REGULATED PRODUCTS SAFETY ASSESSMENT

Safety Assessment on Genetically Modified MS11 Brassica Napus and MS11 × RF3 Brassica Napus for Food and Feed Uses Under Assimilated Regulation (EC) No. 1829/2003 (RP307)

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FSA Research and Evidence

The Food Standards Agency and Food Standards Scotland (FSA/FSS) received an application from BASF Agricultural Solutions Seed US LLC under Regulation (EC) No. 1829/2003 for MS11 and MS11 × RF3 *Brassica napus* (oilseed rape). MS11 *B. napus* is part of a breeding system developed to obtain progeny that benefit from hybrid vigour, a phenomenon by which the progeny of cross-breeding display improved agronomic characteristics. MS11 *B. napus* does not produce viable pollen ("male sterile") and must be pollenated by another line, such as RF3 *B. napus* to produce the hybrid MS11 × RF3 *B. napus*. FSA/FSS undertook a safety assessment of MS11 and MS11 × RF3 *B. napus* and asked the ACNFP (Advisory Committee on Novel Foods and Processes) to provide scientific advice on the data submitted for the authorisation of MS11 and MS11 × RF3 *B. napus*. The molecular characterisation of both MS11 *B. napus* and MS11 × RF3 *B. napus* confirmed the presence of both T-DNA inserts and raised no safety concerns. Genetic stability of both loci, and phenotypic stability of transgenic protein expression, were both confirmed. Due to the inability to produce homozygous MS11 *B. napus* seed for the compositional analyses, a comparative assessment of MS11 × RF3 *B. napus* was provided. The ACNFP considered this approach appropriate, particularly considering MS11 *B. napus* is not a stand-alone product and will not enter the market as a single event. The results from the comparative assessment of MS11 × RF3 *B. napus* did not raise any safety concerns. Studies on the newly expressed proteins found no evidence of potential toxicology. Independent bioinformatics analysis of the allergenicity potential of the newly expressed proteins found no safety concerns. FSA/FSS concluded, based on this advice, that MS11 *B. napus* (in the context of this stack only) and MS11 × RF3 *B. napus* are as safe as their conventional counterpart with respect to its potential effects on human and animal health.

This is a joint FSA and FSS publication

1. Introduction

Oilseed rape (*Brassica napus*) has been developed for human consumption to have low glucosinolate content in its meal, and low erucic acid in its oil. These "double-low" commercial varieties of *B. napus* dominate *Brassica* production in developed countries, and are termed "canola" quality in N. America. Oilseed rape/canola must contain less than 2% erucic acid in its oil, and less than 30 µmol/g glucosinolates in its meal (OECD, 2011). In 2017, over 17,700,000 tonnes of oilseed rape was produced in the EU, and the UK was one of the five largest producers, contributing over 2,000,000 tonnes. The scope of this application is for the authorisation for import, processing, and food and feed use of MS11 and MS11 × RF3 *B. napus*. The application does not cover cultivation and therefore no MS11 or MS11 × RF3 *B. napus* will be grown in the UK.

MS11 *B. napus* is modified by the addition of the *barnase* gene – a dominant gene for male sterility, the *barstar* gene – used here to inhibit leaky expression of Barnase and enhance transformation efficiency, and the *bar* gene conferring tolerance to glufosinate-ammonium, one of the most widely used broad-spectrum herbicides. The sources of these genes are *Bacillus amyloliquefaciens* (*barnase* and *barstar*), and *Streptomyces hygroscopicus* (*bar*). Expression of the *barnase* gene is limited to the tapetum cells during anther development, which results in a lack of viable pollen and male sterility. MS11 × RF3 *B. napus* is a stacked product generated through conventional breeding of MS11 and RF3 *B. napus*, and is a fully fertile hybrid. RF3 *B. napus* also contains the *barstar* gene, but expresses it in the tapetum cells during anther development to inhibit Barnase (from MS11 *B. napus*) and restore fertility in the stacked product. RF3 *B. napus* also contains the *bar* gene. Due to the characteristics of MS11 *B. napus*, it is not intended to be a stand-alone product; it is only used as part of a breeding system for the production of the fully fertile, MS11 × RF3 *B. napus* hybrid.

Brassica napus predominantly reproduces (~70%) by autogamy, also referred to as "selfing". In this method of reproduction, one plant provides both the pollen (male) and the stigma (female). As MS11 *B. napus* is male sterile (due to the expression of Barnase in the tapetum cells), MS11 *B. napus* can produce no homozygous offspring. Instead, it is part of a breeding system that involves two further *B. napus* lines;

- MS11 B. napus – the male sterile line (the A-line)
- A maintainer line (B-line)
- A restorer line such as RF3 which can re-introduce fertility into the F_1 generation (R-line).

MS11 *B. napus* is maintained as a segregating population of 50% A-line and 50% B-line, and the fertile segregants (the B-line) are removed by treatment with glufosinate-ammonium herbicide. The B-line will share the same genetic background as MS11 *B. napus*, but without the male sterility trait. When the A-line is fertilised by the R-line (RF3), 100% of the F_1 hybrid progeny are fertile, and also benefit from hybrid vigour. Hybrid vigour (or heterosis) is a well-known phenomenon in which the progeny of cross-breeding display improved agronomic characteristics such as greater biomass, increased yield, and faster development.

As part of its safety assessment, FSA/FSS asked the ACNFP to provide scientific advice, and consider the data provided for both MS11 *B. napus* and MS11 × RF3 *B. napus*. The biological characteristics of the MS11 transgenic event (male sterility) prevents the comparative assessment being performed on MS11 *B. napus* without deviations from the requirements within Regulation (EU) No. 503/2013, however Article 5(2) of that regulation allows the applicant to provide alternative data where the data as required are not technically possible to supply. The ACNFP deemed the use of this provision, as used by the applicant, appropriate to enable a safety assessment of MS11 *B. napus* and MS11 × RF3 *B. napus* in the context of this application.

