



Notification 6786-01-0147

**Summary of the risk assessment of genetically modified oilseed rape (*Brassica napus*
ssp. oleifera (Metzg.) Sinsk.) with altered fatty acid composition
carried out by the German Competent Authority
within the framework of a proposed deliberate release**

Berlin, 16 May 2003

Explanatory note to this document:

The following text is a summary of the risk assessment of genetically modified organisms intended for use in an experimental field trial (deliberate release) in Germany. The text forms part of the official authorisation issued in response to an application for the deliberate release of genetically modified organisms in Germany in accordance with Directive 2001/18/EC and the German Genetic Engineering Act (Gentechnikgesetz, GenTG). The authorisation was issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [Federal Office of Consumer Protection and Food Safety], as the German Competent Authority under the law on genetic engineering, and comprises the chapters:

- I. Authorisation
- II. Provisions
- III. Justification
 - III.1. Authorisation requirements according to § 16 GenTG [German Genetic Engineering Act]
 - III.1.1. Authorisation requirements according to § 16 (1) No. 1 GenTG
 - III.1.2. Authorisation requirements according to § 16 (1) No. 3 GenTG
 - III.1.3. Authorisation requirements according to § 16 (1) No. 2 GenTG
 - III.1.4. Authorisation requirements according to § (4, 5) GenTG
 - III.2 Appraisal of and response to objections
- IV. Costs
- V. Legal instruction

Only the original German authorisation document is legally binding. The following extract is a courtesy translation of chapter III.1.2., prepared for the Biosafety Clearing House.

III.1.2.1. Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequence

(a) The acyl-[ACP] thioesterase gene (*CIFatB4*)

Acyl-[ACP] thioesterases hydrolyse the thioester ligation between the ACP (acyl carrier protein) and the synthesised acyl chain in fatty acid biosynthesis. Myristic and palmitic-[ACP] are substrates of the *Cuphea lanceolata*-derived enzyme which is encoded by the acyl-[ACP] thioesterase gene (*CIFatB4*). As a result of the formation of this enzyme the oil produced in the seeds of the genetically modified (GM) oilseed rape plants contains up to 20% myristic acid (C_{14:0}), which is not normally present in rapeseed oil, and increased levels of palmitic acid (C_{16:0}).

Expression of the *CIFatB4* gene contained in the GM oilseed rape plants is driven by its own seed-specific promoter and its termination signal. In one of the two constructs used, expression should be amplified by the upstream position of the fourfold enhancer of the CaMV 35S promoter.

In the GM oilseed rape plants the newly formed acyl-[ACP] thioesterase catalyses the same reaction as corresponding enzymes which occur naturally in the seeds of other (wild and cultivated) plant species. Myristic and palmitic acids, the fatty acids which are newly formed or occur in higher concentrations in the rapeseed oil, occur naturally in other plant oils used for human consumption (e.g. coconut oil). There is no evidence to indicate that either the enzyme or the new metabolic product would have a toxic effect.

(b) The *nptII* gene

The *nptII* gene transferred to the GM plants encodes the enzyme neomycin phosphotransferase. It was inserted as a marker gene for selecting transformed plant cell. The neomycin phosphotransferase gene is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which catalyses the ATP-dependent phosphorylation of the 3'-OH group of the aminohexose ring of specific aminoglycoside antibiotics, causing these to become inactivated. The enzyme is characterised by its high substrate specificity. The antibiotics kanamycin, neomycin, geneticin, butirosin, gentamicin A and B, and paromomycin belong to the APH(3')II enzyme substrates. Clinically relevant gentamicins and other aminoglycosides and aminocyclitols used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. Kanamycin and neomycin are, however, widely used in veterinary medicine.

Due to the substrate specificity of neomycin phosphotransferase, no new metabolic products are expected to arise in the GM plants in the absence of substrate under field conditions. Since high concentrations of the relevant antibiotics are not present in soil, the neomycin phosphotransferase does not confer any selection advantage to the GM plants under field conditions. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

(c) Border sequences from Ti-plasmids and regulation sequences

The GM plants contain sequences from the left and right border regions of the T_L-DNA of the plasmid pTiB6S3 from *Agrobacterium tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid pMP90RK present in the *Agrobacterium* strain used for the transformation, which was not transferred to the plants, these sequences effected the integration of the genes located between the border region into chromosomes of the oilseed rape plants. These border regions of the Ti plasmids have no function in the GM oilseed rape plants and are not expected to cause any changes in the plants.

