Import and processing of genetically modified oilseed rape LBFLFK with an altered fatty acid profile

COGEM advice CGM/200210-01

- The present application (EFSA/GMO/DE/2019/157) concerns the authorisation for import and processing for use in food and feed and other products (containing or consisting) of genetically modified (GM) oilseed rape LBFLFK;

- Oilseed rape LBFLFK was produced by *Agrobacterium rhizogenes* mediated transformation. The T-DNA region was inserted at two locations in the oilseed rape genome. The T-DNA consists of thirteen different expression cassettes. Twelve encode enzymes involved in the synthesis of fatty acids. Their expression is driven by seed-specific promoters. As a result of the modification, the seeds of LBFKF contain omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).
  The thirteenth expression cassette encodes a modified AHAS protein, conferring tolerance to imidazolinone containing herbicides;

- Feral oilseed rape populations occur across the Netherlands, with a small number of plants (25 or less) per location, along distribution routes and handling areas as a result of spillage of oilseed rape seeds during transport and transshipment;
- Oilseed rape can hybridise with *Brassica rapa* which is a common plant along Dutch roadsides. To a lesser extent it can also hybridise with *Brassica juncea* and *Brassica oleracea*;
- Stable incorporation (introgression) of genes from *B. napus* into wild populations of *B. rapa* and *B. napus* may be possible;

- The molecular characterisation of LBFLFK meets the criteria of COGEM;
- Apart from a reduced seed germination rate, the phenotypic and agronomic characteristics of LBFLFK are comparable to conventional oilseed rape;
- There are no indications that LBFLFK is able to grow in other habitats than conventional oilseed rape;

- COGEM is of the opinion that import and processing of LBFLFK oilseed rape poses a negligible risk to the environment in the Netherlands;
- COGEM considers the provided PMEM plan adequate for import and processing of LBFLFK oilseed rape;
- COGEM abstains from giving advice on the potential risks of incidental consumption since a food/feed assessment is carried out by other organisations.

1. Introduction

The present application (EFSA/GMO/DE/2019/157), filed by BASF Plant Science Company GmbH, concerns import and processing of genetically modified (GM) oilseed rape (*Brassica napus*) LBFLFK for use in food, feed and other products.

In LBFLFK, a metabolic pathway was introduced to alter the fatty acid composition of its seeds. Twelve expression cassettes that encode seven different fatty acid desaturarases and three different fatty acid elongases were introduced. In addition, LBFLFK expresses a modified *AHAS* gene from *Arabidopsis thaliana* which confers tolerance to imidazolinone containing herbicides.

2. Previous COGEM advices

COGEM issued a generic advice on aspects relevant for import and processing of GM oilseed rape in the Netherlands.[1] In addition, several advices were issued on import and processing of GM oilseed rape lines. In most cases, COGEM advised negatively on import and processing of these lines, because the post-market environmental monitoring (PMEM) plan supplied by the applicant did not meet COGEM's requirements.[2,3,4,5,6,7]

COGEM did not advise previously on applications for import and processing of GM oilseed rape lines with an altered fatty acid composition, or tolerance to imidazolinone containing herbicides, but did advise positively on import and processing of GM soybean lines with an altered fatty acid composition,[8,9,10,11] or tolerance to imidazolinone containing herbicides.[12]

3. Environmental risk assessment

3.1 Aspects of the wild-type crop

Oilseed rape (*Brassica napus*) is a member of the *Brassicaceae* family, which also includes *Brassica rapa*, *Brassica juncea*, *Brassica oleracea* (cabbage), *Brassica nigra* (black mustard) and *Brassica carinata* (Ethiopian mustard). *B. napus* is a hybrid that originates from the interspecific hybridisation of *B. oleracea* and *B. rapa*.[1,13]

B. napus reproduces by self- and cross-pollination. It produces high amounts of pollen, which are dispersed by both wind and insects. In fields, the average rate of cross-pollination is 30%. The seeds of *B. napus* develop in a fruit, and are small, light and produced in large quantities.[1,14,15]

