# Advisory report on import of glyphosate resistent oilseed rape 73496

## COGEM advisory report CGM/130208-02

*This opinion concerns an application for import and processing of genetically modified oilseed rape 73496. Genetically modified oilseed rape 73496 produces the GAT4621* N-*acetyltransferase protein which detoxifies glyphosate by acetylation, leading to* N-*acetyl glyphosate as a residue. As a result, 73496 is resistant to glyphosate containing herbicides. Due to the insertion of the* gat4621 *gene cassette a* tpt *gene located in the 5' border region of the insert was disrupted.*

*COGEM points out that the molecular characterisation of 73496 is incomplete because the insert site was not completely characterised.*

*The establishment of small populations of 73496 oilseed rape on locations where glyphosate is frequently applied to control weeds, e.g. on railway tracks, cannot be excluded. If small 73496 oilseed rape populations become established, cross-fertilisation could occur with oilseed rape and/or wild relatives, in particular turnip. The resulting progeny will not be different from progeny formed after cross-fertilisation with conventional oilseed rape varieties apart from the ability to produce the GAT4621* N-*acetyltransferase protein and the disruption of a* tpt *gene. COGEM considers it unlikely that any resulting progeny will possess a selective advantage unless glyphosate is applied.*

*COGEM recommends to include the monitoring of distribution routes for the occurrence of oilseed rape volunteers in the monitoring plan. It cannot be excluded that cross-fertilisation could lead to the eventual stacking of transgenes in a single oilseed rape plant. COGEM is of the opinion that stacking of the traits in the present oilseed rape varieties will not lead to an environmental risk because these traits are unlikely to lead to an increased fitness or a selective advantage under natural conditions. However, it is important to know whether stacking occurs in order to allow future risk assessments to take into account the presence of established GM oilseed rape with stacked traits. A stacked event would most likely occur in a location where herbicides are frequently used, such as railway tracks. Therefore, COGEM advises to involve railway companies and/or companies in charge of the maintenance of railway tracks in the post-market monitoring plan in order to monitor the occurrence of GM oilseed rape on railway tracks.*

*In summary, COGEM is of the opinion that the molecular characterisation of 73496 is incomplete. Moreover, COGEM is of the opinion that the monitoring plan of 73496 should be improved before a market authorisation is granted. Therefore, COGEM cannot advise positively on the application for import and processing of 73496 oilseed rape. Due to time constraints, COGEM did not assess all elements of the application (e.g. putative effects of* N-*acetyltransferase on other compounds). Therefore, COGEM reserves its right to comment further on this application in the future.*

### Introduction

The present application by Pioneer Hi-Bred International (EFSA/GMO/NL/2012/109), concerns import and processing of genetically modified (GM) oilseed rape 73496 and its use as any other conventional oilseed rape variety with the exception of cultivation. In 2012, 73496 oilseed rape was approved for cultivation, food and feed in Canada and for feed in Mexico.[1]

GM oilseed rape 73496 expresses the *gat4621* gene resulting in resistance to glyphosate containing herbicides. The *gat4621* gene has previously been introduced into the genetically modified maize event 98140.

COGEM previously advised on import and processing of oilseed rape events with similar traits, such as the glyphosate tolerant oilseed rape event MON83302[2] and the glyphosate tolerant and resistant oilseed rape event GT73.[3,4,5,6,7] COGEM also advised on import and processing of the GM maize event 98140 in which the *gat4621* gene was introduced. Due to an incomplete molecular characterisation, COGEM could not advise positively on import and processing of GM maize 98140.[8]

**Considerations**

***Aspects of the crop***

In this opinion, only those biological characteristics of oilseed rape are described that are of immediate concern to the environmental risk assessment of import and processing of 73496 oilseed rape in the Netherlands. In the coming weeks, COGEM will publish an opinion on all elements that are important when assessing the environmental risks of import and processing of oilseed rape in the Netherlands.