The GM oilseed rape plants contain the gene-specific promoter and terminator of the *CIFatB4* gene as well as the 35S promoter and terminator of cauliflower mosaic virus (CaMV) incorporated into the genome. The promoter and termination sequences regulate the expression of the coding sequences of the *CIFatB4* and *nptII* genes located between them in the GM plants. Statements on the effects of the formation of these proteins in the plants are given in III.1.2.1. (a) and (b).

(d) Sequences located outside the T-DNA

As a general rule, only DNA sequences located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformations. However, in isolated cases the transfer of DNA sequences from outside the border regions has been reported and based on the information contained in the application this possibility cannot be ruled out. As a result, the risk assessment also considers those regions on the vector used for transformation of the oilseed rape which are located beyond the T-DNA border regions. These include, in particular, the following DNA fragments:

- The *aadA* gene

The *aadA* gene encodes the enzyme streptomycin adenyltransferase. As a marker gene it is a component of the vectors pNBM99-CIFatB4 and pNBM99-EnCIFatB4 used for transformation of the oilseed rape.

Streptomycin adenyltransferase is an aminoglycoside-3-adenyltransferase (AAD(3'')(9)) which catalyses the adenylation of the 3'-OH-group of the N-methyl-L-glucosamine ring of streptomycin and the 9-hydroxyl group of spectinomycin, causing these to become inactivated. The enzyme is characterised by high substrate specificity. Substrates of the AAD(3'')(9) enzyme include streptomycin and spectinomycin. Today, streptomycin is only used for specific indications in human medicine (e.g. tuberculosis).

The *aadA* gene is controlled by a prokaryotic promoter. As a result, this gene is not expected to be expressed in plants. Hence effects on plant metabolism are just as unlikely to occur as effects on humans or animals as a result of the potential consumption of the GM plants or parts thereof.

- The replication origins of the plasmids pNBM99-CIFatB4 and pNBM99-EnCIFatB4

In addition to the ColE1 replication origin for replication in *E. coli* the plasmids used to generate the GM oilseed rape plants also contain outside the T-DNA the replication region of the plasmid pVS1 from *Pseudomonas aeruginosa*, strain PAT, with the genetic information for replication and stability. The plasmid pVS1 enables replication, for example, in species of *Pseudomonas*, in *Aeromonas formicans*, *Rhizobium leguminosarum* and in *A. tumefaciens*. The plasmid is not stable in *E. coli*. In this particular case the replication region of the plasmid pVS1 is used to replicate the binary vector in *A. tumefaciens*.

There is no evidence that the ColE1 and pVS1 replication regions have any function in plants.

(e) Position effects and context changes; allergenicity

Genes that have been integrated into the plant genome by genetic engineering methods are expressed at varying levels, depending on the site of integration on the chromosome and on the nucleotide sequences neighbouring the integration site ("position effect"). Under field conditions the level of expression may be influenced by environmental factors, for instance, by temperature. In the present case this could mean that the GM oilseed rape plants do not produce the same levels of fatty acids in the field as under greenhouse conditions. This is not expected to pose a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of the plant's own genes at or near the site of insertion. Such processes may alter plant metabolic pathways. However, during propagation of the GM plants in the greenhouse, no observations were made that would point to such an event.

Mobile genetic elements (transposable elements), which when transposed within the genome can exert effects on existing plant genes at the target site, occur naturally in plants. The inactivation of genes or alterations in gene regulation also take place in a range of other naturally occurring processes such as point mutations, deletions or translocations and are traditionally used in plant breeding. Therefore, even in non-GM plants such events can always influence plant metabolic pathways. With regard to these properties the GM plants proposed for release here do not differ fundamentally from non-GM plants.

