was developed by *Agrobacterium tumefaciens*-mediated transformation to express the aryloxyalkanoate dioxygenase-12 (AAD-12) protein, conferring tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and other related phenoxy herbicides, and the phosphinothricin acetyltransferase (PAT) protein, conferring tolerance to glufosinate ammonium-based herbicides. The molecular characterisation data and bioinformatics analyses did not identify issues requiring further assessment for food/feed safety. The agronomic and phenotypic characteristics tested revealed no relevant differences between soybean DAS-68416-4 and its conventional counterpart, except for 'days to 50% flowering'. The compositional analysis identified no differences requiring further assessment, except for an increase (up to 36%) in lectin activity in soybean DAS-68416-4. Such increase is unlikely to raise additional concerns for food/feed safety and nutrition for soybean DAS-68416-4 as compared to its conventional counterpart and the non-GM reference varieties. There were no concerns regarding the potential toxicity and allergenicity of the two newly expressed proteins, and no evidence that the genetic modification might significantly change the overall allergenicity of soybean DAS-68416-4. Soybean DAS-68416-4 is as nutritious as its conventional counterpart and the non-GM reference varieties. There are no indications of an increased likelihood of establishment and spread of occasional feral soybean DAS-68416-4 plants, unless these are exposed to the intended herbicides. The likelihood of environmental effects resulting from the accidental release of viable seeds from soybean DAS-68416-4 into the environment is therefore very low. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of soybean DAS-68416-4. The GMO Panel concludes that the information available addresses the scientific comments of the Member States and that soybean DAS-68416-4, as described in this application, is as safe as its conventional counterpart and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment in the context of the scope of this application.

Keywords: GMO, soybean (*Glycine max*), DAS-68416-4, herbicide tolerance, AAD-12, PAT, Regulation (EC) No 1829/2003

Requestor: Competent Authority of the Netherlands

Question number: EFSA-Q-2011-00052

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Summary

Following the submission of an application (EFSA-GMO-NL-2011-91) under Regulation (EC) No 1829/2003 from Dow AgroSciences LLC, 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 the genetically modified (GM) herbicide-tolerant soybean (*Glycine max* L.) DAS-68416-4 (Unique Identifier DAS-68416-4). The scope of application EFSA-GMO-NL-2011-91 is for import, processing, and food and feed uses of soybean DAS-68416-4 within the European Union (EU), but excludes cultivation in the EU.

The GMO Panel evaluated soybean DAS-68416-4 with reference to the scope and appropriate principles described in its guidelines for the risk assessment of GM plants. 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; the environmental risk assessment; and the post-market environmental monitoring plan.

Soybean DAS-68416-4 was developed by *Agrobacterium tumefaciens*-mediated transformation. It expresses the aryloxyalkanoate dioxygenase-12 (AAD-12) protein, conferring tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and other related phenoxy herbicides, and the phosphinothricin acetyltransferase (PAT) protein, conferring tolerance to glufosinate ammonium-based herbicides. The molecular characterisation data established that soybean DAS-68416-4 contains one insert consisting of two expression cassettes, *aad-12* and *pat*. No other parts of the plasmid used for transformation were detected in soybean DAS-68416-4. Bioinformatic analyses and genetic stability studies were performed and the results did not identify issues requiring further assessment for food/feed safety. The levels of the newly expressed proteins present in soybean DAS-68416-4 were obtained and reported adequately.

The agronomic and phenotypic characteristics of soybean DAS-68416-4 tested revealed no relevant differences between soybean DAS-68416-4 and its conventional counterpart, except for 'days to 50% flowering' for the GM soybean not treated with the intended herbicides. The difference observed in 'days to 50% flowering' was further assessed for its potential environmental impact. No differences in composition requiring further assessment for food/feed safety were found between soybean DAS-68416-4 and its conventional counterpart, except for a higher lectin activity (up to 36%) in two of the four treatments of soybean DAS-68416-4.

The increase in lectin activity is unlikely to raise additional concerns for food/feed safety and nutrition for soybean DAS-68416-4 as compared to its conventional counterpart and the non-GM commercial varieties. The safety assessment identified no concerns regarding the potential toxicity and allergenicity of the newly expressed PAT and AAD-12 proteins in soybean DAS-68416-4, and found no evidence that the genetic modification might significantly change the overall allergenicity of soybean DAS-68416-4. The GMO Panel concludes that soybean DAS-68416-4 is as safe and as nutritious as its conventional counterpart and the non-GM soybean reference varieties. The GMO Panel considers that post-market monitoring of food/feed derived from soybean DAS-68416-4 is not necessary, given the absence of safety concerns identified.

Considering the scope of this application, the environmental risk assessment is concerned with the accidental release into the environment of viable soybean DAS-68416-4 seeds (i.e. during transport and/or processing), and with the exposure of bacteria to recombinant DNA in the gastrointestinal tract of animals fed GM material and those present in environments exposed to their faecal material (manure and faeces).

In the case of accidental release into the environment of viable seeds of soybean DAS-68416-4, there are no indications of an increased likelihood of establishment and spread of occasional feral soybean DAS-68416-4 plants, unless these plants are exposed to the intended herbicides. This will not result in different environmental impacts compared to conventional soybean. Considering the scope of the application EFSA-GMO-NL-2011-91, interactions with the biotic and abiotic environment are not considered to be relevant issues. Bioinformatic analyses of the inserted DNA identified sufficient sequence identity with bacterial DNA which could theoretically facilitate the transfer of a plant codon-optimised *pat* gene onto a plasmid of a soil bacterium. Illegitimate transfer of a plant-optimised *aad-12* gene was also considered. Based on the functional proteins encoded by these genes and their expected prevalence in environmental bacteria, the GMO Panel did not identify a concern in relation to horizontal gene transfer to bacteria. Therefore, considering the introduced traits, the outcome of the

comparative analysis, the routes of exposure and the limited exposure levels, the GMO Panel concludes that soybean DAS-68416-4 would not raise safety concerns in the event of accidental release of viable GM soybean seeds into the environment**.** The scope of the post-market environmental monitoring plan provided by the applicant and the reporting intervals are in line with the intended uses of soybean DAS-68416-4 and the GMO Panel guidelines on the post-market environmental monitoring of GM plants.

In delivering its scientific opinion, the GMO Panel took into account application EFSA-GMO-NL-2011-91, additional information provided by the applicant, scientific comments submitted by the Member States and relevant scientific publications. In conclusion, the GMO Panel considers that the information available for soybean DAS-68416-4 addresses the scientific comments raised by the Member States and that soybean DAS-68416-4, as described in this application, is as safe as its conventional counterpart and the tested non-GM soybean reference varieties with respect to potential effects on human and animal health and the environment in the context of the scope of this application.

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1. Introduction

Soybean DAS-68416-4 was developed to confer tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate ammonium-based herbicides. Tolerance to 2,4-D and other related phenoxy herbicides is provided by the expression of the aryloxyalkanoate dioxygenase-12 (AAD-12) protein from *Delftia acidovorans*. Tolerance to glufosinate ammonium-based herbicides is provided by the expression of the phosphinothricin acetyltransferase (PAT) protein from *Streptomyces viridochromogenes*.[1]

The assessment of potential consumer health risks resulting from 2,4-D residues and its metabolites in soybean DAS-68416-4 is outside the remit of the GMO Panel and needs to be performed upon request of an applicant in the framework of Regulation (EC) No 396/2005.

1.1. Background

On 25 January 2011, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands an application (Reference EFSA-GMO-NL-2011-91) for authorisation of genetically modified (GM) soybean DAS-68416-4 (Unique Identifier DAS-68416-4), submitted by Dow AgroSciences LLC within the framework of Regulation (EC) No 1829/2003 on GM food and feed.[2]

After receiving the application EFSA-GMO-NL-2011-91, 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. On 23 March 2011, 5 May 2011, 27 June 2011 and 19 August 2011 EFSA received additional information requested under completeness check (on 4 March 2011, 12 April 2011, 24 May 2011 and 20 July 2011, respectively). On 8 September 2011, 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 the 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 8 December 2012) to make their opinion known.

The GMO Panel requested additional information from the applicant on 5 December 2011, 30 January 2012, 20 April 2012, 7 September 2012, 1 July 2014, 28 November 2014, 16 February 2015, 19 February 2015, 6 March 2015, 1 April 2015, 24 June 2015, 15 September 2015, 2 October 2015, 23 March 2016, 26 April 2016 (EURL-JRC), 26 May 2016 and 29 September 2016. The applicant provided the requested information on 13 April 2012, 15 May 2012, 18 October 2013, 2 September 2014, 16 December 2014, 19 February 2015, 12 March 2015, 16 March 2015, 11 June 2015, 22 July 2015, 25 September 2015, 26 April 2016 (EURL-JRC), 29 April 2016, 13 May 2016, 13 June 2016 and 26 October 2016. The applicant also spontaneously provided additional information on 7 August 2012, 27 August 2012, 22 December 2015, 31 March 2016 (EURL sequence info) and 13 May 2016.

In the frame of contract OC/EFSA/UNIT/GMO/2013/01 – CT01, the contractor performed preparatory work and delivered reports on the methods applied by the applicant in performing bioinformatic analyses.

On 7 September 2016, the European Union Reference Laboratory (EURL-JRC) submitted to EFSA the report on the verification of sequencing data on event DAS-68416-4 received from the applicant.

In giving its scientific opinion on soybean DAS-68416-4 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.

[1] Dossier: Part I – Section D1.
[2] 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.
[3] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2011-00052
[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.

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 soybean DAS-68416-4 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/environment and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The GMO Panel was not requested to give an opinion on 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-2011-91, 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 soybean DAS-68416-4 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 the appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006a; EFSA GMO Panel, 2011a), the environmental risk assessment (ERA) of GM plants (EFSA GMO Panel, 2010a) and the post-market environmental monitoring (PMEM) of GM plants (EFSA GMO Panel, 2011b).

The comments raised by Member States are addressed in Annex G of EFSA's overall opinion[3] and were taken into consideration during the scientific risk assessment.

3. Assessment

3.1. Molecular characterisation

3.1.1. Evaluation of relevant scientific data

3.1.1.1. Transformation process and vector constructs

Soybean DAS-68416-4 was developed by *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation of cotyledonary nodes of soybean (*Glycine max* (L.) Merr.) line Maverick with the *A. tumefaciens* strain EHA101 containing the binary plasmid pDAB4468. The plasmid pDAB4468 contained two expression cassettes, *aad-12* and *pat*, between the right and left borders of the T-DNA.[5]

The *aad-12* expression cassette contains the following genetic elements: the constitutive *Arabidopsis thaliana* polyubiquitin UBQ10 promoter, 5'-untranslated region and intron; a codon-optimised version of the *aad-12* gene from *D. acidovorans*; and the 3'-untranslated region from the open reading frame (ORF) 23 of *A. tumefaciens* pTi15955 (AtuORF23), which includes a transcription terminator. The RB7-MAR matrix attachment region from *Nicotiana tabacum* was positioned next to the *aad-12* expression cassette to increase expression of the *aad-12* gene.