Oilseed rape (*Brassica napus*) has established itself in the Netherlands.[9] Recently, the assumption that oilseed rape is widespread in the Netherlands was refuted by results from a research project commissioned by COGEM. The researchers studied the distribution of oilseed rape in the Netherlands and found that oilseed rape (*B. napus*) is often confused with turnip (*Brassica rapa*). Oilseed rape was present across the Netherlands, but in general only a small number of plants (25 or less) was found on a single location.[16] Populations of oilseed rape are nearly always located on highly disturbed soil and close to locations where it is cultivated or where seeds are spilled in transport along roads or in transshipment.[16] This indicates that under non-cultivated conditions and without human intervention feral oilseed rape plants are unable to survive for more than a few generations in the Netherlands. These observations are consistent with previous studies that reported that in undisturbed habitats feral populations become extinct over a period of years.[10] Although some feral plants can persist for several years, there is no evidence that oilseed rape is invasive in undisturbed natural habitats in other European countries.[21]

Seeds of oilseed rape are small and produced in large quantities.[19] Oilseed rape seeds display little or no dormancy.[11] When seeds escape harvesting they may germinate immediately when conditions are favourable. Plants emerging from these seeds may overwinter or regenerate from the bottom of stems if died back during winter.[12] When conditions are not favourable for germination and seeds are buried e.g. by tillage, seeds can persist in the soil and form a seed bank. Several publications on the persistence of oilseed rape seeds in soil seed banks have been published.[13,14,15] These studies indicate that the seed bank has a quite rapid turnover, but that a small portion of oilseed rape seeds may remain viable for several (up to ten) years.

Oilseed rape (an allotetraploid with chromosome number 2n = 38, AACC) is a member of the *Cruciferae* or *Brassicaceae* family which originates from interspecific hybridization between the two diploid species *Brassica oleracea* (2n = 18, CC) and *B. rapa* (2n = 20, AA).[16] Oilseed

rape reproduces sexually. It is mainly a self-pollinating species, but outcrossing may occur.[17,18]

Controlled pollination studies have pointed out that oilseed rape can outcross with several of the wild relatives that occur in the Netherlands. These relatives include *B. rapa* (turnip), *Brassica juncea* (Indian mustard), *Hirschfeldia incana* (shortpod mustard), *Raphanus raphanistrum* (wild radish), *B. oleracea* (wild cabbage), *Sinapis arvensis* (charlock) and *Diplotaxis tenuifolia* (perennial wall rocket).[9,19,20,21] The potential for cross-pollination between oilseed rape and its wild relatives is influenced by several factors such as pollen viability, overlapping flowering period, distance, and insect activity. Because of a mismatch in chromosome numbers most hybrids have a severely reduced fertility (very low pollen viability and seed production). Exceptions are hybrids obtained from crosses between oilseed rape and *B. rapa*. Only some of the interspecific embryos develop into viable seeds.[22] F1 hybrids resulting from these seeds can produce pollen and seed, although pollen viability and percentage seed set is typically lower than that of the parental species.[21] In the wild F1 hybrids between the two species are relatively rare, but have been reported for Canada, the United States, New Zealand, Japan, the United Kingdom, Denmark, Czech Republic, and the Netherlands. [21,23,24]

In a study focusing on hybridisation between oilseed rape and *B. rapa* in the Netherlands, Luijten & De Jong (2011) found some F1 hybrids containing nine C chromosomes from oilseed rape in addition to the normal set of A chromosomes. They did not identify any wild *B. rapa* plants with other numbers of C chromosomes nor did they find molecular markers characteristic for oilseed rape in *B. rapa* populations close to crops or feral populations of oilseed rape.[23] Therefore they argue that if hybrids between oilseed rape and *B. rapa* are formed, the progeny arising from crosses between these hybrids and *B. rapa* is probably unfit. This would limit the establishment of hybrids between oilseed rape and wild *B. rapa* to the first generation. Other researchers have also reported that backcross progeny of F1 hybrids exhibited a low survival rate and fertility.[22] Nevertheless, in other countries it has been shown that gene flow from oilseed rape to *B. rapa* can occur.[23,25,26]

***Molecular characterisation***

Oilseed rape 73496 was produced by biolistic transformation of microspores (donor line 1822B). Gold particles were coated with the gel-purified PHP28181A restriction fragment (2112 bp) from plasmid PHP28181. This restriction fragment contains the *UBQ10* promoter, the *gat4621* gene and the *pin*II terminator.

After bombardment of the microspores, double haploid embryos were produced by the addition of Triflurarin which prevents the formation of spindle fibres during cell division.