In the Netherlands, *B. napus* is grown as a crop and its seeds are imported for oil production. Wild *B. napus* populations grow on disturbed soil. *B. napus* is able to form volunteers in distributed environments near roadsides, railways and handling areas. The spillage of oilseed rape seeds during transport and transshipment has led to the establishment of feral populations, with a small number of plants (25 or less) per location, along distribution routes and handling areas.[16]

Oilseed rape can cross-pollinate with its more common wild relative *B. rapa* and to a lesser extent with *B. juncea* and *B. oleracea*.[1,14] Oilseed rape x *B. rapa* hybrid plants have been observed in the Netherlands.[17] Stable incorporation (introgression) of genes from *B. napus* into wild *B. rapa* has not been documented in the Netherlands, but has been reported in Canada.[18]

Conclusion: Wild *B. napus* populations exist in the Netherlands. *B. napus* can hybridise with its wild relative *B. rapa.* Therefore, GM volunteers from spilled seeds can lead to dispersal of genes to wild populations of *B. napus* and *B. rapa.*

3.2 Description of the introduced genes and traits

Oilseed rape LBFLFK was produced by *Agrobacterium rhizogenes* (strain SHA001) mediated transformation of oilseed rape (*B. napus* cultivar Kumily). The T-DNA region of the plasmid used for the transformation (LTM593) is 44,010 bp and contains 13 expression cassettes.

One expression cassette encodes acetohydroxy acid synthase (AHAS). The coding sequence contains two mutations compared to the sequence in the donor organism *A. thaliana*. These mutations result in two amino acid substitutions: [A122T] and [S653N]. In addition, nucleotides were changed to eliminate unwanted restriction sites. These did not alter the amino acid sequence of the protein.
The AHAS protein catalyses the first step in the biosynthesis of branched chain amino acids. The amino acid substitutions impair imidazolinone binding to the AHAS large subunit protein, conferring tolerance to imidazolinone herbicides.

Twelve of the expression cassettes encode enzymes involved in the synthesis of fatty acids, i.e. seven different desaturases and three different elongases. These enzymes are expressed in the seeds of LBFLFK. As a result, omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are produced in its seeds.
The sequences encoding fatty acid synthesis enzymes were optimised for codon usage in *B. napus.* In addition, the following elements (if present) were removed: 1) additional open reading frames (ORFs) longer than 90 bp in sense and anti-sense directions; 2) ORFs within 30 bp after the start codon in sense direction; 3) internal TATA-boxes, chi sequences and ribosomal entry sites; 4) AT-rich or GC-rich sequence stretches, 5) RNA instability motifs, 6) RNA secondary structures and repeat sequences, and 7) possible cryptic intron splice donor and acceptor sites in higher eukaryotes. According to the applicant, analysis of the deduced amino acid sequences showed that these modifications did not alter the amino acid sequences of the encoded proteins, except for one amino acid substitution [P196S] in the delta-6 elongase derived from *Thalassiosira pseudonana*. The applicant states that the substitution is not located in any of the known conserved domains responsible for its function.

Introduced genes (in order of the introduced biosynthesis pathway)	**Encoded proteins**	**Regulatory elements**	**Traits**
D12D(Ps)	Delta-12 desaturase from *Phytophthora sojae*	seed-specific promoter from the gene encoding napin (napA) from *B. napus* intron containing 5'untranslated region (UTR) from *A. thaliana* locus At5g63190 polyadenylation and termination of transcription: E9 3'UTR of the gene encoding ribulose bisphosphate carboxylase (rbcS) from *Pisum sativum*	Converts oleic acid (C18:1n-9) to linoleic acid (C18:2n-6)
D6D(*Ot*)	Delta-6 desaturase from *Ostreococcus tauri*	seed-specific promoter from the gene encoding sucrose binding protein (SBP) from *Vicia faba* intron containing 5'UTR from *A. thaliana* locus At1g65090 polyadenylation and termination of transcription: 3'UTR of the gene encoding cathepsin D inhibitor *(CATHD)* from *Solanum tuberosum*	Converts linoleic acid (C18:2n-6) to gamma-linolenic acid (C18:3n-6)
D6E(*Tp)*	Delta-6 elongase from *T. pseudonana*	seed-specific promoter from the gene encoding	Converts gamma-linolenic acid (C18:3n-6) to dihomo-