[5] Dossier: Part I – Sections C2 and C3.

The *pat* expression cassette consisted of the following elements: the promoter and 5'-untranslated region from the *Cassava vein mosaic virus* (CsVMV); a codon-optimised version of the *pat* gene from the bacterium *S. viridochromogenes*; and the 3'-untranslated region from the ORF1 of *A. tumefaciens* pTi15955 (AtuORF1), which includes a terminator and a polyadenylation site.

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

3.1.1.2. Transgene constructs in the GM plant[6]

Molecular characterisation of soybean DAS-68416-4 was performed by Southern analysis, polymerase chain reaction (PCR) and DNA sequence analysis, in order to determine copy number, size and organisation of the inserted sequences and to confirm the absence of plasmid backbone sequences. The approach used was acceptable both in terms of coverage and sensitivity.

Southern analysis indicated that soybean DAS-68416-4 contains a single insert, which consists of a single copy of the T-DNA in the same configuration as in the pDAB4468 vector. The insert and copy number were confirmed using multiple combinations of restriction endonucleases and 19 probes that covered all elements of the plasmid. No elements from the vector backbone were detected.[7]

The nucleotide sequence of the entire insert of soybean DAS-68416-4, together with 2,730 bp of the 5' and 1,082 bp of 3' flanking regions, was determined. The EURL-JRC checked the compliance of the sequencing data provided for event DAS-68416-4 with the requirements of its guidance.[8] The insert of 6,400 bp is identical to the T-DNA of pDAB4468, except for the insertion of 9 bp at the 3' end of the insert. A comparison of the sequences of the flanking regions with that of the pre-insertion locus indicated that 55 bp were deleted from soybean genomic DNA. No evidence was found for the interruption of any known endogenous gene in the soybean genome.

The results of segregation (Section 3.1.1.4.) and bioinformatic analyses established that the insert is located in the nuclear genome.[9]

Updated bioinformatic analyses of the amino acid sequences of the newly expressed AAD-12 and PAT proteins revealed no significant similarities to toxins and allergens. In addition, updated bioinformatics analyses of the newly created ORFs present within the insert or spanning the junctions between the insert and genomic DNA did not indicate significant similarities to toxins and allergens.[10]

3.1.1.3. Information on the expression of the insert[11]

Protein levels of AAD-12 and PAT were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from field trials performed at eight locations in the USA during the 2009 growing season (also used for comparative assessment, Section 3.2.1). Samples analysed included leaf (V5 and V10–12), root (R3), forage (R3) and seed (R8-maturity) from soybean DAS-68416-4 treated and not treated with 2,4-D, glufosinate ammonium-based herbicides or a combination of the two. The mean values, standard deviations and ranges of protein expression levels of AAD-12 and PAT in seed and forage are summarised in Table 1.

[6] Dossier: Part I – Section D2.
[7] Dossier: Part I – Section D2; additional information: 13/4/2012.
[8] Guideline for the submission of DNA sequences derived from genetically modified plants and associated annotations within the framework of Directive 2001/18/EC and Regulation (EC) No 1829/2003 (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).
[9] Dossier: Part I – Section D2; additional information: 13/4/2012, 16/12/2014 and 22/12/2015.
[10] Dossier: Part I – Section D2; additional information: 22/12/2015.
[11] Dossier: Part I – Section D3.

Table 1: Protein expression data (μg/g dry weight) for AAD-12 and PAT in soybean DAS-68416-4 seed and forage

		Untreated	Glufosinate treated	2,4-D treated	Glufosinate and 2,4-D treated
Seed	AAD-12	22.92[(a)] ± 4.17[(b)]	21.67 ± 4.47	20.19 ± 4.16	21.16 ± 4.63
		(16.29–32.18)[(c)]	(14.21–31.59)	(12.14–29.77)	(11.51–31.97)
	PAT	2.66 ± 0.46	2.66 ± 0.37	2.57 ± 0.4	2.62 ± 0.44
		(1.80–3.71)	(1.81–3.52)	(1.91–3.34)	(1.55–3.41)
Forage	AAD-12	41.95 ± 16.59	46.02 ± 12.73	43.73 ± 13.98	49.32 ± 11.97
		(0.56–75.14)	(28.35–75.67)	(23.63–73.67)	(26.78–68.94)
	PAT	4.01 ± 0.85	8.28 ± 13.64	4.23 ± 1.01	4.93 ± 0.57
		(< 0.06–5.34)	(3.46–58.56)	(0.42–5.58)	(3.04–5.90)

2,4-D: 2,4-dichlorophenoxyacetic acid; AAD-12: aryloxyalkanoate dioxygenase-12; PAT: phosphinothricin acetyltransferase.
Number of seed and forage samples is 28 both for untreated (unsprayed) and for herbicide treated plants.
(a): Mean.
(b): Standard deviation.
(c): Range.

3.1.1.4. Inheritance and stability of inserted DNA[12]

Genetic stability of the soybean DAS-68416-4 insert was assessed by Southern analysis of genomic DNA from three consecutive generations. The restriction enzyme/probe combinations used were sufficient to conclude that all the plants tested retained the single copy of the insert and flanking regions, which were stably inherited in subsequent generations.

Phenotypic stability was observed by segregation analysis of the 2,4-D tolerance trait of soybean DAS-68416-4. The results supported the presence of a single insertion, segregating in a Mendelian fashion.

3.1.2. Conclusions on molecular characterisation

The molecular characterisation data established that soybean DAS-68416-4 contains a single insert consisting of one copy of the *aad-12* and *pat* expression cassettes. Bioinformatic analyses of the sequences encoding the newly expressed proteins and other ORFs present within the insert or spanning the junctions between the insert and genomic DNA did not indicate significant similarities to toxins and allergens. The levels of the AAD-12 and PAT proteins were obtained and reported adequately. The stability of the inserted DNA and of the introduced herbicide tolerance traits was confirmed over several generations.

3.2. Comparative analysis

3.2.1. Evaluation of relevant scientific data

3.2.1.1. Choice of comparator and production of material for the comparative assessment[13]

Application EFSA-GMO-NL-2011-91 presents data on agronomic and phenotypic characteristics, as well as forage and seed composition, of soybean DAS-68416-4 derived from field trials performed at eight sites in the USA in 2009 (Table 2).

Table 2: Overview of comparative assessment studies with soybean DAS-68416-4 provided in application EFSA-GMO-NL-2011-91

Study focus	Study details	Comparators	Commercial reference varieties
Agronomic and phenotypic characteristics; composition	Field trials, 2009, USA (eight locations)	Maverick	Six non-GM varieties
Agronomic and phenotypic characteristics	Seed germination test	Maverick	None

[12] Dossier: Part I – Section D5.
[13] Dossier: Part I – Section D7.2.

The field trials were conducted in major soybean growing areas of the USA,[14] representing regions of diverse agronomic practices and environmental conditions. At each site, the following materials were grown in a randomised complete block design with four replicates: soybean DAS-68416-4 (DAS-68416-4/untreated), the non-GM comparator Maverick and three non-GM soybean reference varieties, all treated with required maintenance pesticides (including conventional herbicides); and soybean DAS-68416-4 treated with 2,4-D (DAS-68416-4/2,4-D), with glufosinate ammonium (DAS-68416-4/glufosinate) and with both 2,4-D and glufosinate ammonium (DAS-68416-4/2,4-D + glufosinate). In total (across sites), six non-GM soybean reference varieties[15] were included in the field trials for agronomic/phenotypic characteristics and composition (Table 2).

Soybean DAS-68416-4 was obtained using the non-GM soybean variety Maverick as recipient variety (Section 3.1.1.1). As documented by the pedigree, the line of soybean DAS-68416-4 used in the field trials was not crossed with other soybean lines. Maverick was used as comparator in the field trials (Table 2), and has the same genetic background as the line of soybean DAS-68416-4 used. The GMO Panel considers that this non-GM line is the appropriate conventional counterpart.

Statistical analysis of field trials data

The statistical analysis of the agronomic, phenotypic and compositional data from the 2009 field trials followed the recommendations of the GMO Panel (EFSA GMO Panel, 2010b, 2011a). This included, for each of the four treatments of soybean DAS-68416-4, the application of a difference test (between the GM soybean and its conventional counterpart) and an equivalence test (between the GM soybean and the set of non-GM soybean reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[16]

3.2.1.2. Agronomic and phenotypic analysis[17]

Agronomic and phenotypic characteristics tested under field conditions[18]

The agronomic and phenotypic parameters evaluated in the 2009 field trials were: (early) stand count, early population (as % planted seeds), days to 50% flowering, days to maturity, plant height, number of pods, number of seeds, final population (plant count), yield, seedling vigour, plant vigour (crop injury from herbicide application, scored at V4, R1 and R2) and lodging. Additionally, visually observable responses to naturally occurring diseases (disease incidence) and arthropod damage were recorded, in order to provide indications of altered stress responses of soybean DAS-68416-4 as compared with its conventional counterpart.

Of the 16 endpoints evaluated, nine[19] could be analysed with the combination of difference and equivalence testing described in Section 3.2.1.1, with the following results:

- For soybean DAS-68416-4/untreated, the test of difference identified statistically significant differences from the conventional counterpart for three endpoints ('number of seeds', 'stand count' and 'days to 50% flowering'). The test of equivalence between soybean DAS-68416-4/untreated and the non-GM soybean reference varieties indicated that 'number of seeds' and 'stand count' fell under equivalence category I, while 'days to 50% flowering'[20] fell under equivalence category III (non-equivalence is more likely than equivalence);
- For DAS-68416-4/2,4-D, a statistically significant difference was identified for the endpoint 'number of seeds', which fell under equivalence category I;
- For DAS-68416-4/glufosinate, statistically significant differences were identified for five endpoints ('days to maturity', 'number of seeds', 'stand count', 'early population' and 'final population'), which all fell under equivalence category I;
- For DAS-68416-4/2,4-D + glufosinate, no statistically significant differences were identified.

[14] One site each in Arkansas, Iowa, Indiana and Nebraska, and two sites each in Illinois and Missouri.
[15] Pioneer 93M62, LG Seeds C3884N, Arise 9E394, Phillips 363, Hisoy 38C60 and Hoffman H387.
[16] 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).
[17] Dossier: Part I – Section D7.4; additional information: 18/10/2013 and 2/9/2014.
[18] Dossier: Part I – Section D7.4; additional information: 7/8/2012, 27/8/2012, 18/10/2013 and 2/9/2014.
[19] The endpoints were: early population, days to 50% flowering, days to maturity, plant height, number of pods, number of seeds, final population, stand count and yield.
[20] Soybean DAS-68416-4/untreated: 946.3 ± 30.9 heat units; conventional counterpart: 958.8 ± 30.9 heat units.