1.1. Background

On February 17th 2021, the Food Standards Agency (FSA) received application RP307 (EFSA-GMO-BE-2016-138 and EFSA-GMO-NL-2107-143) for the authorisation of MS11 *Brassica napus* and MS11 × RF3 *Brassica napus* (unique identifiers: BCS-BNØ12-7 and BCS-BNØ12-7 × ACS-BNØØ3-6 respectively), submitted by BASF Agricultural Solutions Seed US LLC (Florham Park, New Jersey) (hereafter referred to as "the applicant") according to Regulation (EC) No. 1829/2003, as assimilated into UK law.

FSA checked the application for compliance with the relevant requirements of Regulation (EC) No. 1829/2003, and assimilated Regulation (EU) No. 503/2013, and on 24th March 2021, declared the application valid.

1.2. Terms of Reference

According to Articles 6 and 18 of Regulation (EC) No. 1829/2003, the FSA and the Advisory Committee on Novel Foods and Processes Committee (ACNFP) were requested to carry out a scientific safety assessment of genetically modified MS11 *B. napus* and MS11 × RF3 *B. napus* for authorisation in the scope of the application, namely the import, processing, and food and feed use of MS11 *B. napus* and MS11 × RF3 *B. napus*. The scope of the authorisation does not include cultivation in the UK.

Data were submitted relating to both MS11 and MS11 × RF3 *B. napus* as safety assessments of each individual event must be performed, or previously approved events referred to where applicable (EFSA, 2012), before a stacked product can be approved, in accordance with Regulation (EC) No. 503/2013.

FSA/FSS sought safety advice from the ACNFP on genetically modified MS11 and MS11 × RF3 *B. napus*, which will inform the FSA/FSS safety assessment. The FSA/FSS safety assessment is to be seen as the opinion requested under Articles 6(6) and 18(6) of that Regulation.

In addition to the present advice on the safety of genetically modified MS11 *B. napus* and MS11 × RF3 *B. napus*, the FSA and the ACNFP were also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No. 1829/2003. These articles concern details that must be included in positive opinions/outcomes of assessment of GMO foods and feeds, including labelling details, any relevant conditions or restrictions, and monitoring plans.

2. Applicant details

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3. Data and methodologies

3.1. Data

The data for application RP307 submitted according to FSA requirements and provided by the applicant at the time of submission are specified below. The ACNFP assessed the application for the authorisation of genetically modified MS11 *B. napus* and MS11 × RF3 *B. napus* for food and feed uses in accordance with Articles 11 and 23 of Regulation (EC) No. 1829/2003. They considered the requirements described in its guidance for the safety assessment of GM food and feed applications under Regulation (EC) No. 1829/2003, and based their scientific safety assessment on the data within application RP307, additional information provided by the applicant, and any relevant peer reviewed scientific publications.

3.2. Methodologies

The ACNFP conducted their assessment in accordance with the principles described in Regulation (EU) No. 503/2013, applicable guidance, explanatory notes, and statements (EFSA GMO Panel 2010, EFSA GMO Panel 2011, 2015; EFSA GMO Panel, 2017). Independent contractors performed preparatory work and delivered reports on the methods applied by the applicant in performing sequencing and bioinformatics analyses.

4. Assessment

4.1. Molecular characterisation

The molecular characterisation section of the safety assessment considered the methods used to insert the transgenic material, the sequence and structure of the newly expressed protein(s), and the sequences at the insertion locus/loci. Analyses performed by the applicant to determine insertion locus, copy number, and any deletions that occurred during the insertion of transgenic material are assessed. Bioinformatics analyses performed on the transgenic sequences are also

assessed to ensure the newly expressed protein(s) does not raise any safety concerns. Additionally, the expression of the new protein(s) is assessed. Finally, bioinformatics analyses performed on the flanking regions either side of the inserted material (and the junctions between them) are assessed to ensure no sequences occur that could raise safety concerns.

4.1.1. Transformation process and vector constructs

The development of MS11 *B. napus* used embryogenic calli from hypocotyl segments of *B. napus* variety N90-740 which were transformed with pTCO113 (derived from pGSC1700) using *Agrobacterium tumefaciens* strain C58C1. A non-oncogenic helper Ti-plasmid pGV4000 was also used in the transformation process.

The T-DNA region of pTCO113 contains the *barnase* gene (from *Bacillus amyloliquefaciens*), under the control of the anther-specific Pta29 promoter (from *Nicotiana tabacum*) and the 3'nos terminator; the *barstar* gene (also from *Bacillus amyloliquefaciens*), under the control of the Pnos promoter (from *Agrobacterium tumefaciens*) and the 3'g7 terminator; and the *bar* gene (from *Streptomyces hygroscopicus*), under the control of the PssuAt promoter (from *Arabidopsis thaliana*) and the 3'g7 terminator. A second copy of the *barstar* gene is present in the vector backbone.

The *barnase* gene was engineered with an Asp718 restriction site at the start of the coding sequence, substituting alanine and glutamine for valine and proline respectively. This Asp718 restriction site was digested to give a blunt end, and then fused to the initiation codon. The *barnase* gene is under the control of the anther-specific Pta29 promoter, which limits expression of Barnase to the tapetum cells during anther development. Barnase is an extracellular ribonuclease and the expression of Barnase in the tapetum cells results in a lack of viable pollen and male sterility. The *barstar* gene was included to enhance transformation efficiency and protect the plant from leaky expression of Barnase. The *bar* gene, which is modified with an *Nco*I site at the initiation codon (consequently the second codon is mutated from serine to asparagine), is under the control of the PssuAt promoter and is expressed in all green tissues. The *bar* gene product, phosphinothricin acetyltransferase (PAT), metabolises phosphinothricin, the active ingredient in glufosinate-ammonium herbicide, to an inactive, acetylated derivative (N-acetyl glufosinate).

MS11 × RF3 *B. napus* was developed through conventional breeding of MS11 *B. napus* and RF3 *B. napus*. No new genetic modification was used for the development of MS11 × RF3 *B. napus*. The RF3 parental event was produced by means of *Agrobacterium*-mediated transformation using vector pTHW118 and has been assessed previously by EFSA while the UK was an EU member state (EFSA, 2005, EFSA, 2012).