An overview of the introduced sequences is given below:

- Polylinker region
- *UBQ10* promoter; derived from the polyubiquitin 10 gene of *Arabidopsis thaliana*, used to drive expression of the *gat4621* gene
- Polylinker region
- *gat4621* gene; generated by shuffling *gat* gene sequences from three *Bacillus licheniformis* strains. The *gat4621* gene encodes the GAT4621 enzyme which acetylates glyphosate
- Polylinker region
- *pin*II terminator, derived from the proteinase inhibitor II gene of *Solanum tuberosum*

- Polylinker region

The applicant used Southern blot analysis with the *Nco*I and *Ssp*I restriction enzymes to verify the number and integrity of the insert and to confirm the absence of plasmid backbone DNA. The applicant concludes that 73496 contains a single intact copy of the insert. The applicant points out that the probes that were used for Southern blot analysis did not cover the restriction fragment and the plasmid backbone completely. Six sequence regions (between 7 and 78 bp) were not covered by the probes due to constraints in the design of the PCR primers used to generate the probes. Overall, the probes covered >90% of the restriction fragment and >95% of the plasmid. Southern blot analysis showed that 73496 contains a single insert and showed that no backbone DNA is integrated. Based on the Southern blot analysis the insertion of tiny fragments (≤ 78 bp) on other locations in the 73496 genome cannot be excluded.

The applicant performed sequence analysis to determine the full sequence of the insert and its flanking genomic regions (>2 kb). Sequence analysis showed that the insert was identical to the PHP28181A fragment, except for a three basepair deletion at position 1 to 3 of the 5' end of the PHP28181A fragment.

PCR analysis was performed on 73496 and unmodified control canola lines to analyse the 5' and 3' flanking regions of the insert. In addition, the sequence of the flanking regions was determined and subjected to BLAST analyses. These regions aligned to several *Brassica* genomic sequences. The results from these analyses demonstrate that the regions flanking the insert are oilseed rape DNA.

BLAST analysis revealed that the 5' border region shows similarity to a putative triose phosphate translocator (*tpt*) gene. This indicates that the insertion of the *gat4621* gene cassette may have disrupted a *tpt* gene. *Tpt* genes encode TPT translocators which are located in the inner envelope membrane of chloroplasts. They export triose phosphates from the stroma to the cytosol where the triose phosphates are used as precursors for sucrose biosynthesis.[27] During the day TPT translocators are the major factor for photoassimilate export. During the night other transporters export maltose and glucose which are produced by the degradation of transitionary starch.[27]

The applicant analysed whether the identified putative *tpt* gene is transcriptionally active. Southern blot analysis showed that multiple (3-4) copies of the predicted *tpt* gene family are present in conventional *B. napus* genotypes. qRT-PCR results showed that the overall transcript level of the *tpt* gene family was significantly reduced by approximately 50% in leaf and showed that the transcript level of the *tpt* gene located in the 5' border region was significantly reduced by about 7-fold in leaf when compared to that of conventional oilseed rape.

Although the BLAST results of the 5' flanking region indicated that in the 5' border region a putative *tpt* gene was disrupted, in the 3' border region no similarities to this *tpt* gene were detected. The applicant does not provide an explanation for the absence of similarity to the putative *tpt* gene in the 3' border region.

The applicant analysed the nucleotide sequence between the most distal junction spanning stop codons to identify similarities to allergens or toxins. In all six reading frames all possible stop codon bracketed sequences were translated. If the translated sequence was eight or more

amino acids in length the sequence was analysed to identify possible similarities to known or putative allergens, toxins or other proteins. Only nine reading frames that spanned the genome – insert junctions (three frames span the 5' border and six frames span the 3' border) were eight or more amino acids in length. No similarities to known or putative allergens or toxins were identified. As expected from the above described results, the junction spanning the 5' border region showed similarity to triose phosphate translocators and glucose-6-phosphate translocators.