The remaining seven endpoints[21] did not fulfil the assumptions for parametric testing and were analysed with the Wilcoxon Signed Rank (WSR) test. Significant differences with the conventional counterpart were identified for DAS-68416-4/glufosinate ('plant vigour' at stage V4) and DAS-68416-4/2,4-D + glufosinate ('disease incidence' and 'plant lodging'); however, the average values for the GM soybean were within the range of the non-GM commercial reference varieties.

In conclusion, none of the agronomic and phenotypic differences between soybean DAS-68416-4 and its conventional counterpart observed at field level were considered relevant, except for 'days to 50% flowering' for soybean DAS-68416-4/untreated. The difference identified in 'days to 50% flowering' is therefore further assessed for its potential environmental impact in Section 3.4.1.1.

Agronomic and phenotypic characteristics tested under controlled conditions[22]

Seed germination of soybean DAS-68416-4 was compared with that of its conventional counterpart under warm and cold conditions. Four replicates of 100 seeds for each line, in a randomised complete block design, were tested for each of the temperature treatments. The warm treatment consisted of exposure to a constant temperature of 25°C for 5 days, while the cold treatment consisted of exposure to 10°C for 7 days, followed by additional exposure to 25°C for 5 days. The germination rate of soybean DAS-68416-4 seeds under warm and cold conditions did not differ significantly from that of its conventional counterpart.

3.2.1.3. Compositional analysis[23]

Soybean forage and seeds harvested from the field trials in the USA in 2009 were analysed for 87 different constituents (nine in forage[24] and 78 in seeds[25]), including the key constituents recommended by the OECD (OECD, 2001). Considering the data on substrate specificity for AAD-12 (Section 3.3.1.2), the GMO Panel concluded that the spectrum of constituents chosen by the applicant was adequate. Seventeen seed constituents having more than 50% of the observations below the limit of quantification were excluded from the statistical analysis.[26]

Of the remaining 70 constituents, the test of equivalence could not be applied to a forage endpoint (NDF) and to five seed endpoints[27] because the estimated variation among the non-GM reference varieties was too small. Among those six endpoints, only iron level for DAS-68416-4/glufosinate was significantly different from that of the conventional counterpart (Table 3).

The test of difference and the test of equivalence could be applied to the remaining 64 endpoints, with the following results:

- For soybean DAS-68416-4/untreated, the test of difference identified statistically significant differences from the conventional counterpart for 22 constituents (2 in forage and 20 in seeds). The test of equivalence between soybean DAS-68416-4/untreated and the non-GM soybean reference varieties indicated that the level of 18 of the 22 constituents[28] fell under equivalence category I or II, while the level of four seed constituents fell under equivalence category III or IV (Table 3).

[21] The endpoints were: disease incidence, insect damage, plant lodging, seedling vigour and plant vigour.
[22] Additional information: 2/9/2014.
[23] Dossier: Part I – Section D7.1; additional information: 13/4/2012, 7/8/2012, 27/8/2012, 18/10/2013, 2/9/2014 and 16/3/2015.
[24] Proximates (crude protein, crude fat, ash, and moisture), carbohydrates by calculation, fibre fractions (acid detergent fibre (ADF) and neutral detergent fibre (NDF)), calcium, and phosphorus.
[25] Protein, fat, ash, moisture, carbohydrates, acid detergent fibre (ADF), neutral detergent fibre (NDF), total dietary fibre, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1), pentadecanoic acid (15:0), pentadecenoic acid (15:1), palmitic acid (16:0), palmitoleic acid (16:1), heptadecanoic acid (17:0), heptadecenoic acid (17:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), γ-linolenic acid (18:3), arachidic acid (20:0), eicosenoic acid (20:1), eicosadienoic acid (20:2), eicosatrienoic acid (20:3), arachidonic acid (20:4), behenic acid (22:0), β-carotene, thiamine HCl, riboflavin, niacin, pantothenic acid, pyridoxine HCl, folic acid, ascorbic acid, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, total tocopherol, total daidzein equivalent, total genistein equivalent, total glycitein equivalent, lectin (activity), phytic acid, raffinose, stachyose and trypsin inhibitor.
[26] These were: sodium, caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1), pentadecanoic acid (15:0), pentadecenoic acid (15:1), palmitoleic acid (16:1), heptadecanoic acid (17:0), heptadecenoic acid (17:1), γ-linolenic acid (18:3), eicosadienoic acid (20:2), eicosatrienoic acid (20:3), arachidonic acid (20:4), β-carotene and β-tocopherol.
[27] Aspartic acid, proline, serine, NDF and iron.
[28] In forage: ash and moisture. In seeds: isoleucine, leucine, methionine, phenylalanine, raffinose, stachyose, oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), ash, moisture, total fat, riboflavin, pantothenic acid and folic acid.

- For DAS-68416-4/2,4-D, statistically significant differences were identified for 23 constituents (2 in forage and 21 in seeds). The level of 19 of the 23 constituents[29] fell under equivalence category I or II, while the level of four seed constituents fell under equivalence category III or IV (Table 3).
- For DAS-68416-4/glufosinate, statistically significant differences were identified for 26 constituents (2 in forage and 24 in seeds). The level of 19 of the 26 constituents[30] fell under equivalence category I or II, while the level of seven seed constituents fell under equivalence category III or IV (Table 3).
- For DAS-68416-4/2,4-D + glufosinate, statistically significant differences were identified for 20 constituents (1 in forage and 19 in seeds). The level of 16 of the 20 constituents[31] fell under equivalence category I or II, while the level of four seed constituents fell under equivalence category III or IV (Table 3).

Table 3: Compositional endpoints that are further discussed based on the results of the statistical analysis: means (for the conventional counterpart and the GM soybean) and equivalence limits (from the non-GM reference varieties) estimated from the 2009 field trials

Endpoint	Conventional counterpart	Soybean DAS-68416-4				Equivalence limits from non-GM reference varieties
		Untreated[(a)]	2,4-D[(b)]	Glufosinate[(c)]	2,4-D + glufosinate[(d)]	
Moisture[(e)] (%FW)	13.59	13.22*	12.82*	13.13*	12.74*	(12.79, 14.31)
Arginine (%AA)	7.541	7.451*	7.476*	7.466*	7.456*	(7.528, 7.840)
Glutamic acid (%AA)	17.03	16.89*	16.92*	16.83*	16.97	(17.02, 17.80)
Histidine (%AA)	2.661	2.711*	2.693	2.653	2.653	(2.507, 2.699)
Leucine (%AA)	7.714	7.755*	7.754*	7.779*	7.762*	(7.615, 7.767)
Stearic acid (18:0) (%FA)	4.457	4.406	4.374*	4.353*	4.29*	(3.547, 4.186)
Folic acid (mg/kg DM)	2.772	2.514*	2.53*	2.465*	2.589*	(2.477, 3.375)
Lectin activity (HU/mg protein)	48.21	53.69	65.78*	53.54	56.03*	(21.89, 52.77)
Raffinose (%DM)	0.579	0.545*	0.563	0.543*	0.572	(0.544, 0.768)
Calcium (mg/g DM)	2.901	3.253*	3.053*	3.222*	2.999	(2.201, 3.144)
Iron (mg/kg DM)	90.62	102.9	86.95	111.2*	94.01	–

2,4-D: 2,4-dichlorophenoxyacetic acid; FW: fresh weight; DM: dry matter; %AA: percentage of total amino acids; %FA: percentage of total fatty acids; HU: haemagglutination unit; –: the equivalence test was not applied because the estimated variation among the non-GM reference varieties was too small.
For the GM soybean, significantly different entries are marked with an asterisk, while the outcomes of the test of equivalence are differentiated by greyscale backgrounds: white (equivalence categories I and II and iron, for which the test was not applied), light grey (equivalence category III) and dark grey (equivalence category IV).
(a): Sprayed only with conventional herbicides.
(b): Sprayed with 2,4-D.
(c): Sprayed with glufosinate ammonium-based herbicides.
(d): Sprayed with 2,4-D and glufosinate ammonium-based herbicides.
(e): Mean values shown for moisture were re-calculated by EFSA for higher numerical precision.

Regarding the differences in moisture, stearic acid and calcium and in four amino acids (Table 3), no further assessment was deemed necessary owing to the known biochemical roles of the compounds involved and to the small absolute magnitude of the reported changes. The increase in iron content in DAS-68416-4/glufosinate is not of concern (or benefit), considering the low absorption of iron (2% to < 5%) from phytate-rich legumes like soybean (Hurrell, 2003). Folic acid decreased in all GM soybean treatments; however, considering that soybean is not a relevant source of folic acid in

[29] In forage: ash and protein. In seeds: isoleucine, leucine, tryptophan, tyrosine, total glycitein equivalent, oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), eicosenoic (20:1), moisture, protein, total fat, calcium, zinc, pyridoxine HCl and folic acid.
[30] In forage: ash and protein. In seeds: alanine, isoleucine, tryptophan, tyrosine, stachyose, total glycitein equivalent, oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), ash, moisture, protein, total fat, phosphorus, riboflavin and pantothenic acid.
[31] In forage: ash, protein and phosphorus. In seeds: alanine, isoleucine, leucine, total glycitein equivalent, oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), protein, total fat, riboflavin, pantothenic acid and folic acid.

the human diet, and that animal feed is usually supplemented with folic acid, no concern was identified for food and feed safety and nutrition. As raffinose is considered an antinutrient, the decrease in raffinose observed in two out of four treatments of soybean DAS-68416-4 did not pose any food/feed safety concern.

Lectin activity[32] in soybean DAS-68416-4 was significantly different from that in the conventional counterpart for two of the four treatments (36% higher in DAS-68416-4/2,4-D and 16% higher in DAS-68416-4/2,4-D + glufosinate) and fell under equivalence category III or IV. Because of the known antinutritional properties of soybean lectins, the increase in lectin activity is further assessed for potential impact on food and feed safety in Section 3.3.1.

3.2.2. Conclusions on the comparative analysis

The increase in lectin activity (up to 36%) observed in soybean DAS-68416-4 with respect to its conventional counterpart is further discussed in Section 3.3.1. The GMO Panel concludes that none of the other differences identified in forage and seed composition between soybean DAS-68416-4 and the conventional counterpart, and none of those identified in the agronomic and phenotypic characteristics, needs further assessment regarding food and feed safety.

Based on the tested agronomic and phenotypic characteristics of soybean DAS-68416-4, no relevant differences were observed between soybean DAS-68416-4 and its conventional counterpart, except for 'days to 50% flowering' for soybean DAS-68416-4 not treated with the intended herbicides. The difference in 'days to 50% flowering' is further assessed for its potential environmental impact in Section 3.4.1.1.

3.3. Food/feed safety assessment

3.3.1. Evaluation of relevant scientific data

3.3.1.1. Effects of processing

Processed products

Soybean DAS-68416-4 will undergo existing production processes used for conventional soybean. No novel production process is envisaged.