Given the current state of knowledge, it is impossible to make reliable predictions about the possible allergenic action of a protein on the basis of its amino acid sequence. However, the use of the gene's own seed-specific promoter to drive expression of the *CIFatB4* gene is limited to the later stages of seed maturation. In previous studies, among others on GM plants not intended for release, it was demonstrated that the promoter of the *CIFatB4* gene is not active in other parts of the plant (e.g. pollen).

Expression of the *nptII* gene is regulated by the constitutive CaMV 35S promoter. In previous greenhouse trials with these GM plants and in field trials carried out during the period from 1996 to 2002, as well as in deliberate releases of other GM plants that express the *nptII* gene under the control of the 35S promoter, the plants were not found to display increased allergenicity.

Therefore, in comparison to non-GM oilseed rape plants the allergenic potential of the GM plants proposed for release is not expected to be altered.

III.1.2.2. Evaluation of the ability of the GM plants to persist or establish in the environment

In view of the proposed measures to minimise dispersal through pollen or seed, the spread of the GM oilseed rape plants beyond the trial area is not to be expected (see the trial description in the application, provisions II.5. and II.7. to II.9. of the decision on this notification, as well as the statements given in III.1.2.3.).

Summer oilseed rape is an annual, winter oilseed rape a perennial plant. Following the generative phase the plant dies off; new plants can only emerge from the seeds produced. If they become buried deep in the soil and enter secondary dormancy, rape seeds can persist in the ground for over 20 years.

Studies have shown that alterations in the fatty acid composition of the storage lipids of the seeds can affect seed persistence in oilseed rape. At the present time there are no experimental data available on whether the myristic acid produced in the GM oilseed rape plant seeds proposed for release affects the capacity of the seeds to persist. However, the applicant assumes that under the conditions found at the release site the GM seeds are disadvantaged in comparison to normal oilseed rape seeds, since the seeds of the transgenic lines contain 30 – 40% saturated fatty acids, whereas oil from conventional oilseed rape plants contains approximately 90% unsaturated fatty acids. Saturated fatty acids have a significantly higher melting point than unsaturated fatty acids and are mobilised more slowly in our climate conditions due to the relatively low germination temperatures.

The persistence of seeds from the GM oilseed rape and from potentially occurring oilseed rape hybrids can be minimized by taking appropriate measures after every harvest to ensure that any seeds released are brought to germination during the same vegetation period and any plants emerging from these seeds are subsequently destroyed. These measures are planned for the proposed experimental release.

During preparation of the soil for future planned agricultural use, seeds from oilseed rape or oilseed rape hybrids that remain in the soil despite completion of the above measures are brought close to the soil surface where they can germinate. The resulting plants will be identified and destroyed, either within the crop rotation monitoring planned by the applicant or during the cultivation gap (spanning a

number of years) and post-trial monitoring required by provision II.10. If GM oilseed rape plants or oilseed rape hybrids continue to appear during the final post-trial monitoring year, the respective monitoring period will be extended by a further year (provision II.10.). The cultivation gap requirement ensures that any potential re-growth of oilseed rape plants and oilseed rape hybrids can be identified.

The potential emergence of individual GM oilseed rape seedlings or hybrids on or outside the release site after the end of the post-trial monitoring period does not pose a risk with regard to pollen transfer to other plants (see III.1.2.3.) or long-term establishment.

Outside cultivated sites oilseed rape is only found as a weed in or near areas where the crop is grown, e.g. on waysides and other ruderal sites. Oilseed rape is not capable of establishing in natural, intact plant communities. These GM oilseed rape plants are not expected to develop modified plant sociological traits as a result of the introduction of the *CIFatB4* gene nor are they expected to populate other biotopes.

Therefore, even in the event of the emergence of individual GM oilseed rape seedlings and the possible transfer of pollen to non-GM plants, no long-term, sustainable spread of the GM oilseed rape is expected. The temporal and spatial limitation of the release is thus guaranteed.

III.1.2.3. Assessment of the possibility of pollen-mediated transfer of the inserted genes from the GM plants to other plants

Oilseed rape stocks are about two thirds self-pollinating and one third cross-pollinating. The oilseed rape pollen is dispersed by insects (particularly bees) and by wind. To minimise undesirable foreign pollination in agricultural seed production, seed legislation calls for isolation distances of 100 m for certified seed and 200m for basic seed. A minimum separation distance to neighbouring oilseed rape fields is set down in provision II.7. of the decision on this application. However, it should be assumed that to a limited extent oilseed rape pollen may be carried beyond the isolation distance.