		peroxiredoxin-like protein *(PXR)* from *Linum usitatissimum* intron containing 5'UTR from *A. thaliana* locus At1g62290 polyadenylation and termination of transcription: 3'UTR of the gene encoding a peroxiredoxin-like protein (PER1) from *A. thaliana*	gamma-linolenic acid (C20:3n-6)
D6E(*Pp*)	Delta-6 elongase from *Physcomitrella patens*	promoter from a gene encoding a seed protein of unknown function (USP) from *V. faba* intron containing 5'UTR from *A. thaliana* locus At1g01170 polyadenylation and termination of transcription: 35S 3'UTR from *Cauliflower mosaic virus (CaMV)*	idem
D5D(*Tc)1*	Delta-5 desaturase from *Thraustochytrium* sp.	seed-specific promoter from the gene encoding conlinin (CNL) from *L. usitatissimum* intron containing 5'UTR from *A. thaliana* locus At5g63190 polyadenylation and termination of transcription: 3'UTR of the gene encoding octopine	Converts dihomo-gamma-linolenic acid (C20:3n-6) to arachidonic acid (C20:4n-6)

		synthase (OCS) from *Agrobacterium tumefaciens*	
D5D(*Tc)2*	idem	seed-specific promoter from the *SETL* gene from *B. napus* termination of transcription: terminator of the *SETL* gene from *B. napus*	idem
O3D(*Pir*)1	Omega-3 desaturase from *Pythium irregulare*	seed-specific promoter from the *SETL* gene from *B. napus* termination of transcription: terminator of the SETL gene from *B. napus*	Converts arachidonic acid (C20:4n-6) to eicosapentaenoic acid (C20:5n-3)
O3D(*Pir*)2	idem	seed-specific promoter from the gene encoding peroxiredoxin-like protein (PXR) from *L. usitatissimum* intron containing 5'UTR from the gene encoding ARGONAUTE4 (AGO4) from *A. thaliana* polyadenylation and termination of transcription: 3'UTR of the gene encoding a peroxiredoxin-like protein (PER1) from *A. thaliana*	idem
O3D(*Pi*)	Omega-3 desaturase from *Phytophthora infestans*	promoter from a gene encoding a seed protein of unknown function (USP) from *V. faba* intron containing 5'UTR	idem

		from *A. thaliana* locus At1g01170 polyadenylation and termination of transcription: 35S 3'UTR from *CaMV*	
D5E(*Ot*)	Delta-5 elongase from *Ostreococcus tauri*	seed-specific promoter from a gene encoding a fatty acid elongase (FAE1) from *B. napus* intron containing 5'UTR from *A. thaliana* locus At1g62290 polyadenylation and termination of transcription: 3'UTR of a gene encoding a fatty acid elongase (FAE1) from *A. thaliana*	Converts eicosapentaenoic acid (C20:5n-3) to docosapentaenoic acid (C22:5n-3)
D4D(*Tc*)	Delta-4 desaturase from *Thraustochytrium* sp	seed-specific promoter from the gene encoding Arcelin-5 (Arc5) from *Phaseolus vulgaris* polyadenylation and termination of transcription: 3'UTR of the gene encoding Arcelin-5 (Arc5) from *P. vulgaris*	Converts docosapentaenoic acid (C22:5n-3) to docosahexaenoic acid (C22:6n-3)
D4D(*PI*)	Delta-4 desaturase from *Pavlova lutheri*	seed-specific promoter from the gene encoding conlinin (CNL) from *L. usitatissimum* intron containing 5'UTR from *A. thaliana* locus At1g65090	idem

		polyadenylation and termination of transcription: 3'UTR of the gene encoding octopine synthase (OCS) from *A. tumefaciens*	
AHAS(*At*)	Modified acetohydroxy acid synthase from *A. thaliana*	constitutive promoter from the gene encoding ubiquitin (Ubi4) from *Petroselinum crispum* intron containing 5'UTR from the gene encoding ubiquitin (Ubi4) from *P. crispum* polyadenylation and termination of transcription: 3'UTR of the gene encoding acetohydroxy acid synthase large subunit (AHAS) from *A. thaliana*	Confers tolerance to imidazolinone herbicides