Compositional analysis identified an increase in lectin activity (up to 36%) in DAS-68416-4 seeds compared to the conventional counterpart. Food/feed processing (e.g. soaking, heating, fermentation) is known to reduce the content and/or activity of soybean endogenous antinutrients, including lectins (Liener, 1994; Duranti and Gius, 1997; OECD, 2012). The applicant provided data on toasted meal, showing that levels of lectin activity in toasted meal from DAS-68416-4 were strongly reduced compared to those in unprocessed DAS-68416-4 seeds (Table 3).[33]

Newly expressed proteins

a) Effect of temperature on newly expressed proteins[34]

The thermal stability of the bacterial AAD-12 protein was evaluated by heating protein solutions for 30 min at 50, 70 and 95°C in a phosphate-based buffer solution. At all heating conditions (50–95°C) the enzymatic activity was eliminated and the protein lost more than 99% of its immunoreactivity. The molecular mass (~ 32 kDa) was unchanged. The temperature dependence of bacterial AAD-12 protein activity was examined after 6 min at different temperatures (1–60°C), using 2,4-D as a substrate, revealing considerable activity up to 40°C, and significantly decreased activity at 50 and 60°C.

The thermal stability of the bacterial PAT protein was evaluated by heating protein solutions for 30 min at different temperatures (25–95°C) in a buffer solution. The molecular mass of the PAT protein (~ 20 kDa) was unchanged at temperatures ≤ 55°C. At temperatures ≥ 55°C, > 99% of the enzymatic activity was lost with no residual activity detected above 75°C. At temperatures ≥ 37°C, the soluble PAT protein lost ≥ 91% of its immunoreactivity.

[32] The biological activity of soybean lectins was quantified using a haemagglutination assay with rabbit red blood cells (RBCs) (Liener, 1955). The activity was measured in haemagglutination units (HU): one HU corresponds to the level of test solution (serially diluted) that gives agglutination of 50% of the RBCs.
[33] Dossier: Part I – Section D7.10.2; additional information: 29/4/2016.
[34] Dossier: Part I – Section D7.8.1; additional information: 18/10/2013.

b) Effect of pH on newly expressed proteins[35]

The effect of pH on the *in vitro* activity of the bacterial AAD-12 was assessed using 2,4-D as a substrate and a mixed buffer system with pH varying from 5.5 to 9.5. Considerable activity after 6 min was observed over a narrow window, between pH 6 and 7.5 with an optimum at 7.

The effect of pH on the *in vitro* activity of the bacterial PAT was assessed using acetyl-CoA and glufosinate as substrates and a mixed buffer system with pH at 3, 8 and 11. The enzyme activity was significantly reduced after 10 min, at pH 3 and 11, showing highest activity at pH 8; the molecular mass (~ 20 kDa) was unchanged at acidic, neutral, and basic pH's.

3.3.1.2. Toxicology

Soybean DAS-68416-4 expresses two new proteins, AAD-12 and PAT (see Section 3.1.1).

Proteins used for safety assessment

Given the technical restraints in producing large enough quantities of the proteins from plants for safety testing, these proteins were recombinantly produced in *Pseudomonas fluorescens.* Prior to safety studies, a set of biochemical methods was employed to demonstrate the equivalence between soybean- and microbe-derived proteins. Purified proteins from these two sources were characterised and compared in terms of their physicochemical, structural and functional properties.

a) AAD-12 characterisation and equivalence[36]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that plant- and microbe-derived AAD-12 proteins had the expected molecular weight of ~ 32 kDa and were comparably immunoreactive to AAD-12 protein specific antibodies. In addition, glycosylation detection analysis demonstrated that the AAD-12 proteins were not glycosylated. Amino acid sequence analysis by mass spectrometry methods showed that both proteins matched their expected sequence. These data also showed that the N-terminal methionine of both proteins was truncated, while alanine 2 of the plant protein was also acetylated. Additional variants of the plant protein were identified that were truncated up to threonine 9. Such modifications are common in eukaryotic proteins (e.g. Polevoda and Sherman, 2000). The C-termini of the plant- and microbial-derived proteins were identical and fully matched the theoretical AAD-12 sequence. Functional equivalence was demonstrated by a biochemical *in vitro* activity assay which showed that both proteins had comparable activity for the intended herbicide. Plant- and microbial-produced AAD-12 proteins were also screened for their ability to utilise certain endogenous plant substrates and none of them were metabolised by AAD-12.

b) PAT characterisation and equivalence[37]

The equivalence between the plant- and microbe-derived PAT proteins was demonstrated by SDS-PAGE and western blot analysis. The results from these analyses showed that both proteins migrated to the expected molecular weight of ~ 20.5 kDa. In addition, western blot analysis showed that both proteins were comparably immunoreactive to PAT specific antibodies. Functional equivalence was demonstrated by a biochemical *in vitro* activity assay which showed that both proteins had comparable activity for the intended herbicide.

The protein characterisation data comparing the structural, biochemical and functional properties of plant- and microbial-derived AAD-12 and PAT proteins indicate that these proteins are equivalent. Therefore, the GMO Panel accepts the use of the AAD-12 and PAT proteins expressed in bacteria for the safety studies.

Toxicological assessment of newly expressed proteins

The PAT protein has been previously assessed by the GMO Panel (e.g. EFSA 2007; EFSA GMO Panel, 2011c, 2013a,b), and no safety concerns for humans and animals were identified. Updated bioinformatics analyses did not reveal similarities of the PAT protein to known toxins.[38] The GMO Panel is not aware of any new information that would change these conclusions. The GMO Panel concludes that the PAT protein does not raise safety concerns.

[35] Additional information: 18/10/2013.
[36] Dossier: Part I – Section D7.8.1; additional information: 12/3/2012, 11/6/2015 and 13/5/2016.
[37] Dossier: Part I – Section D7.8.1; additional information: 13/5/2016.
[38] Dossier: Part I – Section D7.8.1; additional information: 18/10/2013, 2/9/2014 and 22/12/2015.

The newly expressed AAD-12 protein is assessed below.

a) Bioinformatic studies[38]

Bioinformatic analysis of the amino acid sequence of the AAD-12 protein expressed in DAS-68416-4 soybean revealed no relevant similarities to known toxic proteins (Section 3.1.1.2).

b) *In vitro* degradation studies[39]

The resistance to degradation by pepsin of the bacterial AAD-12 and PAT proteins was investigated in solutions at pH ~ 1.2 in two independent studies. The integrity of the test proteins in probes taken at various time points was analysed by SDS-PAGE followed by protein staining or western blot. The AAD-12 protein was degraded by pepsin within 30 s. The PAT protein was degraded by pepsin within 1 min.

c) Acute oral toxicity testing

The bacterial AAD-12 protein was administered by oral gavage at a dose of 2,000 mg/kg body weight (bw) to male and female Crl:CD1(ICR) mice. No adverse effects related to the AAD-12 protein were observed.[40]

A bacterial PAT protein was administered by oral gavage at a dose of 5,000 mg/kg bw to male and female Crl:CD1 mice. No adverse effects related to the PAT protein were observed.[41]

The GMO Panel is of the opinion that acute toxicity testing of the newly expressed proteins is of little additional value for the risk assessment of the repeated consumption of food and feed from GM plants by humans and animals.

d) 28-Day repeated dose toxicity study

The applicant provided a 28-day oral repeated dose toxicity study in mice[40] to investigate the potential toxicity of the AAD-12 protein. However, the GMO Panel did not consider the overall study design adequate to identify the potential hazard of the AAD-12 protein, because of the low doses of AAD-12 protein tested (highest target dose level approximately 47 mg/kg bw per day, corresponding to an actual dose of approximately 17 mg/kg bw per day) and the limited number of animals used in treatment groups (5 per sex per group) which is not considered sufficient to obtain an adequate statistical power (EFSA GMO Panel, 2011a).

The GMO Panel requested another 28-day oral repeated dose toxicity study in rodents, to support the safety assessment of the AAD-12 protein, with a sufficient number of animals to obtain an adequate statistical power, selecting the doses according to OECD TG 407[42] in order to induce adverse effects at the highest dose, or following a limit test approach if toxicity is not expected. In the second 28-day repeated dose toxicity study using mice,[43] the number of animals per treatment group was in line with EFSA GMO Panel (2011a), but the highest target dose selected (142 mg/kg bw per day) was too low for an adequate hazard identification, as also was the case in the first 28-day study submitted by the applicant. Therefore, the GMO Panel did not consider this study in the risk assessment. The study was withdrawn by the applicant.[44]

The applicant provided a new 28-day oral repeated dose toxicity study in mice,[45] which was conducted in accordance with OECD TG 407 and in compliance with the principles of Good Laboratory Practice (GLP). Groups of singly caged Crl:CD1(ICR) mice (11 per sex per group, approximately 8 weeks old at study start) were administered by gavage the AAD-12 protein (in 0.5% METHOCEL™) at a targeted nominal dose of 1,100 mg/kg bw per day (AAD-12 protein group), the vehicle alone (vehicle control group), or bovine serum albumin (BSA) at a targeted nominal dose of 1,100 mg/kg bw per day (BSA control group).

[39] Dossier: Part I – Section D7.9.1; additional information: 18/10/2013 and 2/9/2014.
[40] Dossier: Part I – Section D7.8.1.
[41] Dossier: Part I – Section D7.8.1; additional information: 19/2/2015. The bacterial recombinant expression system was not specified in the study report.
[42] OECD (Organisation for Economic Co-operation and Development), Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4.
[43] Additional information: 23/11/2015 and 13/5/2016.
[44] Applicant to EFSA letter – 2/3/2016.
[45] Additional information: 13/5/2016.

The ADD-12 protein and BSA protein dosing formulations were prepared daily. Samples of the ADD-12 protein and BSA protein dosing formulations along with vehicle were taken from the first mix, from a mix near the middle, and from a mix towards the end of the study for dose confirmation and homogeneity analyses.

Feed and water were provided *ad libitum*. During the treatment period, the animals were checked daily for mortality and general clinical signs. Detailed clinical observations were conducted on all animals pretreatment and then weekly. Ophthalmoscopy was carried out before the start and at the end of the treatment period. Body weights were recorded on test days 1, 2, 3, 4, 8, 15, 22 and 29 (terminal body weight) and body weight gains were calculated relative to test day 1. Feed consumption was determined on test days 1–2, 2–3, 3–4, 4–8, 8–15, and 22–29. At the end of the treatment period, blood samples were taken and haematological, coagulation and clinical chemistry analyses were performed. All animals were sacrificed and underwent a detailed necropsy examination with selected organs weighed. Organs and tissues from all animals were subjected to a comprehensive histological examination.

The GMO Panel noted that the AAD-12 protein formulations were prepared daily from the powdered test material stored at approximately 4°C until use. Stability tests on the powdered test material (i.e. the lyophilised AAD-12 protein) were not performed as part of this study. According to the study report,[45] the lyophilised AAD-12 protein was determined to be stable for 81 months under refrigerated storage conditions, as part of previous studies[45]*;* however, the GMO Panel noted that stability of the AAD-12 protein was not documented in these previous studies. Therefore, the concentration of AAD-12 protein of 33.1% in the powdered test material following storage has not been confirmed. However, as dose confirmation analyses were performed on the dosing formulations both at the start and towards the end of the study, the GMO Panel considered that this is not a major limitation compromising the 28-day study.

