

Import and processing of genetically modified RF3 Canola Quality (CQ) *Brassica juncea*

COGEM advice CGM/201126-02

- The present application (EFSA/GMO/NL/2019/158) concerns the authorisation for import and processing for use in feed and food of genetically modified (GM) RF3 Canola Quality (CQ) *Brassica juncea*;
- RF3 CQ *B. juncea* was developed via conventional crossing of GM *B. napus* (oilseed rape) RF3 and *B. juncea*;
- RF3 CQ *B. juncea* expresses the *barstar* gene, which restores male fertility and is part of a pollination control system. It also expresses the *bar* gene, which confers tolerance to glufosinate-ammonium containing herbicides;
- *B. juncea* is used as a catch crop or green manure in the Netherlands, but the presence of *B. juncea* outside agricultural fields is rare. *B. juncea* is considered an adventive species;
- Under natural conditions, *B. juncea* can hybridise with *B. napus*, and possibly also with *B. rapa* and *B. nigra*;
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- There are no indications that the introduced traits alter the fitness of RF3 CQ *B. juncea* under natural conditions. Herbicide tolerance gives the plant advantage in places where glufosinate-ammonium herbicides are used for weed control, however, glufosinate ammonium herbicides are no longer used along transport routes;
- The molecular characterisation of RF3 CQ *B. juncea* meets the criteria of COGEM;
- COGEM is of the opinion that the chance of gene flow from spilled RF3 CQ *B. juncea* seeds to wild relatives is negligible, because of the temporary presence of GM *B. juncea*, and low hybridisation rates;
- COGEM considers the provided PMEM plan adequate for import and processing of RF3 CQ *B. juncea*;
- COGEM is of the opinion that import and processing of RF3 CQ *B. juncea* poses a negligible risk to the environment in the Netherlands;
- 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/NL/2019/158), filed by BASF Agricultural Solutions Seeds US LLC, concerns the import and processing of genetically modified (GM) RF3 Canola Quality (CQ) *B. juncea*, for use in feed and food. RF3 CQ *B. juncea* was developed via conventional crossing of GM *B. napus* (oilseed rape) RF3 and non-GM *B. juncea*, followed by a series of backcrosses

resulting in the incorporation of the transgenic sequence into an elite CQ *B. juncea* line. This GM *B. juncea* expresses the *barstar* gene, which encodes the Barstar protein. This protein inhibits the Barnase protein which functions as a pollination control system and confers male sterility in certain GM lines. Presence of the Barstar protein can therefore restore male fertility in GM lines which carry the *barnase* gene. RF3 CQ *B. juncea* also expresses the *bar* gene, which encodes the phosphinothricin acetyl transferase (PAT/*bar*) protein and confers tolerance to glufosinate-ammonium containing herbicides.

2. Previous COGEM advice

COGEM has not previously advised on import and processing of GM *B. juncea*. However, COGEM did advise on import and processing of the parental line GM *B. napus* RF3.^{1,2,7,8} Following a generic COGEM advice in 2013 on aspects relevant for import and processing of GM oilseed rape in the Netherlands,³ COGEM issued several opinions in which it advised negatively on import and processing of GM oilseed rape RF3 or stacked events which include RF3, because the post-market environmental monitoring (PMEM) plan supplied by the applicant did not meet COGEM's requirements.^{4,5,6,7,8}

3. Environmental risk assessment

3.1 Characteristics of *Brassica juncea*

Brassica juncea, also known as Indian mustard, is a member of the *Brassicaceae* family, which also includes *Brassica napus* (oilseed rape), *Brassica rapa*, *Brassica oleracea* (cabbage), *Brassica nigra* (black mustard) and *Brassica carinata* (Ethiopian mustard). *B. juncea* originated from the interspecific hybridisation of *B. nigra* (BB, $2x=16$) and *B. rapa* (AA, $2x=20$), and contains the diploid chromosome set of both parents (AABB, $2n=4x=36$).⁹ The original distribution of *B. juncea* is unclear, but it is thought to range from Eastern Europe to China, where both parental species grow.¹⁰ Based on research using genetic markers, hybridisation events may have occurred several times throughout history.¹¹ Compared to *B. napus* and *B. rapa*, *B. juncea* is considered to be more tolerant to heat and drought stress and displays less seed shattering.¹²

B. juncea can be divided into four subspecies; *ssp. integrifolia*, *ssp. juncea*, *ssp. napiformis* and *ssp. taisai*, which differ in morphology and use.¹³ Most often, *B. juncea* is cultivated for its seeds, from which oil is extracted and processed meal is used for animal feed, but the crop can also be grown for use as leaf vegetable.