The applicant performed Southern Blot analysis on oilseed rape plants from several generations to assess the genetic stability of the inserted sequences. Two bands should be visible when Southern blot analysis was carried out with the *Ssp*I restriction enzyme and the *UBQ10* promoter or *pin*II terminator probes. The applicant reported that one of the two bands is faint and may not be visible on the presented image. However, COGEM noted that two bands are visible in some but not all analysed individuals, and that the visibility of the faint band did not seem to correlate to the strength of the other band. In addition, a deviation from the expected segregation ratio (1:1) was observed in the BC1F1[*1] and the BC1F1[*3] generations. The applicant attributes this deviation to the inadverted self-pollination of some of the flower buds. In view of the above, COGEM is of the opinion that the information on the genetic stability of the inserted sequences is inconclusive. It is however questionable whether this information is of importance to the risk assessment.

In conclusion, COGEM is of the opinion that the molecular characterisation of 73496 is incomplete because the insert region was not completely characterised. The applicant does not provide a rationale for the absence of similarity to the putative *tpt* gene in the 3' border region. As the absence of similarity could be caused by a deletion in the 3' border region COGEM is of the opinion that the applicant should provide more information to assure that the insert region is completely characterised.

***Properties of the gene conferring glyphosate resistance***

Glyphosate inhibits an enzyme involved in the biosynthesis of aromatic amino acids. Application of glyphosate therefore results in a lack of amino acids essential for growth and development of plants. [28]

73496 oilseed rape expresses the *gat4621* gene which is a shuffled variant of *gat* genes from three different *B. licheniformis* strains. GAT enzymes detoxify glyphosate by acetylating the secondary amine of glyphosate, thus generating the non-toxic compound N-acetyl glyphosate.[28] The GAT4621 protein which is produced by 73496 detoxifies glyphosate with improved efficiency in comparison to the native GAT proteins of *B. licheniformis*.

The GAT4621 enzyme has been shown to acetylate not only glyphosate but also certain amino acids *in vitro*. These amino acids include a.o. aspartic acid, glutamic acid, threonine, serine and glycine.[29] To determine whether these amino acids were acetylated in 73496 the applicant compared the level in seed of the acetylated derivates of these amino acids i.e. N-acetylaspartate, N-acetylglutamate, N-acetylthreonine, N-acetylserine and N-acetylglycine, to the level in the conventional near isoline control. According to the applicant the levels of the latter two were not significantly different from the near isoline. The levels of N-

acetylaspartate, N-acetylglutamate and N-acetylthreonine were higher than in the near isoline or in other conventional oilseed rape varieties.

Because the elevated level of the acetylated derivates of amino acids may influence the level of free amino acids, the concentration of twenty-two free amino acids in 73496 oilseed rape seeds has also been measured. In addition, the nutrient composition (including 18 proteinogenic amino acids) of 73496 seed was analysed. The level of some free and proteinogenic amino acids was significantly different from the near isoline. According to the applicant, the variability in the amino acid levels in the six selected reference oilseed rape lines was estimated to be zero for some amino acid levels. Therefore, the applicant also used commercial oilseed rape lines in addition to the near isoline and the reference oilseed rape lines. The applicant reported that the level of free and proteinogenic amino acids was always within the range (the 'tolerance interval') observed in the tested commercial oilseed rape lines. In addition, the applicant concluded from these data that the elevated levels of N-acetylaspartate, N-acetylglutamate and N-acetylthreonine did not influence the level of the corresponding free and proteinogenic amino acids.

***Analysis of biological characteristics***

The applicant compared the agronomic and seed germination characteristics of 73496 oilseed rape to the near isoline. 73496 was significantly different from the near isoline, but equivalent to other commercial conventional oilseed rape varieties with regard to 'early population', 'final population', 'flowering duration', 'plant height' and seed germination under warm conditions.

As the phenotype of 73496 is similar to the phenotype of other oilseed rape varieties, COGEM has no indication that 73496 is intrinsically more (or less) fit than other oilseed rape varieties. Apparently, the disruption of the *tpt* gene does not affect the phenotype of 73496. It has previously been reported that a decreased TPT transport capacity can be compensated by an increased starch turnover during daytime.[27]

***Environmental risk assessment***

This application concerns an authorisation for import and processing of GM oilseed rape. Therefore, COGEM will only focus on the environmental risks associated with incidental spillage of oilseed rape.

As mentioned earlier, COGEM is of the opinion that the molecular characterisation of 73496 is incomplete. The applicant should provide more information to assure that the insert region is completely characterised.