The GMO Panel noted that haematology and clinical chemistry analyses were conducted on six mice/gender per group and coagulation (prothrombin time) was conducted on the remaining five animals in each group. The reasoning provided by the applicant was practical limitations in obtaining sufficient quantities of blood from mice for haematology and clinical chemistry, and coagulation examinations in the same animal. However, it is well known that when mice are used as the test animal, additional animals may be needed in each dose group to conduct all required determinations. The GMO Panel also noted that the animals were not fasted prior to necropsy and blood collection, as recommended in OECD TG 407.

The AAD-12 protein group was statistically compared to the BSA control group; the latter was also compared to the vehicle control group in order to assess potential effects of the higher protein intake. For all the continuous parameters a two-way analysis of variance (ANOVA; factors: sex and dose) for the two sexes combined, in order to account for sex-dose interactions, and a one-way ANOVA (factor: dose) separately for each sex were performed. For all parameters, in case a statistically significant dose effect was found with the one- or two-way ANOVA, each individual dose group was compared to the control group using Dunnett's test. For body weight gains, globulin, albumin/globulin ratio, red blood cell (RBC) indices, and differential white blood cell (WBC) counts only descriptive statistics were reported.

The results of the dose confirmation analyses revealed that the average recoveries for AAD-12 and BSA protein in 0.5% METHOCEL™ were 71.7% and 73.2%, respectively, based on nominal dosing suspensions at 110 mg/ml. The average recoveries were therefore within the acceptable experimental variation (70–120%). The GMO Panel noted that based on the measured concentration of AAD-12 protein in the dosing suspension, the actual dose administered was 789 mg/kg bw per day. The results of the homogeneity analyses indicated that the preparations were homogeneously mixed.

The few statistically significant differences between the BSA control and vehicle control groups in the examined parameters were considered by the GMO Panel to be within normal biological variability; therefore, both the vehicle control and the BSA control groups were considered suitable to be used as the control groups for the comparison and evaluation of data from the AAD-12 protein group.

No mortality occurred during the treatment period. The GMO Panel considered that the isolated clinical findings and the few ophthalmic changes observed at the end of the study in the AAD-12 protein group were not treatment-related.

No statistically significant differences in body weight or body weight gain were observed in the AAD-12 protein group compared to the BSA control group.

In comparison with the BSA control group, males of the AAD-12 protein group showed statistically significantly lower feed consumption during specific time periods (days 1–2, 2–3, 15–22, 22–29); these

differences were not considered as an adverse treatment-related effect by the GMO Panel as there were no statistically significant differences in body weights and body weight gains in animals of the AAD-12 protein group when compared with the BSA control group.

Haematology analysis showed statistically significantly higher WBC in males of the AAD-12 protein group when compared with the BSA control group. This finding was largely due to changes in two individual animals (one with an abscess in the neck region and a granulomatous inflammation around the oesophagus; the other showed chronic inflammation of the mediastinal tissue). The changes which largely resulted from (not significantly) higher values in neutrophil counts[46] were attributed by the applicant to inadvertent trauma associated with repeated oral gavage and not to treatment with the AAD-12 protein. The GMO Panel agrees with the interpretation by the applicant.

The platelet (PLT) count in males treated with the AAD-12 protein was statistically significantly higher compared with the BSA control group. This finding was, according to the applicant,[46] attributed to one animal with inadvertent trauma (abscess in the neck region and a granulomatous inflammation around the oesophagus) related to repeated oral gavaging procedure and not related to the treatment with the AAD-12 protein. After removing this animal from the statistical analysis, the increase in the PLT count showed no statistical significance. The increase was considered by the applicant not to be related to the treatment with the AAD-12 protein. The GMO Panel noted that even after removing this single animal with the highest PLT count ($1{,}836 \times 10^3/\mu L$) the mean PLT count in the male AAD-12 protein group ($1{,}499 \times 10^3/\mu L$ vs $1{,}555 \times 10^3/\mu L$) was still higher compared to both the BSA protein group ($1{,}321 \times 10^3/\mu L$) and the vehicle control group ($1{,}302 \times 10^3/\mu L$). The GMO Panel could not exclude that the increased PLT count was related to the treatment with the ADD-12 protein for the following reasons: (1) the PLT counts for the individual animals in the male AAD-12 protein group (range $1{,}386–1{,}836 \times 10^3/\mu L$) were either comparable to or higher than the two highest PLT counts for individual animals in the BSA protein group (1,454 and $1{,}510 \times 10^3/\mu L$) and the vehicle control group (1,398 and $1{,}511 \times 10^3/\mu L$); (2) the SD (87) for the AAD-12 protein group after removing the single animal with the highest PLT count was lower than that for the control groups (BSA protein: 168, vehicle control: 143); and (3) the PLT counts for five out of six of the individual animals in the male AAD-12 protein group (range $1{,}466–1{,}836 \times 10^3/\mu L$) were higher than the historical control range ($1{,}205–1{,}417 \times 10^3/\mu L$) presented in the study report (five studies between 2012 and 2016). However, as the increase was slight and not statistically significant after removing the single animal with the highest PLT count the GMO Panel considered that this difference was not toxicologically relevant.

In females treated with the AAD-12 protein, the reticulocyte count (RET) was slightly, but statistically significantly lower compared with the BSA control group; the mean value was very close to that of the vehicle control group and within the historical control range. This difference was not considered to be toxicologically relevant by the GMO Panel as there were no differences in related haematological parameters.

There were no other significant differences in haematological and clinical chemistry parameters, or in the prothrombin time. However, the GMO Panel noted that the haematological, clinical chemistry and coagulation examinations were only performed on six or five animals per gender per group, and thus, the GMO Panel recommendation to use a higher number of animals (EFSA GMO Panel, 2011a) was not fulfilled for the examination of these parameters.

Organ weight determinations showed no statistically significant differences except for a lower absolute (but not relative) epididymides weight (7.6%) in males treated with the ADD-12 protein compared with the BSA control group. This difference was not considered as toxicologically relevant by the GMO Panel as there was no difference in the relative epididymides weight.

Macroscopic examinations at necropsy revealed no gross pathological findings related to the treatment with the AAD-12 protein. Microscopic examinations of selected organs and tissues identified no treatment-related differences in the incidences and severity of the histopathological findings between the groups.

The GMO Panel noted that the haematological, clinical chemistry and coagulation examinations were only performed on six or five animals per gender per group (in line with OECD TG 407 minimum requirements), and thus, the GMO Panel recommendation to use a higher number of animals to ensure appropriate statistical power (EFSA GMO Panel, 2011a) was not fulfilled for the examination of these parameters. Nevertheless, the GMO Panel concluded that no adverse effects were observed in this

[46] Additional information: 26/10/2016.

study after a 28-day administration of the AAD-12 protein to mice at the dose tested (789 mg/kg bw per day).

Toxicological assessment of components other than newly expressed proteins

No new constituents other than the AAD-12 and PAT proteins are expressed in soybean DAS-68416-4.

With the exception of an increased lectin activity (up to 36%) in soybean DAS-68416-4 for two out of four treatments, no relevant changes in the composition of the GM soybean were detected in the comparative compositional analysis (Table 3).

Lectins are a superfamily of proteins selectively binding carbohydrates, and function as recognition molecules in cell–molecule and cell–cell interactions in a variety of biological systems (Sharon and Lis, 2004; Miyake et al., 2007). Lectins are natural components in plants used for food and feed (Nachbar and Oppenheim, 1980; Peumans and Van Damme, 1996; Vasconcelos and Oliveira, 2004) and are widely distributed among Leguminosae, including soybean (Gupta, 1987). The ingestion of legumes containing high levels of certain lectins may be associated with gastrointestinal effects in humans and animals (Noah et al., 1980; Rodhouse et al., 1990; Bardocz et al., 1995; Grant et al., 1995).

In its toxicological assessment of the increase in lectin activity, the GMO Panel took into account the following:

1) The toxicity of raw soybean lectins is low compared to other commonly consumed legumes (Nasi et al., 2009), consisting of reduced growth performance and transient small intestine hypertrophy in experimental feeding studies (Grant et al., 1995).
2) Current industrial and traditional home processing practices are known to considerably reduce lectin content and/or activity in legumes, including soybean (Liener, 1994; Duranti and Gius, 1997; OECD, 2012), and the safe use of soybean depends on such practices (König et al., 2004). However, it cannot be excluded that residual lectin activity is still present in processed soybean products (Peumans and Van Damme, 1996; Rizzi et al., 2003; Vasconcelos and Oliveira, 2004).
3) The observed increase was considered in the context of the high variability reported for lectin activity and lectin protein content in raw soybean (Becker-Ritt et al., 2004; OECD, 2012; Maria John et al., 2017). Regarding any possible impact of the observed increase on the levels of residual activity in processed products, it was considered that (measurable) residual activity is also characterised by high variability (across different products (Calderon de la Barca et al., 1991) and between different samples of the same product (Maenz et al., 1999)).

Based on these considerations, the GMO Panel is of the opinion that the observed increase (up to 36%) in lectin activity in raw soybean DAS-68416-4 is unlikely to raise additional toxicological concerns for soybean DAS-68416-4 with respect to conventional soybean varieties.

The nutritional impact of the increase in lectin activity in soybean DAS-68416-4 will be discussed in Section 3.3.1.5.

3.3.1.3. Animal studies with the food/feed derived from GM plants

The applicant provided a 42-day feeding study with a total of 600 (half male and half female) chickens for fattening (day-old Ross 708).[47] The birds were randomly allocated to five dietary treatment groups with 120 chicks per treatment (12 pens per treatment, six pens for male and six for female, ten birds per pen). Birds fed diets containing soybean DAS-68416-4 (verified by PCR) were compared to those fed diets containing the conventional counterpart (control) or each of three non-GM commercial soybean varieties (LG C3540, Pioneer 93B82 and HiSoy 38C60). The starter (1–14 days), grower (15–28 days) and finisher (29–42 days) diets consisted of 40.4%, 36.4% and 31.5% soybean meal, respectively. The main other component was a commercial corn meal. Before feed formulation, all soybean varieties were analysed for proximates, fibre fractions, isoflavones, minerals, amino acids, antinutrients and mycotoxins. The metabolisable energy was calculated for each maize grain source. All diets were balanced for metabolisable energy, crude protein and amino acids. Feed in mash form and water were provided to the birds for *ad libitum* intake. The presence of AAD-12 and PAT proteins was confirmed analytically in unprocessed seeds and toasted meal. Chickens were observed twice daily for clinical signs; deaths were recorded and necropsy performed in all cases. Body weight and feed intake were measured on days 1, 14, 28 and 42. At the end of the study, four birds per pen were taken for carcass evaluation (yield, dressing percentage, weight of thighs, breast, wings, legs, abdominal fat and

[47] Dossier: Part I – Section D7.10.2.

whole liver). ANOVA (pen was considered as the experimental unit, dietary treatment and gender as fixed factors) was applied for statistical evaluation. Four pairwise comparisons between the GM group and each of the non-GM groups were made using Dunnett's test. Overall mortality was low (2.3%) with no significant difference between the groups. Significant treatment-by-sex interactions were detected for final weight, daily gain and daily feed intake. No significant differences between the GM group and the control group were detected within genders for final weight and daily gain, except for daily feed intake. Feed:gain ratio showed similar differences (average 1.57 for females and 1.47 for males). Carcass characteristics and liver weight did not show significant differences between the GM and the control group; significantly higher relative breast weight was found in birds fed diets containing one of the commercial varieties; and relative higher thigh weight was found in birds fed diets containing another commercial variety.