In the Netherlands, *B. juncea* is used as green manure or as a catch crop.^{14,15} It is also grown in vegetable gardens by amateur growers.¹⁶ The presence of *B. juncea* outside agricultural fields is rare in the Netherlands, and it is considered an adventive species.^{17,18,19,20}

B. juncea is an annual species which 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 similar to *B. napus* and is considered to be 30%.^{9,10} The seeds of *B. juncea* develop in a pod, and are small, light and produced in large quantities. Seeds from *B. juncea* are generally smaller than *B. napus* seeds.⁹

Under artificial conditions, interspecific and intergeneric crosses with *B. juncea* have been described for about 15 different species,²¹ including related (cultivated) *Brassica* species; *B. napus*, *B. carinata* and native species *B. nigra* or *B. rapa*.^{9,13,21,22,23,24,25,26} Reports of cross pollination between *B. juncea* and other species under natural conditions are rare.²¹ Cross pollination between *B. juncea* and *B. napus* under natural conditions occurs, but hybridisation rates are generally low.^{9,13,21,24} Pollen and seed fertility of *B. juncea* and *B. napus* hybrids are often less than 30%, but spontaneous backcrossing resulting in progeny with improved fertility has been reported.²² Although cross pollination between *B. juncea* and native *Brassica* species *B. rapa* and *B. nigra* has only been observed following hand pollination, it cannot be excluded to also occur under natural conditions.²¹ Hybrids with *B. rapa* produce viable seed.¹³ Hybridisation between *B. juncea* and *Sinapis arvensis* (pollen recipient) was detected during field co-cultivation, but at a very low frequency ($1,8 \times 10^{-5}$).^{9,13} One of the two resulting hybrids was able to set seed when self-pollinated, and the next generation plants showed good growth and pollen fertility. However, backcrossing of the hybrids to *S. arvensis* was unsuccessful, indicating that gene flow from *B. juncea* to *S. arvensis* is unlikely.¹³

Conclusion: *B. juncea* is cultivated in the Netherlands, but the presence of *B. juncea* outside agricultural fields is rare. In the Netherlands, *B. juncea* is an adventive species. Under natural conditions, *B. juncea* can hybridise with *B. napus*, and possibly also with native species *B. rapa*, *B. nigra*.

3.2 Description of the introduced genes, traits and regulatory elements

RF3 CQ *B. juncea* was developed by crossing *B. juncea* (ssp. *juncea*) and GM *B. napus* RF3, followed by a series of backcrosses resulting in the incorporation of the RF3 trait into CQ *B. juncea*. RF3 CQ *B. juncea* therefore contains the same transgenes as present in GM *B. napus* RF3. A description of the inserted genetic elements is listed in the table below. The list is limited to information on the introduced genes, corresponding traits, and regulatory elements (promoters and terminators).

Introduced genes	Encoded products	Traits	Regulatory elements
<i>Barstar</i> (origin <i>Bacillus amyloliquefaciens</i>)	Encodes the Barstar ribonuclease-inhibitor protein, which is expressed in the anther tapetal cell layer during pollen development ²⁷	Restores male fertility	Pta29 promotor from <i>Nicotiana tabacum</i> and the terminator of the <i>nopaline synthase</i> gene from <i>Agrobacterium tumefaciens</i>
<i>bar</i> (origin <i>Streptomyces hygroscopicus</i>)	Encodes the Phosphinothricin N-acetyltransferase (PAT) enzyme ²⁸	Tolerance to glufosinate-ammonium herbicides	PssuAt promotor from <i>Arabidopsis thaliana</i> and the Tg7 terminator derived from <i>A. tumefaciens</i>
See references for detailed descriptions of the traits.			

3.3 Molecular characterization and bioinformatic analysis

RF3 CQ *B. juncea* was obtained by crossing conventional *B. juncea* with GM RF3 *B. napus*. The molecular characterisation and bioinformatic analysis of RF3 *B. napus* were previously deemed adequate by COGEM, and based on the molecular characterisation and bioinformatic analysis no indications for potential environmental risks of RF3 *B. napus* were identified.⁷ The applicant compared the RF3 CQ *B. juncea* transgenic sequence (using overlapping PCR fragments and sequencing these fragments) and 1 kb flanking genomic regions with the previously determined transgenic locus sequence of RF3 *B. napus*, and found the sequences to be identical. Using Southern blot analysis, the applicant also confirmed the RF3 CQ *B. juncea* insert organization as determined in RF3 *B. napus* and the absence of vector backbone sequences.