4.1.2. Molecular studies performed on MS11 *B. napus* and MS11 × RF3 *B. napus*

Southern blot analysis of leaf material from the T_2 generation of MS11 *B. napus* was used to confirm the presence of a single insertion locus containing the *bar*, *barnase*, and *barstar* gene cassettes. Southern blot hybridisation analysis also confirmed the absence of any backbone sequences and PCR analysis was used to confirm the absence of *barstar* sequences originating from the vector backbone.

The inserted sequence, and at least 1 kb of both flanking regions, were sequenced by Sanger sequencing of PCR fragments. The insertion locus was also sequenced as one fragment. The sequenced MS11 *B. napus* transgenic locus consisted of 8209 bp, 5778 bp of which was identical to the T-DNA region of pTCO113, 1129 bp corresponded to the 5' flanking region, and 1302 bp to the 3' flanking region, both identical to the corresponding sequence in the insertion locus. A target site deletion of 40 bp was observed.

Bioinformatics analysis located the MS11 insertion locus to chromosome A03. An endogenous gene was identified within the 3' flanking region of the MS11 insertion locus, however its coding sequence was not interrupted. Additional bioinformatics analyses of all putative ORFs at the insertion site and both junction sites found no biologically relevant similarities with known toxins and/or allergens.

The MS11 and RF3 insertion loci of MS11 × RF3 *B. napus* were characterised by Southern blot hybridisation analysis. Since MS11 × RF3 *B. napus* is derived from breeding hemizygous MS11 *B. napus* with homozygous RF3 *B. napus*, approximately half of MS11 × RF3 *B. napus* samples are expected to show no fragments for the MS11 insertion locus. Southern blot analysis was performed on 18 MS11 × RF3 *B. napus* plants that scored positive for MS11, and 18 plants that scored negative and the Southern blot analysis confirmed the stability of the transgenic loci of the parental lines in MS11 × RF3 *B. napus.*

Updated bioinformatics analysis of MS11 × RF3 *B. napus* confirmed the location of the MS11 insertion locus to chromosome A03. Analysis of the RF3 event found strong similarities with different regions of chromosomes C06 and A07, however no region had 100% identity. In a previous safety assessment (EFSA, 2005), analysis of the RF3 event in an RF3 line found one T-DNA copy in an inverted repeat structure and a second incomplete T-DNA copy. The incomplete T-DNA copy contained a functional part of the Pta29 promoter, the coding region of *barstar*, the 3' nos and a non-functional part of the PssuAra promoter. Bioinformatics analyses indicated that it is unlikely that any endogenous genes are interrupted by the RF3 insertion locus.

Table 1. Protein expression of Barnase, Barstar, and PAT/*bar* in grain matrices of MS11, RF3, and MS11 × RF3 *B. napus* (µg/g FW and (µg/g DW))

	MS11 × RF3 *B. napus*		MS11 *B. napus*		RF3 *B. napus*	
Herbicide treatment	CHM[a]	TIH[b]	CHM	TIH	CHM	TIH
Barnase	<LLOQ	<LLOQ	<LLOQ	<LLOQ	-	-
Barstar	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
PAT/bar	0.53 ± 0.24 (0.59 ± 0.26)	0.55 ± 0.28 (0.60 ± 0.30)	0.30 ± 0.17 (0.34 ± 0.18)	0.44 ± 0.18 (0.49 ± 0.18)	0.66 ± 0.08 (0.74 ± 0.09)	0.76 ± 0.24 (0.83 ± 0.25)

[a] Treated with conventional herbicide only

[b] Treated with Liberty 280 SL (279.2 g/L glufosinate-ammonium) at BBCH stage 12-14 at a target rate of 500 g ai/ha

Results reported as mean ± standard deviation (n = 15, except MS11 CHM = 9)

Bioinformatics analyses of the insertion sites found no sequences likely to contribute to horizontal gene transfer with bacterial species.

4.1.3. Transgenic protein expression

Expression levels of Barnase, Barstar, and PAT/*bar* were determined by ELISA on whole plant, root, raceme, and grain samples from tissues harvested from MS11 × RF3 *B. napus* plants grown in the USA and Canada in 2014 and compared to the expression levels in MS11 *B. napus* and RF3 *B. napus* (grown at the same locations at the same time). MS11, RF3, and MS11 × RF3 *B. napus* were treated with the conventional herbicide treatment and the trait-specific herbicide (glufosinate-ammonium). Barnase and Barstar expression was <LLOQ in all matrices treated with the conventional and intended herbicides. PAT/*bar* expression in grain matrices from MS11 × RF3 *B. napus* was 0.53 µg/g FW (0.59 µg/g DW) after conventional treatment, and 0.55 µg/g FW (0.60 µg/g DW) after the intended treatment (Table 1), higher than MS11 *B. napus*, but lower than RF3 *B. napus*.

4.1.4. Genetic stability

Southern blot hybridisation analysis confirmed the genetic stability of the MS11 insert over five generations of MS11 *B. napus* (T2, T3, F1, BC1, and BC2). For all individual plants, expected fragments were obtained demonstrating genetic stability.

Southern blot analysis was also used to demonstrate the structural stability of the transgenic loci in MS11 × RF3 *B. napus.* Additionally, overlapping PCR fragments prepared from MS11 × RF3 *B. napus* gDNA were sequenced and assembled into the consensus sequences for the MS11 and RF3 transgenic loci. These sequences were found to be identical to the corresponding sequences in the parental lines. The sequences had complete bi-directional read coverage and included the inserted sequences and at least 1 kb of both host flanking regions.

Phenotypic stability of PAT/*bar* expression was determined for five generations of MS11 *B. napus* (T3, T4, T5, BC4, and BC5) using lateral flow strip analysis. PAT/*bar* expression was consistent across all generations tested. PCR was used to test for the presence or absence of the MS11 transgenic locus in the same five generations to calculate the segregation ratios. Chi-square analysis of the segregation data confirmed that the MS11 insert is inherited in a predictable manner, consistent with Mendelian principles.

4.1.5. Conclusion on the molecular characterisation

The molecular characterisation data presented confirm that MS11 *B. napus* contains a single transgenic insert. Bioinformatics analyses of this insert, and the flanking sequences, raised no safety concerns. MS11 × RF3 *B. napus* was developed through conventional breeding of MS11 *B. napus* and RF3 *B. napus* and as such no addition genomic changes were made.