The GMO Panel concludes that administration of diets containing up to 40.4% soybean DAS-68416-4 to broilers, up to 42 days, did not cause adverse effects. Moreover, the measured performance endpoints were similar between groups fed balanced diets containing GM and non-GM soybean.

3.3.1.4. Allergenicity

The strategies to assess the potential risk of allergenicity focus on the source of the recombinant protein, on the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons and on whether the transformation may have altered the allergenic properties of the modified plant.

Assessment of allergenicity of the newly expressed proteins[48]

A weight-of-evidence approach was followed, taking into account all of the information obtained on the newly expressed proteins, as no single piece of information or experimental method yield sufficient evidence to predict allergenicity (EFSA, 2006a; Codex Alimentarius, 2009; EFSA GMO Panel, 2010c).

The *aad-12* gene originates from *D. acidovorans* and the *pat* gene originates from *S. viridochromogenes*, microorganisms which are not considered to be common allergenic sources.

Updated bioinformatic analyses of the amino acid sequences of the AAD-12 and PAT proteins, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no significant similarities to known allergens. In addition, the applicant also performed analyses searching for matches of eight contiguous identical amino acid sequences between the AAD-12 and PAT proteins and known allergens, which confirmed the outcome of the previous bioinformatic analysis.

The study on resistance to degradation of the AAD-12 and the PAT proteins by pepsin has been described in Section 3.3.1.2.b.

The GMO Panel has previously evaluated the safety of the PAT protein in the context of several other applications and no concerns about allergenicity were identified (e.g. EFSA 2007; EFSA GMO Panel, 2011c, 2013a,b).

There is no information available on the structure or function of the newly expressed AAD-12 and PAT proteins that would suggest an adjuvant effect of the individual proteins or their simultaneous presence in soybean DAS-68416-4 resulting in or increasing an eventual specific immunoglobulin E (IgE) response to a bystander protein.

In the context of the present application, the GMO Panel considers that there are no indications that the newly expressed AAD-12 and PAT proteins, individually or simultaneously present, in soybean DAS-68416-4 may be allergenic.

Assessment of allergenicity of the whole GM plant or crop[49]

Soybean is considered to be a common allergenic food (OECD, 2012).[50] Therefore, any potential change in the endogenous allergenicity of the GM plant when compared with that of the conventional non-GM variety should be assessed in line with the applicable GMO Panel guidance document (EFSA, 2006a). The applicant performed *in vitro* allergenicity studies with extracts of soybeans DAS-68416-4, its conventional counterpart (Maverick) and three non-GM commercial soybean varieties.

Specifically, the applicant performed two-dimensional (2D) electrophoresis of extracts of soybean DAS-68416-4 and its conventional counterpart followed by western blotting using individual sera from

[48] Dossier: Part I – Section D7.9.1; additional information: 18/10/2013, 2/9/2014 and 22/12/2015.
[49] Dossier: Part I – Section D7.9.2.
[50] Directive 2007/68/EC of the European Parliament and of the Council of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. OJ L 310, 27.11.2007, p. 11–14.

six allergic humans to soybean. In addition, immunoglobulin E (IgE) inhibition ELISA studies using individual sera from four allergic humans to soybean were also carried out. These studies showed no meaningful differences in the IgE-binding patterns between the extracts of proteins derived from soybean DAS-68416-4 and its conventional counterpart.

The applicant also performed one-dimensional (1D) electrophoresis of extracts of soybean DAS-68416-4, its conventional counterpart and three commercial non-GM soybean varieties followed by western blot analysis using individual as well as pooled sera from 20 individuals allergic to soybean. Inhibition ELISA studies were also carried out using pooled sera from 20 individuals allergic to soybean. The GMO Panel has previously indicated the limitations of the 1D-PAGE gels and the use of pooled sera for the allergenicity assessment (see Annex 4 and Annex 5 of EFSA GMO Panel, 2010c).

In the context of this application and considering the information above, the GMO Panel is of the opinion that there is no evidence that the genetic modification might significantly change the overall allergenicity of soybean DAS-68416-4 when compared with that of its conventional counterpart.

3.3.1.5. Nutritional assessment of GM food/feed

Comparison of the composition of soybean DAS-68416-4 with its conventional counterpart and non-GM reference varieties identified differences in the lectin activity in seeds (up to 36% increase in the GM soybean, Table 3). Lectins are known to be antinutritional factors for humans and animals (Section 3.3.1.2).

The presence of lectins in common plant foods, such as Leguminosae, is well known (Vasconcelos and Oliveira, 2004; Peumans and Van Damme, 1996). Current industrial and traditional home processing practices are used to considerably reduce lectin content and/or activity in legumes, including soybean (Liener, 1994; Duranti and Gius, 1997; OECD, 2012). It cannot be excluded that residual lectin activity is still present in processed soybean food products (Calderon de la Barca et al., 1991; Peumans and Van Damme, 1996; Rizzi et al., 2003); hence, dietary exposure to functionally active lectins at residual levels in processed products is considered a common event (Nachbar and Oppenheim, 1980; Vasconcelos and Oliveira, 2004).

Soybean oil is the predominant soybean product for human consumption. The oil is obtained by fractionation of soybean seeds and is almost entirely composed of fat, with a negligible non-fat fraction (0.3%) consisting of moisture, insoluble, and volatile matter (Gandhi, 2009).[51] Even in the worst-case scenario, assuming that the entire non-fat fraction of the oil derived from soybean DAS68416-4 are lectins, the observed increase in lectin activity in soybean DAS-68416-4 (up to 36%) would not raise concerns compared to oil from non-GM soybeans. Other processing procedures, such as soaking, heating and fermentation, are known to considerably reduce active lectin content in soybean (Gupta, 1987; Codex Alimentarius, 1989, 2013, 2015; Reddy and Pearson, 1994; Duranti and Gius, 1997; Lajolo and Genovese, 2002; OECD, 2012). Soybean sprouting is also known to be accompanied by a considerable decrease in active lectin content (Rizzi et al., 2003). In the context of this application, it was also demonstrated that toasting is effective in lowering lectin activity of DAS-68416-4 soybean seeds to negligible levels.[47] The GMO Panel considers that the additional intake of active lectins from soybean DAS-68416-4 food products (deriving from the 36% increase in the raw material) is likely to be negligible compared to the habitual dietary intake of lectins from non-GM soybean food products.

Soybean meal is the by-product of the extraction of soybean oil and it is the most important protein source used to feed farm animals. In the solvent extraction process, the soybeans are cracked, heated and flaked, and the oil is extracted by solvent (usually hexane). The obtained by-product is then subjected to treatments that improve its nutritional value by a decrease in the activity of antinutritional factors (e.g. lectins and enzyme inhibitors). Current conditions during the commercial processing of soybean into commodity oil, meal and other products, and on-farm processing conditions (e.g. soaking, roasting, extrusion and micronisation) have consistently shown the ability to reduce the presence and/or activity of lectins and enzyme inhibitors to the extent that they can be consumed by monogastric animals. Quality processes and testing such as urease activity are in place, mainly in the commercial production, to assure that many of these commercial products have undergone the proper processing procedures for use in feed applications.[52] Urease activity is highly positively correlated with lectin and trypsin inhibitor activity (Fasina et al., 2003). The GMO Panel considers that, within the

[51] Additional information: 29/4/2016.

[52] Commission Regulation (EU) No 68/2013, on the Catalogue of feed materials http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0068&from=EN

frame of good farming practices described above and considering current processing practices, the observed increase in lectin activity is unlikely to influence animal nutrition.

The GMO Panel concluded that food and feed derived from soybean DAS-68416-4 are expected to have no adverse nutritional impact, as compared to those from its conventional counterpart and commercial non-GM reference varieties.

3.3.1.6. Post-market monitoring of GM food/feed

There was no indication that food/feed products derived from soybean DAS-68416-4 are less safe or nutritious than those derived from its conventional counterpart or the non-GM commercial varieties (Sections 3.2.1.3, 3.3.1.2 and 3.3.1.5). Therefore, in line with EFSA (2006a) and EFSA GMO Panel (2011a), the GMO Panel is of the opinion that post-market monitoring of the GM food/feed is unnecessary.

3.3.2. Conclusions on the food/feed safety assessment

The safety assessment identified no concerns regarding the potential toxicity of the AAD-12 and PAT proteins newly expressed in soybean DAS-68416-4, considering their structural and functional properties, the results of bioinformatic analyses and the results of a sub-acute 28-day toxicity study on AAD-12. The GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity with the AAD-12 and PAT proteins or regarding the overall allergenicity of DAS-68416-4. The observed increase in lectin activity in raw soybean DAS-68416-4 is unlikely to raise additional concerns for food/feed safety and nutrition for the GM soybean as compared to its conventional counterpart and the non-GM commercial varieties. No other changes in soybean DAS-68416-4 composition relevant for food/feed safety and nutrition were identified. Considering current soybean processing, soybean DAS-68416-4 is as safe and nutritious as its conventional counterpart and the non-GM commercial varieties.

3.4. Environmental risk assessment and monitoring plan

3.4.1. Evaluation of relevant scientific data

Considering the scope of the application EFSA-GMO-NL-2011-91 (which excludes cultivation), the ERA of soybean DAS-68416-4 is mainly concerned with: (1) the exposure of bacteria to recombinant DNA in the gastrointestinal tract of animals fed GM material and bacteria present in environments exposed to their faecal material (manure and faeces), and (2) the accidental release into the environment of viable soybean DAS-68416-4 seeds during transportation and/or processing (EFSA GMO Panel, 2010a).

3.4.1.1. Environmental risk assessment

Persistence and invasiveness of the GM plant[53]

Cultivated soybean (*G. max* (L.) Merr.) is a species in the subgenus *Soja* of the genus *Glycine*. The species originated from eastern Asia and is a highly domesticated crop (Lu, 2005). Cultivated soybean seeds rarely display any dormancy characteristics and can grow as volunteers in the year after cultivation only under certain environmental conditions. If volunteers occur, they do not compete well with the succeeding crop, and can easily be controlled mechanically or chemically (OECD, 2000). The presence of volunteers of *G. max* was occasionally reported in some areas of Italy where soybean is intensively cultivated (Celesti-Grapow et al., 2010). However, soybean seeds usually do not survive during the winter owing to herbivory, rotting and germination, or owing to management practices prior to planting the subsequent crop (Owen, 2005). Also, survival of soybean plants outside cultivation areas is limited mainly by a combination of low competitiveness, absence of a dormancy phase, and susceptibility to plant pathogens and cold climatic conditions.