The applicant also performed BLASTn and BLASTx analyses to determine the position of the transgenic sequence in the parental RF3 *B. napus* genome (chromosome C6 of *B. napus* cultivar ZS11) and concluded that it is unlikely that endogenous genes are disrupted by the insertion of the transgenic sequence. Because RF3 CQ *B. juncea* was obtained by conventional crossing, and the transgenic sequence was found to be identical to GM RF3 *B. napus*, the applicant draws the same conclusion for RF3 CQ *B. juncea*.

The applicant screened the transgenic locus, containing the insert and 5' and 3' flanking sequences for potential newly created open reading frames (ORFs) between stop codons. The applicant identified 353 ORFs with a size > 8 amino acids and compared these to known allergens and toxins, but no potential allergenicity or toxicity concerns were discovered. The applicant also performed an identity search for amino acid sequence homology of the Barstar and PAT/bar protein sequences to protein sequences in a general protein database and searched for sequence homology with known toxins. Most alignments corresponded to expected sequences and no indications of toxicity were found for both proteins.

The molecular characterisation was conducted according to the criteria previously laid down by COGEM.²⁹ The results from the molecular characterisation and bioinformatic analysis do not provide indications that RF3 CQ *B. juncea* could pose a risk to the environment.

<p>Conclusion: The molecular characterisation of RF3 CQ <i>B. juncea</i> is adequate and no indications for potential environmental risks were identified.</p>

3.4 Phenotypic and agronomic characteristics

The applicant performed field trials to analyse the phenotypic and agronomic characteristics of RF3 CQ *B. juncea*. Statistically significant differences were observed between RF3 CQ *B. juncea* treated with conventional herbicides (CHM) or trait-intended herbicides (TIH) and the *B. juncea* conventional counterpart (CHM) for 5 out of 11 agronomic and phenotypic parameters, i.e., days to flowering, plant height, days to maturity, seed moisture, and final stand count (for TIH only) or flowering duration (for CHM only). However, no statistically significant differences were found for all agronomic and phenotypic parameters when comparing CHM or TIH RF3 CQ *B. juncea* with (seven) non-GM *B. juncea* reference varieties (CHM, selected to represent a range of genetic backgrounds and phenotypic characteristics). This indicates that the agronomic and phenotypic

parameters fall within the natural range of genetic variation of *B. juncea* reference varieties. The differences with the conventional counterpart are therefore not considered biologically relevant by the applicant.

Herbicide tolerance gives RF3 CQ *B. juncea* an advantage in places where glufosinate-ammonium herbicides are used for weed control. However, the use of herbicides is phased out, and glufosinate-ammonium herbicides are no longer used along transport routes.^{30,31} In view of the above, COGEM is of the opinion that the introduced traits do not give reason to assume that RF3 CQ *B. juncea* has an altered survivability compared to its conventional counterparts and commercial reference *B. juncea* varieties under natural conditions. Therefore, there are no indications that RF3 CQ *B. juncea* poses an environmental risk.

<p>Conclusion: There are no indications that the introduced traits alter the fitness of RF3 CQ <i>B. juncea</i> under natural conditions.</p>
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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 other organisations (EFSA, WFSR) 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. For previous applications for import and processing of GM oilseed rape (*B. napus*) events, COGEM has expressed concerns with regard to the PMEM plan. 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 newly generated stacked events may have an adverse effect, COGEM is of the opinion that in case of GM oilseed rape an elaborate PMEM plan is needed. Although *B. juncea* is not an established species in the Netherlands, it can grow as an adventive species. Adventive RF3 CQ *B. juncea* may grow where spillage of seeds occurs during transshipment and transport in the Netherlands. Cross-pollination between *B. juncea* and other related (native) *Brassica* species cannot be excluded. However, considering the temporary presence of GM *B. juncea*, and the low hybridization rates,²¹ COGEM is of the opinion that the chance of gene flow from spilled RF3 CQ *B. juncea* seeds to wild relatives is negligible. In view of the aforementioned, COGEM considers the submitted general surveillance plan for RF3 CQ *B. juncea* adequate.

6. Overall conclusion

There are no indications that expression of the introduced traits will alter the fitness of RF3 CQ *B. juncea* under natural conditions. COGEM is of the opinion that the chance of gene flow from spilled RF3 CQ *B. juncea* seeds to wild relatives is negligible. COGEM concludes that import and processing of RF3 CQ *B. juncea* poses a negligible risk to the environment in the Netherlands. COGEM abstains from giving advice on the potential risks of incidental consumption since other organisations carry out a food/feed safety assessment.

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