The genetic stability of the insert was confirmed over five generations. The expression levels of the transgenic proteins in MS11 × RF3 *B. napus* grain were determined using suitable methodologies, and do not cause a safety concerns.

4.2. Comparative analysis

The purpose of the comparative analysis is to compare the GM plant with its conventional counterpart, a non-GM plant with a similar genetic background. This comparison takes two forms; firstly, a comparison of the agronomic characteristics of the plant as it grows in the field which looks at the yields derived from the plants, as well as their observable characteristics such as height and colour, and secondly a comparison of the composition of the plant after harvest which considers the nutritional value and safety of the genetically modified plant.

4.2.1. Experimental field trial design

The physical characteristics of field trials for a comparative assessment means that trait-specific treatment (glufosinate-ammonium) of MS11 *B. napus* will remove the fertile segregants and expose the MS11 *B. napus* plants to pollen from neighbouring plots resulting in 100% cross-pollination. The donor pollen would be a mix of the three non-GM conventional reference varieties and the B-line (see section 1), and as such, the genotype of the seeds used in the comparative assessment will not be homogeneous. Plants treated with the conventional treatment will primarily cross-pollinate with the B-line, but the genotype will also not be known for certain. Therefore, the comparative assessment of MS11 × RF3 *B. napus* is presented as a means of assessing the MS11 single event, as well as the stacked product. MS11 *B. napus* is maintained as a segregating

population (50% A-line and 50% B-line), with the fertile segregants (B-line) removed by treatment with the intended herbicide, glufosinate-ammonium. The remaining A-line plants are fertilised by a restorer line (such as RF3 *B. napus*), resulting in fully fertile, homogeneous F_1 hybrid seed which is planted by the grower.

MS11 × RF3 *B. napus*, along with the non-GM conventional counterpart (N90-740 – the genetic background for MS11 × RF3 *B. napus*) and six non-GM reference varieties (46A65, AC Elect, AC Excel, Peace, Spectrum, and Westar) were grown at nine sites in the USA and Canada in 2014 (ten sites were initially used but one suffered from flooding and poor seed emergence). The generation of the MS11 × RF3 *B. napus* seed used in the study was F_1. The field trials consisted of six entries replicated four times in a randomised complete block design. The six entries were:

- Non-GM conventional counterpart with conventional herbicide treatment
- MS11 × RF3 *B. napus* with conventional herbicide treatment
- MS11 × RF3 *B. napus* with trait-specific herbicide treatment
- Three of the six reference varieties with conventional herbicide treatment

The agronomic/phenotypic data and compositional data from these field trials were analysed as specified previously in guidance provided by EFSA (EFSA GMO Panel 2010, EFSA GMO Panel 2011, EFSA GMO Panel, 2015). This includes the application of a test of difference between MS11 × RF3 *B. napus* and the conventional counterpart, and a test of equivalence between MS11 × RF3 *B. napus* and the non-GM reference varieties. Due to the inability to produce suitable MS11 *B. napus* seed for the compositional analyses, the ACNFP considers it appropriate to use the comparative analysis of MS11 × RF3 *B. napus* as a surrogate to determine the safety of the composition of both MS11 *B. napus* and MS11 × RF3 *B. napus*, particularly considering MS11 *B. napus* is not a stand-alone product and will not enter the market as a single event.

4.2.2. Suitability of field trials and test materials

The field trial sites were representative of the commercial *B. napus* growing sites in the USA and Canada. The sites represented a range of soil types, diverse cropping systems, and variable meteorological conditions.

Average monthly maximum and minimum temperatures and total monthly precipitation were recorded for each site. Excessive rainfall was reported at one site leading to poor seed emergence. Additionally, grain samples could not be collected from six plots at this site so agronomic and phenotype data was not considered for this site.

MS11 × RF3 *B. napus* seeds, the conventional counterpart, and the non-GM reference varieties (Peace seeds were obtained from a certified Canadian seed producer) were produced under standard agronomic practices and with quality assurance mechanisms to ensure genetic identity, purity, and health. All seed lots were tested for the presence or absence of the transgenic insert and PCR analysis determined all seed lots were true to type. Good seed germination potential was observed for all seed lots.

The ACNFP are satisfied that the field trials, and the materials used in the field trails are appropriate for the comparative assessment. The geographical locations, soil conditions, meteorological conditions, and the management practices used were all considered typical of the receiving environments where MS11 × RF3 *B. napus* could be grown.

4.2.3. Comparative analysis (agronomic characteristics)

In the comparative assessment of agronomic characteristics, tests between MS11 × RF3 *B. napus* not treated with the intended herbicide and the conventional counterpart found no statistically significant differences for any of the seven parameters tested (early stand count, final stand count, days to flowering, days to maturity, days to flowering – 10% remains, average plant height, and yield). In the test between MS11 × RF3 *B. napus* treated with the intended herbicide, no statistically significant differences were observed for any of the parameters tested except average plant height. However, equivalence between MS11 × RF3 *B. napus* and the reference varieties was demonstrated for average plant height.

The Cochran-Mantel-Haenszel test was used to compare categorical data (seedling vigour, lodged plants, pod shattering, abiotic disease stress, and insect stress ratings) from MS11 × RF3 *B. napus* with the conventional counterpart. No statistically significant differences were observed for any of parameters tested, and all mean values were within the range of the non-GM reference varieties. No analysis for abiotic disease stress at BBCH 30-39 could be performed due to a lack of variability in the data (all plots were rated as “1”).

Table 2. Outcome of the comparative compositional analysis of MS11 × RF3 *B. napus* grain.