The applicant presented agronomic and phenotypic data on soybean DAS-68416-4 gathered from field trials conducted in soybean growing areas in the USA (Table 2). The data showed a statistically significant reduction in 'days to 50% flowering', for which non-equivalence between soybean DAS-68416-4/untreated and the non-GM soybean reference varieties was more likely than equivalence. No relevant differences in the other measured plant characteristics were identified (Section 3.2.1.2). Due

[53] Dossier: Part I – Sections D4, D9.1 and D9.2; additional information: 18/10/2013 and 2/9/2014.

to the low survival capacity of soybean, the observed difference in 'days to 50% flowering' is unlikely to change the fitness (e.g. survival, fecundity, competitiveness) or invasiveness characteristics of soybean DAS-68416-4 plants.

As the general characteristics of soybean DAS-68416-4 remain unchanged compared to its conventional counterpart, it is considered very unlikely that soybean DAS-68416-4 will differ from conventional soybean 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 soybean DAS-81419-2 seeds during transportation and processing.

The GMO Panel is not aware of any scientific report of increased survival capacity, including overwintering, of existing GM soybeans varieties (Dorokhov et al., 2004; Owen, 2005; Bagavathiannan and Van Acker, 2008; Lee et al., 2009). Therefore, the GMO Panel is of the opinion that the likelihood of environmental effects of occasional feral soybean DAS-68416-4 plants in Europe will not be different from that of conventional soybean varieties.

Effects of gene transfer[54]

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

1) Plant-to-bacteria gene transfer[54]

Genomic DNA is a component of many food and feed products derived from soybean. It is well documented that DNA present in food and feed becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, a low level of exposure of fragments of ingested DNA, including the recombinant fraction of such DNA, to bacteria in the digestive tract of humans, domesticated animals, and other environments exposed to the GM plant or plant material is expected.

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

A successful horizontal gene transfer would require stable insertion of the recombinant DNA sequences into a bacterial genome and a selective advantage to be conferred to the transformed host. The only mechanism known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes is homologous recombination. This requires the presence of stretches of DNA sequences that are similar in the recombining DNA molecules. The similarity between the plant and bacterial sequences can be situated in the coding region of a recombinant protein (transgene), or in the border regions of the recombinant gene cassettes inserted into the plant genome. In the case of sequence identity with the transgene itself, recombination would result in gene replacement. In the case of identity with border regions, recombination could result in the insertion of additional DNA sequences in bacteria and thus confer the potential for new properties.

Soybean DAS-68416-4 contains genetic elements originating or derived from bacteria (Section 3.1.1.1). These are: (1) a plant codon-optimised version of the *aad-12* gene from the bacterium *D. acidovorans* conferring tolerance to 2,4-D; (2) a plant codon-optimised version of the *pat* gene from the bacterium *S. viridochromogenes* conferring tolerance to herbicides containing glufosinate-ammonium; (3) two untranslated regions (UTR) comprising the transcriptional terminator and polyadenylation site of ORF23 and ORF1 from plasmid pTi15955 (AtuORF23 and AtuORF1, respectively), from *A. tumefaciens*; and (4) an intervening sequence from the Ti plasmid C58 of *A. tumefaciens*.

Bioinformatic analyses of the inserted DNA confirmed that the plant codon-optimised bacterial genes *aad-12* and *pat* did not provide sufficient sequence identity to facilitate homologous recombination. However, sufficient sequence identity with bacterial DNA was found for the AtuORF23 and AtuORF1 sequence, flanking the *pat* gene. Double homologous recombination of transgenic plant DNA with the plasmid of *A. tumefaciens* could result in an insertion of the *pat* gene onto a pTI15955 or highly similar plasmid.

A. tumefaciens occurs in soil, water and in the plant rhizosphere. It is therefore not expected to be prevalent in the main receiving environments, i.e. the gastrointestinal tract of humans or animals. However, occurrence of the recombinant genes outside of the immediate receiving environment

[54] Dossier: Part I – Section D6.

(through faecal material) in soils cannot be ruled out, and is therefore also considered when assessing the risks associated with a horizontal gene transfer.

The theoretically possible double homologous recombination between both sequences in soybean DAS-68416-4 and pTi15955 in *A. tumefaciens* could result in the insertion of the plant-codon optimised *pat* gene including its plant viral promoter. Such a recombination event, however, is expected to be expressed at low levels by the plant viral promoter. Compared to the natural bacterial variants of the *pat* gene, it is likely that the plant-optimised version results in a less functional enzyme in bacteria. The insertion of the *pat* gene on the Ti plasmid would result in a dysfunctional plasmid due to the loss of a large fragment between ORF23 and ORF1. Furthermore, the double homologous recombinant and insertion of the *pat* gene would delete the bacterial potential for crown gall formation, due to the loss of plasmid encoded genes between ORF23 and ORF1.

In addition to homology-based recombination processes, non-homologous (illegitimate) recombination that does not require the presence of DNA similarity between the recombining DNA molecules is theoretically possible. However, the transformation rates for illegitimate recombination were considered to be 10^{10}-fold lower than for homologous recombination (Hülter and Wackernagel, 2008; EFSA, 2009) and have not never been detected for GM plants and bacteria, even in studies that have directly exposed bacteria to high concentrations of GM plant DNA (EFSA, 2009).

In summary, the GMO Panel identified a potential for an increased likelihood for horizontal gene transfer of a plant-optimised *pat* gene from soybean DAS-68416-4 to a plasmid of a soil bacterium by double homologous recombination. This recombination would occur at the cost of inefficient expression of a plant-optimised protein and loss of the potential of the bacterial host for crown-gall formation. Also, considering the natural abundance of *pat* as well as *aad-12* genes in environmental bacteria, the unlikely, but theoretically possible, transfer of the recombinant plant-codon optimised genes and promoter sequences from soybean DAS-68416-4 to bacteria does not give rise to a safety concern.

2) Plant-to-plant gene transfer[55]

Considering the scope of the application EFSA-GMO-NL-2011-91 and the biology of soybean, the potential of occasional feral GM soybean plants originating from seed import spills to transfer recombinant DNA to sexually cross-compatible plants and the environmental consequences thereof were considered.

The genus *Glycine* is divided into two distinct subgenera: *Glycine* and *Soja*. The subgenus *Glycine* contains 16 perennial wild species, while the cultivated soybean, *G. max*, and its wild and semiwild annual relatives, *Glycine soja* and *Glycine gracilis*, are classified in the subgenus *Soja* (OECD, 2000). Owing to the low level of genomic similarity among species of the genus *Glycine*, *G. max* can cross with only other members of the *Glycine* subgenus *Soja* under natural conditions (Singh et al., 1987; Hymowitz et al., 1998; Lu, 2005). Hence, the three species of the subgenus *Soja* are capable of cross-pollination and the hybrid seed that is produced can germinate normally and produce plants with fertile pollen and seed (Abe et al., 1999; Nakayama and Yamaguchi, 2002). Since *G. soja* and *G. gracilis* are indigenous to China, Taiwan, Korea, Japan, the far-east region of Russia, Australia, the Philippines and the South Pacific, and since they have not been reported in other parts of the world where the cultivated soybean is grown (Dorokhov et al., 2004; Lu, 2005), the plant-to-plant gene transfer from soybean is restricted to cultivated areas and occasional soybean plants resulting from seed spillage in the EU.

Soybean is an annual, almost completely self-pollinating crop with a percentage of cross-pollination usually below 1% (OECD, 2000; Ray et al., 2003; Lu, 2005; Yoshimura et al., 2006; Abud et al., 2007). Soybean pollen dispersal is limited because the anthers mature in the bud and directly pollinate the stigma of the same flower (OECD, 2000).

However, cross-pollination rates as high as 6.3% have been reported for closely spaced plants (Ray et al., 2003), suggesting the potential for some within-crop gene flow in soybean. These results indicate that natural cross-pollination rates can fluctuate significantly among different soybean varieties under particular environmental conditions, such as favourable climate for pollination and an abundance of pollinators (Caviness, 1966; Gumisiriza and Rubaihayo, 1978; Kikuchi et al., 1993; Ahrent and Caviness, 1994; Ray et al., 2003; Lu, 2005).

For plant-to-plant gene transfer to occur, imported soybean DAS-68416-4 seeds need to be processed outside the importing ports, transported into regions of soybean production in Europe, spilled during transportation, germinate and develop into plants in the very close vicinity of soybean

[55] Dossier: Part I – Sections D6 and D9.3.

fields, and there needs to be an overlap of flowering periods and environmental conditions favouring cross-pollination. It must be noted that most soybean DAS-68416-4 seeds are processed in the countries of production or in ports of importation. The overall likelihood of cross-pollination between occasional feral GM soybean plants and cultivated soybean is therefore extremely low.

In conclusion, 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 soybean plants in Europe will not differ from that of conventional soybean varieties.

Interactions of the GM plant with target organisms[56]

Considering the scope of application EFSA-GMO-NL-2011-91, potential interactions of occasional feral soybean DAS-68416-4 plants arising from seed import spills with target organisms are not considered a relevant issue by the GMO Panel.

Interactions of the GM plant with non-target organisms[57]

Considering the scope of the application EFSA-GMO-NL-2011-91, and the low level of exposure to the environment, potential interactions of spilled seeds or occasional feral soybean DAS-68416-4 plants arising from seed import spills with non-target organisms are not considered a relevant issue by the GMO Panel.

Interactions with the abiotic environment and biogeochemical cycles[58]

Considering the scope of the application EFSA-GMO-NL-2011-91, and the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles are not considered a relevant issue by the GMO Panel.

3.4.1.2. Post-market environmental monitoring[59]

The objectives of a PMEM plan according to Annex VII of Directive 2001/18/EC are to: (1) 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 environmental risk assessment.

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 content of the PMEM plan provided by the applicant (EFSA, 2006b; EFSA GMO Panel, 2011b).

The PMEM plan proposed by the applicant for soybean DAS-68416-4 includes: (1) the description of an approach involving operators (federations involved in soybean 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 newly established by EuropaBio for the collection of the information recorded by the various operators; and (3) the use of networks of existing surveillance systems (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 consent period.

The GMO Panel considers the scope of the PMEM plan provided by the applicant is consistent with the scope of soybean DAS-68416-4. As the ERA does not cover cultivation and did not identify potential adverse environmental effects from soybean DAS-68416-4, no case-specific monitoring is necessary. The GMO Panel agrees with the reporting intervals proposed by the applicant in the PMEM plan.