Pollination of individual flowers of non-GM oilseed rape would result in the temporary appearance of isolated oilseed rape plants, the seeds of which would exhibit an altered fatty acid profile. This is not expected to pose a risk to the environment or to agriculture. During extraction of the rapeseed oil from any seeds that might emerge from the pollination of individual oilseed rape flowers with pollen from the GM oilseed rape plants, the enzyme acyl-[ACP] thioesterase would be separated from the oil along with the rest of the proteins. The proteins would remain in the pressing residue, the so-called "press cake", which is used in animal feed. Myristic acid, the newly expressed fatty acid, and palmitic acid, which occur in higher proportions in the seeds of these oilseed rape plants, are already found in existing products, some of which are used for human consumption. Therefore, the potential consumption of rapeseed oil with a higher myristic and palmitic acid content or products made therefrom does not pose a threat to human health.

Swede (*Brassica napus* var. *napobrassica*) belongs to the same species as oilseed rape. It can be assumed that oilseed rape and swede are cross-compatible.

Swede is a biennial plant which develops a tuberous hypocotyl in the first year, but only flowers in the second year. When cultivated for sale and consumption the plants are harvested in the first year. The possibility of fertilisation with pollen from GM oilseed rape is given when swede is brought to flowering for the purpose of harvesting seeds (e.g. for the cultivator's own requirements). Although they belong to the same species, swede and oilseed rape differ significantly in terms of morphology (oilseed rape does not develop a tuberous hypocotyl). It can be assumed that hybrids resulting from the pollination of swede by oilseed rape pollen would be markedly different in appearance from swede. Since untypical plants would not be cultivated for the further propagation of swede, GM hybrids are not expected to be consumed or used for further seed production.

Several species in the *Brassicaceae* family are closely related to oilseed rape; these are potential crossing partners. Oilseed rape (*Brassica napus*) is a hybrid of wild turnip (*Brassica rapa*) and wild cabbage (*Brassica oleracea*) and is therefore, in principle, cross-compatible with these species – with the following limitations.

Experimental hybrids of *Brassica napus* and *B. oleracea* were generated by extracting embryos from the ovules and regenerating these to plants on culture media (embryo rescue). However to date the spontaneous development of such hybrids under field conditions has not been observed.

Winter turnip rape (*Brassica rapa* ssp. *oleifera*) is cultivated as a crop plant for oil production and as a catch crop. Outside of cultivated areas it is also found growing wild on sites influenced by human activity (ruderal sites, waysides, field edges). Hybrids of *B. napus* x *B. rapa* appear sporadically in oilseed rape fields if fertilisation with pollen from *B. rapa* took place when the oilseed rape seeds were propagated.

With regard to the possible consequences of pollination of individual flowers of non-GM winter turnip rape plants, the above statements on oilseed rape apply correspondingly. In addition, the fertility of primary hybrids of *B. rapa* and *B. napus* is generally limited. They are anorthoploid and are characterised by a marked reduction in the function of the gametes resulting from irregular meiotic chromosome distribution. The progeny of such gametes are aneuploid; they are generally of low vigour and also display low fertility.

Other *Brassicaceae*, such as leaf mustard (*Brassica juncea*), black mustard (*Brassica nigra*), white mustard (*Sinapis alba*), wild mustard (*Sinapis arvensis*), species of common radish (*Raphanus sativus*), wild radish (*Raphanus raphanistrum*) and shortpod mustard (*Hirschfeldia incana*) are potential crossing partners for oilseed rape. Because of the low chromosomal homology between these plant species and oilseed rape, the above statements on *Brassica rapa* and *Brassica oleracea* apply to an even greater extent to hybrids of these plants and oilseed rape. Amphidiploid hybrids obtained by experimental crossing of oilseed rape with related *Brassicaceae* species represent the only exception. These hybrids, which probably arise from unreduced gametes of the parent plants, exhibit only slightly reduced pollen fertility. Even if isolated cases of hybridisation between the GM plants and these species of *Brassicaceae* were to occur, it is highly unlikely that the genetic material transferred to the GM plants would spread to into wild plant populations.