		Test of difference[a]			
		Intended herbicide treatment[c]		Conventional herbicide treatment[c]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[b]	Category I	25[d]	26	26	28
	Category II	3[e]	2[f]	0	2[g]
	Category III	1[h]	0	1[h]	0
	Category IV	0	0	0	0
	No category	0	0	0	0
	Total endpoints	57[d]		57	

[a] Comparison between MS11 × RF3 *B. napus* and the conventional counterpart (N90-740)

[b] The test of equivalence with the reference varieties is categorised into four different outcomes; category I (equivalence with the reference varieties is demonstrated), category II (equivalence is more likely than not), category III (equivalence is less likely than not), and category IV (non-equivalence is demonstrated)

[c] The intended herbicide treatment was one application of Liberty 280 SL (280 g ai/L glufosinate-ammonium) at BBCH 12-14

[d] See Annex A for all parameters tested

[e] Copper, zinc, and glucobrassicin

[f] Acid detergent fibre and C16:1 palmitoleic acid

[g] C16:1 palmitoleic acid and copper

[h] Aspartic acid

4.2.4. Compositional analysis of grain

In the compositional analysis of MS11 × RF3 *B. napus* grain, statistically significant differences between MS11 × RF3 *B. napus* not treated with the intended herbicide and the conventional counterpart were found in 30 of the composition parameters tested (Table 2). However, equivalence (or equivalence more likely than not) with the reference varieties was demonstrated for all of these parameters.

Statistically significant differences between MS11 × RF3 *B. napus* treated with the intended herbicide and the conventional counterpart were found in 28 of the composition parameters tested (Table 2). In all cases, equivalence (or equivalence more likely than not) was demonstrated.

A statistically significant difference was only observed for copper in the comparison of between MS11 × RF3 *B. napus* not treated with the intended herbicide and the conventional counterpart, however the difference was less than the standard deviation of the conventional counterpart. Copper is an essential trace mineral in plants and its content is an exogenic factor dependent on its availability in the soil, however *B. napus* is not a copper-sensitive plant.

Statistically significant differences were observed for C16:1 palmitoleic acid in MS11 × RF3 *B. napus* treated, and not treated, with the intended herbicide. C16:1 palmitoleic acid is a non-essential fatty acid and can be synthesised by humans and is not an essential component of the diet.

Equivalence with the non-GM reference varieties was less likely than not for aspartic acid for MS11 × RF3 *B. napus* treated, and not treated, with the intended herbicide, however there was not a significant difference with the conventional counterpart in either, and aspartic acid is a non-essential amino acid and can be synthesised by humans.

A statistically significant difference was only observed for acid detergent fibre in the comparison of between MS11 × RF3 *B. napus* treated with the intended herbicide and the conventional counterpart, however the difference was less than the standard deviation of the conventional counterpart.

Additionally, the mean values of all these parameters were within the range of the reference varieties. The mean values of C16:1 palmitoleic acid and copper were also within the ranges published by the OECD (OECD, 2011) and ILSI (ILSI, 2018), however the mean value of aspartic acid exceeded the OECD range, even though aspartic acid levels were not different between intended herbicide treatment and conventional herbicide management.

4.2.5. Conclusion on the comparative analysis

Due to the inability to produce suitable MS11 *B. napus* seed for the compositional analyses, it is not possible to complete the comparative assessment in the manner specified by the requirements as laid out in Regulation (EU) No. 503/2013. However, Article 5(2) of that regulation allows the applicant, by derogation from the specific requirements for the risk assessment of genetically modified foods and/or feeds, to submit an application that does not satisfy all the requirements, if “it is not scientifically necessary, or technically possible to supply such information”, and “reasoned justification is given for the derogation”.

The ACNFP considers a comparative assessment of MS11 *B. napus* according to the requirements of Regulation (EC) No. 503/2013 not technically possible, and considers the scientific rationale provided by the applicant as an acceptable, reasoned justification for using the comparative assessment of MS11 × RF3 *B. napus* as an appropriate alternative to that of MS11 *B. napus*. Their decision was based upon several characteristics of MS11 *B. napus* and MS11 × RF3 *B. napus;*

- The results of the molecular characterisation;
- MS11 *B. napus* is only to be used in the production of MS11 × RF3 *B. napus* and will not go to market as a single event;

- The previous assessment of MS8 × RF3 *B. napus* (EFSA, 2012), which has been on the European market with no reported negative consequences. The safety assessment of MS8 × RF3 *B. napus* took place alongside that of MS8 *B. napus* and RF3 *B. napus*, and was presented as a single EFSA opinion in 2012.

The ACNFP assessed the field trials used to generate material for the comparative assessment and considered the locations selected were representative of commercial oilseed rape production, and that the meteorological conditions and management practices used during the field trails were appropriate.

The ACNFP also assessed the results from the comparative assessment, including all the significant differences between MS11 × RF3 *B. napus* and its conventional counterpart, and found the information provided did not raise any safety concerns.

4.3. Food/feed safety assessment

The food/feed safety assessment covers the likelihood that the newly expressed protein(s), or the whole genetically modified food or feed, will cause safety concerns when consumed by humans and/or animals. This includes looking at the concentrations of newly expressed protein(s) in the final products that will be consumed, as well as the anticipated rates of consumption by humans and animals to understand the anticipated magnitude of exposure to any newly introduced proteins. Any toxicological or allergenic risks that can be identified and any effects on nutritional quality were also assessed.

4.3.1. Effects of processing

Traditional oilseed rape products are unsuitable as a source of food due to the presence of two naturally occurring anti-nutrients; glucosinolates and erucic acid. Modern “canola” quality cultivars have been developed to reduce the amounts of glucosinolates in their meal, and of erucic acid in their oil, and are hence sometimes referred to as “double-low” cultivars. Oilseed rape is primarily used in the human diet when it has been processed into vegetable oil and can be found in products including frying oil, salad dressings, and spreads such as margarine. Whilst oilseed rape meal is not widely used by humans due to the presence of glucosinolates, an emerging use of oilseed rape in humans is as a protein isolate, an enriched protein product derived from oilseed rape meal. Oilseed rape protein isolates can be used as a protein source (found in meal replacements, protein and nutrition drinks, and plant protein products (meat analogues)), as a texture improver, as an egg yolk replacement, or as a replacement for fat.

Table 3. Expression levels of PAT/*bar* in MS11 *B. napus* grain and processed fractions.