73496 produces the glyphosate *N*-acetyltransferase GAT4621 enzyme which enables 73496 to detoxify glyphosate, but which may also lead to the N-acetylation of certain amino acids. The applicant showed that the level of N-acetylaspartate, N-acetylglutamate and N-acetylthreonine in seed was elevated. According to the applicant the level of the corresponding (and other) free and proteinogenic amino acids was within the range of other oilseed rape varieties.

Almost all import of oilseed rape seeds concerns the use of these seeds for oil production. A small part of the imported oilseed rape seed is used in pet food, particularly for birds and rodents.[30] The estimated seed loss during transport ranges from 0.1 to 3.0%.[23] Spillage of

oilseed rape seeds during transport could lead to growth and establishment of oilseed rape. In the Netherlands, oilseed rape populations usually consist of a small number of plants (25 or less) and are nearly always located on highly disturbed soil associated to areas were oilseed rape is cultivated or where seed spillage occurred (along roads or in transhipment areas).[16] Although some feral oilseed rape plants can persist for several years, there is no evidence that oilseed rape is invasive in undisturbed natural habitats.[21]

As mentioned earlier, COGEM has no indication that 73496 is intrinsically more (or less) fit than other oilseed rape varieties. 73496 oilseed rape plants do not possess a selective advantage unless glyphosate is applied. In the Netherlands, the policy of the road maintenance authority is to use non-chemical methods to control weeds on the verges of roads.[31] Glyphosate is, however, one of the few herbicides available to control weeds along railway tracks in the Netherlands. Spilled GM oilseed rape may be able to form small volunteer populations along railway tracks where glyphosate is applied. These populations may be controlled by use of other herbicides, flame weeding or steam weeding. However, the establishment of small populations of spilled 73496 oilseed rape cannot be excluded in disturbed environments where glyphosate is frequently applied.

If small 73496 populations become established, cross-fertilisation could occur with oilseed rape (*B. napus*) and/or wild relatives, in particular *B. rapa*. The resulting progeny will not be different from progeny arising from cross-fertilisation with conventional oilseed rape varieties apart from the ability to produce the GAT4621 *N*-acetyltransferase protein and the disruption of a *tpt* gene. COGEM considers it unlikely that any resulting progeny will possess a selective advantage unless glyphosate is applied.

***Incidental consumption***

Since 2008 COGEM abstains from giving advice on the potential risks of incidental consumption in case a food/feed assessment is already carried out by other organisations.[32] This application is submitted under Regulation (EC) 1829/2003, therefore a food/feed assessment is carried out by EFSA. Other organisations who advise the competent authorities can perform an additional assessment on food safety although this is not obligatory. In the Netherlands a food and/or feed assessment for Regulation (EC) 1829/2003 applications is carried out by RIKILT. Regarding the risks for food and feed, the outcome of the assessment by other organisations (EFSA, RIKILT) was not known at the moment of the completion of this advice.

***Post-market environmental monitoring (PMEM)***

General surveillance (GS) has been introduced to be able to observe unexpected adverse effects of GM crops on the environment. In general, the setting or population in which these effects might occur is either not, or hardly predictable. GS for 73496 oilseed rape focuses on the import, handling and processing of viable 73496 oilseed rape.

In the European Union, a few GM oilseed rape varieties are authorised for import and processing. Because there is a small, but non-negligible possibility that cross fertilisation between *B. napus* plants arising from spilled seed could occur, there is a chance that several transgenes could be ‘stacked’ in a single oilseed rape plant.

Apart from tolerance or resistance to glyphosate containing herbicides, the traits that have been introduced in the currently authorised GM oilseed rape varieties are tolerance to

glufosinate ammonium containing herbicides and male sterility as well as the restoration of male sterility. Stacking of traits may influence the fitness of the resulting progeny. Depending on the type of traits introduced and the combination of parental genomes in the absence of selective pressure under natural conditions fitness could increase, remain the same,[33] or decrease.[34] COGEM is of the opinion that stacking of the above mentioned traits in combination with glyphosate tolerance will not lead to an environmental risk, because under natural conditions these traits are unlikely to lead to an increased fitness or a selective advantage.

However, in view of future applications and to increase knowledge on the occurrence of stacked events, it is important to know whether stacked events arise in order to allow future risk assessments to take the putative presence of established GM oilseed rape with stacked traits into account.