3.3 Molecular characterisation

Next Generation Sequencing (NGS) of LBFLFK (third generation after transformation) showed that LBFLFK contains two copies of the T-DNA at different locations of the nuclear genome and does not contain vector backbone sequences.

The two inserts and their flanking regions (>1kb) were sequenced. Sequence analyses demonstrated that insert 1 consists of 43,818 bp. Both the 5' end of the right border region (184 bp) and the 3' end of the left border region (72 bp) of the T-DNA region were trimmed. In addition, sequence analyses revealed that the first 64 bp of the right border region in insert 1 contains a rearrangement consisting of short repeats from the right border region of the T-DNA. Eight base pairs of the host genome were deleted upon integration of the T-DNA.

Insert 2 consists of 43,779 bp. Both the 5' end of the right border region (184 bp) and the 3' end of the left border region (53 bp) of the T-DNA region were trimmed. At both sides of the insertion site base pairs were added: two base pairs at the 5' end, and four base pairs at the 3' end. Thirty-one base pairs of the host genome were deleted upon integration of the T-DNA.

According to the applicant, there are no indications that endogenous genes were disrupted by insertion of the T-DNA regions. Bioinformatic analyses did not identify any endogenous genes at the sites of integration.

A comparison of the sequence of the two inserts to the sequence of the T-DNA region present in plasmid LTM593 identified three nucleotide changes. At insert 1, the promoter sequence of one of the genes encoding an omega-3 desaturase (O3D(Pir)2) was changed. An adenine was present instead of a cytosine. In addition, in the coding sequence of the gene encoding delta-12 desaturase (D12D(Ps)) an adenine was present instead of a cytosine. This results in a phenylalanine to leucine substitution [F83L]. At insert 2, in the coding sequence of one of the genes encoding a delta-4 desaturase (D4D(Pl)) a thymine was present instead of a guanine. This results in a alanine to serine substitution [A102S]. According to the applicant, these substitutions do not have an impact on the function or the activity of the two desaturases.

The applicant analysed the rearranged region in insert 1 and the four junctions between the insertion sites and the oilseed rape genome to identify all potential newly created open reading frames (ORFs) between stop codons. 37 putative ORFs were identified in the six reading frames and translated in silico into amino acid sequences. Sequences of eight or more amino acids were subjected to bio-informatic analyses. According to the applicant, no potential allergenicity or toxicity concerns were discovered by the bio-informatic analyses.

The molecular characterisation was conducted according to the criteria previously laid down by COGEM.[19] The results from the updated molecular characterisation do not provide indications that LBFLFK could pose a risk to the environment.

Conclusion: The molecular characterisation of oilseed rape LBFLFK is adequate and no indications for potential environmental risks were identified.

3.4 Biological characteristics of LBFLFK

The applicant evaluated the phenotypic and agronomic characteristics of oilseed rape LBFLFK and assessed the germination and dormancy of its seeds. The phenotypic and agronomic evaluation indicated that LBFLFK is comparable to conventional oilseed rape, but seed germination assays show that seed germination is reduced. Dormancy of LBFLFK seeds is comparable to conventional oilseed rape seeds. The evaluation of phenotypic and agronomic characteristics carried out by the applicant did not provide any indications that LBFLFK has an increased fitness under natural conditions. In places where imidazolinone containing herbicides are used for weed control, herbicide tolerance may give LBFLFK an advantage over other plants. In the Netherlands, imidazolinone containing herbicides may only be used on agricultural fields.[20]