	Fresh weight (µg/g)	Dry weight (µg/g)
Grain	0.23	0.25
Press cake[a]	0.23 ± 0.02	0.26 ± 0.02
Solvent extracted meal[a]	0.27 ± 0.03	0.31 ± 0.03
Toasted meal	<LLOQ	<LLOQ
Crude oil	<LLOQ	<LLOQ
RBD oil	<LLOQ	<LLOQ

[a] Results reported as mean ± standard deviation (n = 2)

Oilseed rape seeds are processed into a refined oil through a process of heating, crushing, and solvent extraction to separate the oil from the meal. Further refining steps improve the colour, flavour, and shelf life of extracted oils. After the oil has been removed, the meal is toasted to remove any remaining solvent. No new methods for the production of oilseed rape oil and meal will be used in the processing of MS11 × RF3 *B. napus*.

The concentrations of the newly expressed proteins were determined by ELISA on MS11 *B. napus* grain and processed fractions. The expression levels of Barnase and Barstar were <LLOQ in all fractions tested. The levels of PAT/bar were <LLOQ in toasted meal and oil (Table 3).

The levels of PAT/*bar* in any alternative protein products derived from MS11 × RF3 *B. napus* were determined by multiplying the PAT/*bar* content of MS11 × RF3 *B. napus* grain (see Table 1) by a conversion factor of four. Oilseed rape grain is approximately 25% protein, and protein extraction from grain into an alternative protein product would increase protein content by weight up to four-fold (i.e. 100% protein) (EFSA, 2019). This method estimated the PAT/bar content of alternative protein products derived from MS11 × RF3 *B. napus* as 2.2 µg/g FW, below the limit dose level of 2000 mg/kg body weight. The levels of both Barnase and Barstar were <LLOQ in MS11 × RF3 *B. napus* grain.

4.3.2. Activity and stability of the newly expressed protein

The studies on all three newly expressed proteins were performed with bacterially-produced recombinant proteins rather than the proteins extracted directly from the plants due to the low levels of expression and the limitations on protein quantity that can be extracted from plant material. Structural and functional equivalence between bacterially-produced and plant-produced proteins was demonstrated by comparing molecular weight, immuno-reactivity, and biological activity;

- Peptide mapping of the bacterially-produced Barnase and Barstar proteins demonstrated 100% coverage of the amino acid sequences of the Barnase and Barstar proteins predicted by the nucleotide sequences of the *barnase* and *barstar* genes in the MS11 transgenic insert.
- The bacterially-produced Barnase gene had demonstrable enzymatic activity.
- The N-terminal sequence of PAT/*bar* was consistent with the amino acid sequence expected from the nucleotide sequence of the *pat* gene, however acetylation was observed. The intact molecular mass confirmed the molecular mass of an acetylated PAT/*bar* protein (21 kDa).
- The immuno-reactivity of the bacterially-produced PAT/*bar* protein was confirmed and the activity of the plant-produced and bacterially-produced proteins were equivalent.

The effect of temperature on the newly expressed proteins was determined using functional assays to assess activity, and SDS-PAGE to assess stability and protein integrity. Barnase lost activity and stability upon heating above 55 °C, Barstar lost activity and stability upon heating to 75 °C, and PAT/bar was heat stable when incubated at 90 °C for up to an hour, but lost activity upon heating to 55 °C. SDS-PAGE analysis suggested oligomers of Barstar, and the Barnase/Barstar complex, form upon heat treatment.

All proteins (including the Barnase/Barstar complex) were degraded very rapidly (within 30 seconds) in SGF (simulated gastric fluid containing pepsin at pH 1.2). Barnase and the Barnase/Barstar complex were stable after incubation for 60 minutes in SIF (simulated intestinal fluid containing pancreatin at pH 7.5), Barstar was degraded after 10 minutes in SIF, and PAT/*bar* was degraded very rapidly (within 30 seconds).

4.3.3. Toxicology assessment of the newly expressed proteins

The ACNFP considered the toxicological safety of the newly expressed proteins during their safety assessment using the molecular characterisation data, bioinformatic analyses, and any *in vitro* or *in vivo* studies performed by the applicant.

Neither of the source organisms of the newly expressed proteins (*Bacillus amyloliquefaciens* and *Streptomyces hygroscopicus*) are considered to be pathogenic. They are both ubiquitous in nature and have been utilised in biotechnological applications for many years.

Bioinformatic analyses were performed for the newly expressed proteins using the FASTA algorithm, with the BLOSUM50 scoring matrix. No biologically relevant similarities were identified (most matches corresponded to sequences from the ribonuclease family of proteins from different origins with no known toxic properties).

To determine any potential toxic effects of the newly expressed proteins (including the Barnase/Barstar complex but not PAT/*bar*), 28-day repeated dose toxicity studies in mice were performed in compliance with OECD TG 407 (OECD, 2008). The PAT/*bar* protein has been previously evaluated by EFSA (EFSA, 2005, EFSA, 2012) and has a history of safe use when expressed in MS8 × RF3 *B. napus*, so no 28-day repeated dose toxicity studies were deemed necessary. Neither the Barnase protein (at 0.95, 2.85, and 9.50 mg/kg/day), Barstar (at 1, 3, and 10 mg/kg/day), nor the Barnase/Barstar complex (at 2, 6, and 20 mg/kg/day) induced any treatment-related changes.

4.3.4. Toxicology assessment of new constituents other than the newly expressed proteins

No new constituents other than the newly expressed proteins, Barnase, Barstar, and PAT/*bar*, were identified in MS11 *B. napus* and MS11 × RF3 *B. napus*, therefore no assessment of any constituents other than the newly expressed proteins is required.

4.3.5. Toxicology assessment of the whole genetically modified food or feed

In accordance with Regulation (EU) No. 503/2013, the applicant provided 90-day feeding studies of Sprague Dawley (Crl:CD(SD)) rats fed diets consisting of 15% (w/w) toasted MS11 *B. napus* meal, 15% (w/w) toasted RF3 *B. napus* meal, 15% (w/w) conventional counterpart, or 15% (w/w) non-GM *B. napus*. The studies were performed in accordance with OECD TG 408 (OECD, 2018).

No effects on any of the parameters tested were observed during the study.