EFSA has stated in its guidance document that monitoring plans should address relevant exposure pathways.[35] Given the above considerations, COGEM points out that in the monitoring plan for 73496 not all relevant exposure pathways for monitoring of GM oilseed rape are included. In 2010, COGEM remarked that GS for crops that have outcrossing potential should cover handling areas and distribution routes.[36] In the Netherlands, populations of oilseed rape have been observed on several occasions near roads, railway tracks and railway stations.[16,30] Therefore, monitoring should pay special attention to these areas.

Also, since glyphosate application is the most common method of weed control along railway tracks in the Netherlands, railway companies and/or companies in charge of the maintenance of railway tracks (such as ProRail in the Netherlands) should be enlisted by the authorisation holder to monitor the occurrence of GM oilseed rape along railway tracks.

In 2010, COGEM formulated compliance criteria for GS plans concerning Dutch applications for import and cultivation of GM crops.[36] In addition to the criteria mentioned above, the two following criteria are applicable to the GS plan of 73496 oilseed rape.

In the EFSA guidance document, EFSA states that raw data and analysis of monitoring data should be made available by the applicant to the Competent Authorities and the European Commission.[35] COGEM agrees with this request and points out that the GS plan of 73496 oilseed rape could be improved by a statement of the applicant on this point.[37]

The GS plan states that if the authorisation holder identifies an unexpected adverse effect caused by the GM plant, he will inform the European Commission immediately. COGEM is of the opinion that Member States should also be directly informed of these effects by the authorisation holder, to ensure that appropriate measures for protection of humans and the environment can be implemented immediately.

COGEM concludes that the PMEM plan for import and processing of 73496 oilseed rape could be improved on several points. Most importantly, COGEM advises to include monitoring of roadsides and railway beddings near oilseed rape transhipment and transport sites for spillage of GM oilseed rape and stacking of event 73496 oilseed rape.

**Conclusions and advice**

COGEM cannot advise positively on the application for import and processing of 73496 oilseed rape due to the incomplete molecular characterisation of 73496 oilseed rape. In COGEM's view the applicant should provide more information to assure that the insert region is completely characterised.

Due to time constraints, COGEM did not assess all elements of the application (e.g. putative effects of N-acetyltransferase on other compounds). Therefore, COGEM reserves its right to comment further on this application in the future.

COGEM advises to include in the PMEM plan that roadsides and railway beddings near oilseed rape transhipment and transport sites will be monitored for spillage of GM oilseed rape and stacking of event 73496. COGEM is of the opinion that the PMEM plan for import and processing of 73496 oilseed rape should be improved before a market authorisation for this event is granted.

**Additional remarks**

COGEM notes that the applicant did not provide information on the seed persistence (dormancy) of 73496. The applicant states that the question on 'seed persistence leading to volunteer occurrence' is not applicable as the application does not concern cultivation. COGEM points out that information on seed persistence is also important when an application concerns import and processing, because seed spillage may occur during transport and/or processing. It is known that oilseed rape seeds may persist in the soil and are able to form a seed bank. Under normal agricultural circumstances it was shown that oilseed rape seeds can persist for over four years,[13] and another study reported the occurrence of oilseed rape seedlings after a dormancy period of ten years.[38] When assessing the environmental risk assessment of import of 73496 oilseed rape, COGEM took notice of the possible lengthy persistence of oilseed rape seeds.

COGEM also noted that the data on the agronomic characteristics (annex 17) contains anomalies. The annex contains tables reporting the mean of the observed characteristics and the outcome of either the difference or the equivalence test. The mean of several characteristics differs between the two tables. The applicant does not provide an explanation for the deviation between the values reported in the two tables. Since the differences are very small, there is no reason to assume that the anomalies would change the outcome of the environmental risk analysis.

In addition, COGEM noticed that the applicant used the Baker list with weed characteristics to provide information on *B. napus*. In 2012, COGEM commissioned a research project that examined the usability of the Baker list to predict the weediness potential of several species.[39] The results from this project indicate that even if one quantifies all the characteristics on the Baker list using botanical databases it is not possible to predict weediness. Therefore, COGEM is of the opinion that not too much emphasis should be placed on this type of information.

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