COGEM notes that there are indications that the presence of some fatty acids may alter the attractiveness of seeds to predators[21] and could affect the seed predation rate. As the fatty acid composition of LBFLFK's seeds is altered, COGEM is of the opinion that the applicant should have discussed the likelihood of an altered predation rate and potential consequences thereof. COGEM evaluated whether an altered predation rate could allow LBFLFK to become invasive. If predators avoid LBFLFK's seeds, the number of LBFLFK plants in disturbed areas may increase. Oilseed rape

grows on disturbed soil and does not compete well with other plants.[22] It is virtually absent in areas with dense grass.[23] There are no indications that *B. napus* cultivars or *B. rapa* accessions with seeds which are avoided by rodents are invasive in the Netherlands.[21] Therefore, COGEM considers it unlikely that an altered predation rate would enable LBFLFK oilseed rape to become invasive and grow in other habitats than conventional oilseed rape.
In summary, COGEM is of the opinion that there are no indications that LBFLFK oilseed rape poses an environmental risk.

Conclusion: There are no indications that LBFLFK has an increased fitness under natural conditions.

4. Food/feed assessment

This application is submitted under Regulation (EC) 1829/2003, therefore a food/feed assessment is carried out by EFSA and national organisations involved in the assessment of food safety. In the Netherlands, a food and/or feed assessment for Regulation (EC) 1829/2003 applications is carried out by Wageningen Food Safety Research (WFSR). The outcome of the assessment by these other organisations was not known when this advice was completed.

5. Post-market environmental monitoring (PMEM)

The applicant supplied a general surveillance plan as part of the PMEM. On several occasions, COGEM has expressed concerns with regard to the PMEM plan of GM oilseed rape events. Feral oilseed rape populations can arise from GM oilseed rape seeds spilled during transshipment and transport, and prolonged use of the corresponding herbicide may lead to the establishment of feral herbicide tolerant GM oilseed rape. Gene flow between different GM oilseed rape events could give rise to stacked GM oilseed rape events with a new combination of GM traits. As it cannot be excluded beforehand that such a newly generated stacked event may have an adverse effect, COGEM is of the opinion that in case of GM oilseed rape an elaborate PMEM plan is needed.
The GM oilseed rape event under consideration, LBFLFK, is tolerant to imidazolinone containing herbicides. In the Netherlands, imidazolinone containing herbicides are authorised for use in agricultural fields,[20] but not at transshipment areas and along transport routes where spillage of LBFLFK seeds may occur. In practice, LBFLFK plants arising from spilled seeds will not have an advantage over other plants. In view of the above, COGEM is of the opinion that the chance that a new stacked event arises from spilled LBFLFK seeds is negligible. Therefore, in this particular case COGEM consider the submitted general surveillance plan adequate.

Conclusion: COGEM is of the opinion that the provided PMEM plan is adequate for import and processing of LBFLFK oilseed rape.

6. Overall conclusion

COGEM is of the opinion that import and processing of oilseed rape LBFLFK poses a negligible to the environment in the Netherlands and considers the provided PMEM plan adequate for import and processing of LBFLFK oilseed rape.
COGEM abstains from giving advice on the potential risks of incidental consumption since other organisations carry out a food/feed safety assessment.

7. Additional remarks

COGEM notes that the applicant does not discuss all aspects that are relevant for the environmental risk assessment in its application. Potential direct effects of LBFLFK on non-target organisms are not discussed even though repeated incidental spillage of seeds could result in the presence of feral LBFLFK populations for a prolonged period. However, the feral oilseed rape populations that are found along distribution routes and handling areas in the Netherlands are small, and COGEM is not aware of any reports on adverse effects on non-target organisms resulting from an altered fatty acid composition.
Also, as mentioned previously, the applicant does not discuss the likelihood and consequences of an altered seed predation rate even though this could affect fitness. However, there are no indications that *B. napus* cultivars or *B. rapa* accessions with seeds which are avoided by rodents are invasive in the Netherlands.[21] Although COGEM considers it an omission that the applicant does not discuss these aspects, they do not alter the outcome of the risk assessment.

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