4.3.6. Allergenicity assessment

In accordance with Regulation (EU) No. 503/2013, the applicant used a weight-of-evidence approach to assess the allergenicity potential of the newly expressed proteins as no single method is sufficient to predict allergenicity (Codex Alimentarius, 2009). The PAT/*bar* protein has been previously evaluated, and it is not considered to be allergenic (EFSA, 2005, EFSA, 2012).

Two *in silico* searches (an overall identity search and an 80-mer sliding window search) against the AllergenOnline database (www.allergenonline.org) found no biologically relevant matches with known allergenic proteins.

The ACNFP considered the bioinformatics analyses performed by the applicant and found no safety concerns for either newly expressed protein.

4.3.7. Anticipated intake/extent of use

In accordance with Regulation (EU) No. 503/2013, the applicant provided anticipated dietary intake of the newly expressed proteins by using protein expression data in MS11 × RF3 *B. napus* and EU data on the consumption of oilseed rape.

The anticipated human dietary intake of the newly expressed proteins is considered negligible, as the newly expressed proteins will not be present in oil, the main food product derived from oilseed rape.

Anticipated animal dietary intakes were calculated using a worst-case scenario approach, assuming all rape forage and oilseed rape meal products used for animal feed were derived from MS11 × RF3 *B. napus*, the maximum percentages of rape forage and oilseed rape meal were used to prepare animal feed, and that the PAT/*bar* content in the oilseed rape meal was not lower than the PAT/*bar* levels in the oilseed rape seeds. The highest chronic dietary exposures were found in beef cattle in Australia fed up to 100% rape forage (40 g/kg body weight/day rape forage, 2375.2 µg/kg bw/day PAT/*bar*, and 18.8 µg/kg bw/day Barstar).

4.3.8. Nutritional assessment

The intended traits of MS11 × RF3 *B. napus* are for agronomic purposes only and not intended to change the nutritional characteristics of MS11 × RF3 *B. napus*. Compositional comparisons with the conventional counterpart and non-GM reference varieties found no biologically relevant differences, so no change in the nutritional value of the product is expected.

4.3.9. Conclusion of the food/feed safety assessment

The ACNFP assessed the food/feed safety of the newly expressed proteins in terms of their toxicological potential, allergenic potential, and nutritional quality. They concluded that the newly expressed proteins shared no biologically relevant identity with known toxins and allergens, and the overall allergenicity of MS11 *B. napus* and MS11 × RF3 *B. napus* was not different to conventional oilseed rape. The ACNFP concluded that based on

the comparative analysis and the nutritional assessment, MS11 *B. napus* and MS11 × RF3 *B. napus* are not nutritionally disadvantageous, and are as safe as conventional oilseed rape varieties.

4.4. Environmental risk assessment and monitoring plan

4.4.1. Environmental risk assessment

The environmental risk assessment (ERA) of MS11 *B. napus* and MS11 × RF3 *B. napus* was considered by the Advisory Committee on Releases to the Environment (ACRE).

The scope of the application does not include cultivation and only covers the import, processing, and food and feed use of MS11 and MS11 × RF3 *B. napus*. No deliberate release of viable plant material or derived products is expected. Therefore, only accidental release of viable GM seeds or propagating material during import, transportation, storage, handling, and processing will be considered.

ACRE considered the ability of MS11 and MS11 × RF3 *B. napus* to persist under GB environmental conditions, interaction of feral MS11 and MS11 × RF3 *B. napus* with the environment, and the potential for horizontal gene transfer (HGT) to the environment. ACRE concluded that MS11 and MS11 × RF3 *B. napus* would not raise safety concerns in the event of accidental release of viable seeds or propagating material into the environment.

ACRE's advice is available on the GOV.UK website.

4.4.2. Post-market environmental monitoring (PMEM) plan

The PMEM plan provided by the applicant proposes general surveillance to identify the occurrence of unanticipated adverse effects due to the unintended release of MS11 and MS11 × RF3 *B. napus*. Exposure (via accidental release) can be controlled by clean-up measures, and the application of current practices used for the control of any adventitious oilseed rape plants, such as manual or mechanical removal, and the application of herbicides.

General surveillance will be predominantly based on collaboration with third parties, such as operators involved in the import, handling, and processing of MS11 and MS11 × RF3 *B. napus*. These third parties will report any potential unanticipated adverse effects to the authorisation holder, who will investigate.

The authorisation holder will submit an annual report including results of the general surveillance and any unanticipated adverse effects. If information that confirms an adverse effect becomes available, the authorisation holder will investigate, and based on a scientific evaluation, define, and implement management measures to protect human and animal health, or the environment, as necessary.

ACRE considered the PMEM plan provided by the applicant, in conjunction with the ERA. As the ERA did not identify potential adverse effects to the environment, it was not considered necessary for case-specific monitoring to be implemented. The proposed PMEM plan and monitoring intervals are appropriate for the intended uses of MS11 and MS11 × RF3 *B. napus*. Assessing any proposals for the PMEM plan is within the remit of ACRE, and their assessment will form part of the final safety assessment published by FSA/FSS.

5. Analytical methods

The FSA and FSS have decided, where appropriate, to make use of the European Union Reference Laboratory (EURL) laboratory reports completed prior to the end of the transition period for a GMO for which an application has also now been made to GB.

The FSA and FSS accepted the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) report, showing that the detection methods for the events BCS-BNØ12-7 and ACS-BNØØ3-6 were validated.

The methods and validation reports for MS11 B. napus and MS11 × RF3 *B. napus* are available on the European Commission website.

6. Overall conclusions and recommendations

To support the safety assessment by FSA/FSS, the ACNFP was asked to provide advice on the data submitted for the authorisation for import, processing, and food and feed use of genetically modified MS11 *B. napus* and MS11 × RF3 *B. napus* in accordance with Regulation (EU) No. 1829/2003. MS11 *B. napus* and MS11 × RF3 *B. napus* are modified by the addition of the Barnase, Barstar, and PAT/*bar* proteins. Expression of Barnase in the tapetum cells during anther development results in male sterility in MS11 *B. napus,* and expression of Barstar under the same promoter in MS11 × RF3 *B. napus* restores male fertility. MS11 *B. napus* is not intended to be a stand-alone product that enters the market; it is only used for the

production of the fully fertile, MS11 × RF3 *B. napus* hybrid. MS11 *B. napus* and MS11 × RF3 *B. napus* also express the PAT/*bar* protein which confers tolerance to glufosinate-ammonium herbicides.