3.4.2. Conclusions on the environmental risk assessment and monitoring plan

In the case of accidental release into the environment of viable seeds of soybean DAS-68416-4, there are no indications of an increased likelihood of establishment and spread of occasional feral soybean DAS-68416-4 plants, unless these plants are exposed to the intended herbicides. The GMO Panel is of the opinion that the latter will not result in different environmental impacts compared to conventional soybean. Considering the scope of the application EFSA-GMO-NL-2011-91, interactions with the biotic and abiotic environment are not considered to be relevant issues. The unlikely, but

[56] Dossier: Part I – Sections D8 and D9.4.
[57] Dossier: Part I – Section D9.5.
[58] Dossier: Part I – Sections D9.8 and D10.
[59] Dossier: Part I – Section D11.

theoretically possible, transfer of the recombinant *pat* gene from soybean DAS-68416-4 to bacteria does not give rise to a safety concern as these genes will not confer a selective advantage to bacteria. Therefore, considering the introduced traits, the outcome of the comparative analysis, the routes of exposure and the limited exposure levels, the GMO Panel concludes that soybean DAS-68416-4 would not raise safety concerns in the event of accidental release of viable GM soybean seeds into the environment**.**

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of soybean DAS-68416-4 and the GMO Panel guidelines on the PMEM of GM plants. In addition, the GMO Panel acknowledges the approach proposed by the applicant to put in place appropriate management systems to restrict environmental exposure in cases of accidental release of viable seeds of soybean DAS-68416-4.

4. Conclusions

The GMO Panel was asked to carry out a scientific assessment of soybean DAS-68416-4 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data and bioinformatics analyses did not identify issues requiring further assessment for food/feed safety.

The agronomic and phenotypic characteristics of soybean DAS-68416-4 tested revealed no relevant differences between soybean DAS-68416-4 and its conventional counterpart, except for 'days to 50% flowering' for the GM soybean not treated with the intended herbicides. The compositional analysis identified no differences between soybean DAS-68416-4 and its conventional counterpart that required further assessment for food/feed safety, except for a higher lectin activity (increased up to 36%) in soybean DAS-68416-4. The increase in lectin activity is unlikely to raise additional concerns for food/feed safety and nutrition for soybean DAS-68416-4 as compared to its conventional counterpart and the non-GM commercial varieties. No concerns were identified regarding the potential toxicity or allergenicity of the newly expressed AAD-12 and PAT proteins, and no evidence was found that the genetic modification might significantly change the overall allergenicity of soybean DAS-68416-4. The GMO Panel concludes that soybean DAS-68416-4, assessed in this application, is as safe and as nutritious as its conventional counterpart and the non-GM soybean reference varieties tested. The GMO Panel considers that post-market monitoring of food/feed derived from soybean DAS-68416-4 is not necessary, given the absence of safety concerns identified.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable seeds from soybean DAS-68416-4 into the environment. Considering the scope of the application with regard to food and feed uses, interactions with the biotic and abiotic environment are not considered an issue. Risks associated with an unlikely, but theoretically possible, horizontal gene transfer from soybean DAS-68416-4 to bacteria have not been identified. The scope of the PMEM plan provided by the applicant is in line with the intended use of soybean DAS-68416-4.

In conclusion, the GMO Panel considers that the information available for soybean DAS-68416-4 addresses the scientific comments raised by the Member States and that soybean DAS-68416-4, as described in this application, is as safe as its conventional counterpart and the tested non-GM soybean reference varieties with respect to potential effects on human and animal health and the environment in the context of the scope of this application.

Documentation provided to EFSA

1) Letter from the Competent Authority of the Netherlands received on 19 January 2011 concerning a request for placing on the market of genetically modified soybean DAS-68416-4 submitted by Dow AgroSciences LLC in accordance with Regulation (EC) No 1829/2003 (application reference EFSA-GMO-NL-2011-91).
2) Acknowledgement letter dated 7 February 2011 from EFSA to the Competent Authority of the Netherlands.
3) Letter from EURL-JRC dated 15 February 2011 requesting additional information under completeness check.
4) Letter from EFSA to applicant dated 4 March 2011 requesting additional information under completeness check.
5) Letter from applicant to EFSA received on 23 March 2011 providing additional information under completeness check.

6) Letter from EFSA to applicant dated 12 April 2011 requesting additional information under completeness check.
7) Letter from EURL-JRC dated 18 April 2011 requesting additional information under completeness check.
8) Letter from applicant to EFSA received on 5 May 2011 providing additional information under completeness check.
9) Letter from EFSA to applicant dated 24 May 2011 requesting additional information under completeness check.
10) Letter from applicant to EFSA received on 27 June 2011 providing additional information under completeness check.
11) Letter from EFSA to applicant dated 20 July 2011 requesting additional information under completeness check.
12) Letter from applicant to EFSA received on 19 August 2011 providing additional information under completeness check.
13) Letter from EFSA to applicant dated 8 September 2011 delivering the 'Statement of Validity' of application EFSA-GMO-NL-2010-91 for placing on the market of genetically modified soybean DAS-68416-4 submitted by Dow AgroSciences LLC in accordance with Regulation (EC) No 1829/2003.
14) Letter from EFSA to applicant dated 5 December 2011 requesting additional information and stopping the clock.
15) Letter from EFSA to applicant dated 30 January 2012 requesting additional information and maintaining the clock stopped.
16) Letter from EFSA to applicant dated 6 March 2012 asking for timeline for submission of responses.
17) Letter from applicant to EFSA received on 16 March 2012 extending the timeline for submission of responses.
18) Letter from applicant to EFSA received on 13 April 2012 providing additional information.
19) Letter from applicant to EFSA received on 13 April 2012 providing additional information.
20) Letter from EURL-JRC to EFSA dated 16 April 2012 requesting EFSA to stop the clock.
21) Letter from EFSA to applicant dated 20 April 2012 requesting additional information (EURL-JRC) and maintaining the clock stopped.
22) Email from Applicant to EURL-JRC dated 17 April 2012 providing additional information requested under completeness check on 18 April 2011 and under risk assessment on 20 April 2012.
23) Email from EURL-JRC to EFSA dated 15 May 2012 requesting EFSA to re-start the clock.
24) Letter from EFSA to applicant, dated 9 July 2012, re-starting the clock on behalf of EURL-JRC.
25) Letter from applicant to EFSA, received on 7 August 2012, providing additional information spontaneously.
26) Letter from applicant to EFSA, received on 27 August 2012, providing additional information spontaneously.
27) Letter from EFSA to applicant dated 7 September 2012 requesting additional information and stopping the clock.
28) Letter from applicant to EFSA received on 19 November 2012 providing a timeline for submission of responses.
29) Letter from applicant to EFSA received on 19 April 2013 extending (1) the timeline for submission of responses.
30) Letter from applicant to EFSA received on 25 July 2013 extending (2) the timeline for submission of responses.
31) Letter from applicant to EFSA received on 18 October 2013 providing additional information
32) Letter from EFSA to applicant dated 1 July 2014 requesting additional information and maintaining the clock stopped.
33) Letter from applicant to EFSA received on 2 September 2014 providing additional information.
34) Letter from applicant to EFSA, received on 4 November 2014, providing additional information spontaneously.
35) Letter from EFSA to applicant dated 28 November 2014 requesting additional information and maintaining the clock stopped.
36) Letter from applicant to EFSA received on 16 December 2014 providing additional information.

37) Letter from EFSA to applicant dated 16 February 2015 requesting additional information and maintaining the clock stopped.
38) Letter from EFSA to applicant dated 19 February 2015 requesting additional information and maintaining the clock stopped.
39) Letter from applicant to EFSA received on 19 February 2015 providing additional information
40) Letter from EFSA to applicant dated 6 March 2015 requesting additional information and maintaining the clock stopped.
41) Letter from applicant to EFSA received on 12 March 2015 providing additional information.
42) Letter from applicant to EFSA received on 16 March 2015 providing additional information.
43) Letter from EFSA to applicant dated 1 April 2015 requesting additional information and maintaining the clock stopped.
44) Letter from applicant to EFSA received on 11 June 2015 providing additional information.
45) Letter from EFSA to applicant dated 24 June 2015 requesting additional information and maintaining the clock stopped.
46) Letter from applicant to EFSA received on 22 July 2015 providing additional information.
47) Letter from EFSA to applicant dated 15 September 2015 requesting additional information and maintaining the clock stopped.
48) Letter from applicant to EFSA received on 25 September 2015 providing additional information.
49) Letter from EFSA to applicant dated 2 October 2015 requesting additional information and maintaining the clock stopped.
50) Letter from applicant to EFSA received on 23 November 2015 providing additional information.
51) Letter from applicant to EFSA, received on 7 December 2015, providing clarifications related to information received on 23 November 2015.
52) Letter from applicant to EFSA received on 22 December 2015 providing additional information spontaneously.
53) Letter from applicant to EFSA received on 4 March 2016, requesting EFSA to disregard the information regarding EFSA's request of 2 October 2015 (received on 23 November 2015).
54) Letter from EFSA to applicant dated 23 March 2016 requesting additional information and maintaining the clock stopped.
55) Email from applicant to EFSA received on 31 March 2016 providing sequencing information.
56) Letter from EURL-JRC to EFSA dated 22 April 2016 requesting EFSA to stop the clock.
57) Email from EFSA to applicant dated 26 April 2016 requesting additional information (EURL-JRC) and maintaining the clock stopped.
58) Letter from applicant to EFSA received on 29 April 2016 providing additional information.
59) Letter from applicant to EFSA received on 13 May 2016 providing additional information; this letter contains information submitted spontaneously.
60) Letter from EFSA to applicant dated 26 May 2016 requesting additional information and maintaining the clock stopped.
61) Letter from applicant to EFSA received on 13 June 2016 providing additional information.
62) Email from EURL-JRC to EFSA dated 7 September 2016 requesting EFSA to re-start the clock.
63) Letter from EFSA to applicant, dated 28 September 2016, re-starting the clock.
64) Letter from EFSA to applicant dated 29 September 2016 requesting additional information and stopping the clock.
65) Letter from applicant to EFSA received on 26 October 2016 providing additional information.
66) Email from EFSA to applicant, dated 3 November 2016, re-starting the clock.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AA	amino acid
AAD-12	aryloxyalkanoate dioxygenase-12
ADF	acid detergent fibre
ANOVA	analysis of variance
AtuORF	Agrobacterium tumefaciens open reading frame
bp	base pair
BSA	bovine serum albumin
bw	body weight
CsVMV	Cassava vein mosaic virus
DM	dry matter
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
EURL-JRC	European Union Reference Laboratories-Joint Research Centre
FA	fatty acid
FAO	Food and Agricultural Organisation of the United Nations
FW	fresh weight
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HGT	horizontal gene transfer
HU	haemagglutination unit
IgE	immunoglobulin E
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PLT	platelets
PMEM	post-market environmental monitoring

RET	reticulocyte
RBC	red blood cells
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T-DNA	transfer-deoxyribonucleic acid
UTR	untranslated region
WBC	white blood cells
WSR	Wilcoxon Signed Rank