The molecular characterisation data established that MS11 *B. napus* contains a single transgenic insert. Bioinformatics analyses of this insert, and the flanking sequences, raised no safety concerns. The stability of the insert was confirmed over five generations. MS11 × RF3 *B. napus* is generated through conventional breeding of MS11 *B. napus* and RF3 *B. napus* and no new genetic material is inserted. The expression levels of the transgenic proteins in MS11 *B. napus* and MS11 × RF3 *B. napus* grain were determined using suitable methodologies, and do not cause a safety concern.

Due to the inability to produce suitable MS11 *B. napus* seed for the compositional analyses, the ACNFP considers it appropriate to use the comparative assessment of MS11 × RF3 *B. napus* to determine the safety of the composition of both MS11 *B. napus* (in the context of this stack only) and MS11 × RF3 *B. napus*. The field trials used to generate material for the comparative analyses were deemed appropriate, and the locations selected were considered representative of commercial oilseed rape production. The ACNFP assessed the results from the comparative assessment, including all the significant differences between MS11 × RF3 *B. napus* and its conventional counterpart, and found no safety concerns when compared to the non-GM reference varieties.

The food/feed safety of the newly expressed proteins was assessed, and no safety concerns were raised in terms of their toxicological potential, allergenic potential, and nutritional quality. Based on the comparative assessment and the nutritional assessment, MS11 *B. napus* and MS11 × RF3 *B. napus* do not cause any nutritional concerns.

Overall, FSA/FSS concluded, based on ACNFP advice, that MS11 *B. napus* (in the context of this stack only) and MS11 × RF3 *B. napus* are as safe as its conventional counterpart with respect to its potential effects on human and animal health.

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Abbreviations

Abbreviation	Definition
ACNFP	Advisory Committee on Novel Foods and Processes
ACRE	Advisory Committee on Releases to the Environment
ADF	Acid Detergent Fiber
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
BW	Body Weight
DNA	Deoxyribonucleic acid
DW	Dry weight
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERA	Environmental risk assessment
EU	European Union
FW	Fresh weight
FSA	Food Standards Agency
FSS	Food Standard Scotland
gDNA	Genomic DNA
GM	Genetically modified
GMO	Genetically modified organism
HGT	Horizontal gene transfer
HR	Homologous recombination
ISLI	International Life Sciences Institute
kb	Kilobase
KDa	Kilodalton
LLOQ	Lower limit of quantification
NGS	Next generation sequencing
OECD	Organisation for Economic Co-operation and Development
ORFs	Open reading frames
PAT	Phosphinothricin-N-acetyltransferase
PCR	Polymerase chain reaction
PMEM	Post-market environmental monitoring
PMM	Post-Market Monitoring
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
T-DNA	Transfer-deoxyribonucleic acid
UTR	Untranslated region

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Appendix 1

Results of the statistical analyses performed on all analytes tested in the comparative assessment of MS11 × RF3 *B. napus*

Intended herbicide treatment

Category I (equivalence demonstrated)[a]

Not significantly different

Ash, fat, neutral detergent fibre, alanine, phenylalanine, threonine, tryptophan, tyrosine, valine, C16:0 palmitic acid, C18:3 linolenic acid, C20:1 eicosenoic acid, iron, magnesium, manganese, phosphorus, α-tocopherol, γ-tocopherol, 4-hydroxyglucobrassicin, progoitrin, total glucosinolates, phytic acid, insoluble tannins, soluble tannins, and total condensed tannins (25)

Significantly different

Moisture, carbohydrates, protein, arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid, C20:0 arachidic acid, C22:0 behenic acid, C24:0 lignoceric acid, C24:1 nervonic acid, calcium, potassium, vitamin K, gluconapin and sinapine (26)

Category II (equivalence more likely than not)

Not significantly different

Copper, zinc, and glucobrassicin (3)

Significantly different

Acid detergent fibre and C16:1 palmitoleic acid (2)

Category III (equivalence less likely than not)

Not significantly different

Aspartic acid (1)

Significantly different

N/A

Category IV (non-equivalence demonstrated)

Not significantly different

N/A

Significantly different

N/A

Conventional herbicide treatment

Category I (equivalence demonstrated)

Not significantly different

Moisture, ash, acid detergent fibre, alanine, phenylalanine, threonine, tryptophan, tyrosine, valine, C16:0 palmitic acid, C18:1 oleic acid, C18:3 linolenic acid, iron, magnesium, manganese, phosphorus, zinc, α-tocopherol, γ-tocopherol, 4-hydroxyglucobrassicin, glucobrassicin, progoitrin, phytic acid, insoluble tannins, soluble tannins, and total condensed tannins (26)

Significantly different

Carbohydrates, fat, protein, neutral detergent fibre, arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, C18:0 stearic acid, C18:2 linoleic acid, C20:0 arachidic acid, C20:1 eicosenoic acid, C22:0 behenic acid, C24:0 lignoceric acid, C24:1 nervonic acid, calcium, potassium, vitamin K, gluconapin, total glucosinolates, and sinapine (28)

Category II (equivalence more likely than not)

Not significantly different

N/A

Significantly different

C16:1 palmitoleic acid and copper (2)

Category III (equivalence less likely than not)

Not significantly different

Aspartic acid (1)

Significantly different

N/A

Category IV (non-equivalence demonstrated)

Not significantly different

N/A

Significantly different

N/A

[a] *The comparative analysis comprises a test of equivalence with the non-GM reference varieties and a test of difference with the conventional counterpart, in this case N90-740, the genetic background for MS11 × RF3 B. napus. The results of the test of equivalence are categorised into four groups; equivalence with the reference varieties is demonstrated, equivalence with the reference varieties is more likely than not, equivalence with the reference varieties is less likely than not, and non-equivalence with the reference varieties is demonstrated.*