With regard to all theoretically possible hybrids between the GM plants and non-GM crop plants or wild plants, the *CIFatB4* gene would not cause the development of altered plant sociological characteristics in these hybrids.

III.1.2.4. Assessment of the possibility of horizontal gene transfer of the inserted foreign genes from the GM plants to microorganisms

The inserted sequences are stably integrated into the chromosome of the recipient organism. There is no evidence to suggest that a transfer of genetic information from plants and its expression in microorganisms takes place under natural field conditions. Studies on the transformation capacity of soil bacteria under natural conditions do however suggest that a transfer of plant genetic material to soil microorganisms is theoretically possible, although it is assumed that such a gene transfer would constitute an extremely rare event.

Insofar as we assume that a genetic exchange between organisms that are so distantly related in terms of taxonomy as plants and microorganisms is actually possible, it could be concluded that the occurrence of an exchange of heterologous genetic material does not in itself represent a safety criterion, since such an exchange could always result in the uptake of all forms of heterologous genetic material, including all forms of plant DNA.

The acyl-[ACP] thioesterase gene is expressed under the control of its own seed-specific regulation sequences. The gene contains 6 introns. Therefore, even in the event of a transfer of this gene to microorganisms, formation of the corresponding enzyme in the microorganisms is not to be expected. Moreover, genes that code for enzymes with the same function occur naturally in a number of plant species.

As already described in III.1.2.1. (b), the antibiotics that are inactivated by the neomycin phosphotransferase are of little relevance in human medicine, but they are widely used in veterinary medicine. It was therefore necessary to examine whether the therapeutic use of the relevant antibiotics would be affected by a potential horizontal gene transfer of the *nptII* gene.

The inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism in soil microorganisms. APH(3')II enzymes have also been found in human clinical isolates. The prevalence of genes which confer resistance to aminoglycoside antibiotics can be explained by the frequent application of these antibiotics, and by the fact that these genes are often located on plasmids, thus enabling effective transfer by conjugation. Even in the event of a horizontal gene transfer from the GM oilseed rape to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

The same applies to the *aad* gene located outside the T-DNA border regions, for which, according to the information provided in the application dossier, a possible transfer into the plant genome cannot be ruled out. The *aad* gene codes for the enzyme streptomycin adenylyltransferase.

The sequences inserted into the oilseed rape plants to regulate the *nptII* gene were derived from CaMV. The theoretical possibility of a transfer of the CaMV-derived sequences from these plants would not represent a new situation in comparison to the naturally occurring situation, since CaMV is already found in plants as a double-stranded plant-infecting DNA virus.

In addition to the ColE1 replication origin for replication in *E. coli*, the plasmids pNBM99-CIFatB4 and pNBM99-EnCIFatB4 used to generate the GM oilseed rape plants contain outside the T-DNA the replication region of the plasmid pVS1 from *Pseudomonas aeruginosa*, strain PAT, with the genetic information for replication and stability. These replication origins may have been chromosomally integrated into the GM oilseed rape plants (see III.1.2.1 (d)). These nucleic acid sequences are non-functional in the GM plants; however, they are expected to function as a replication origin in certain bacteria.

The host range of the ColE1 replicon is limited to certain gram-negative bacteria. Basically, the replicon can replicate in *E. coli* and closely-related bacterial species, such as *Serratia* and *Salmonella*. Replication does not occur in the majority of gram-negative soil bacteria. ColE1 plasmids are quite frequently present in enterobacteria. Gene transfer from enterobacteria to other bacteria is considered far more likely than a horizontal gene transfer from the GM plants to bacteria. Therefore there is no reason to expect that the potential presence of the replication origin of ColE1 in the plant chromosome would contribute to an increase in the overall frequency of horizontal gene transfer.

In this particular case the replication region of the plasmid pVS1 is used for replication of the binary vector in *A. tumefaciens*. The vectors used for the transformation are mobilisable but do not have their own transfer system. Since the conjugative exchange of nucleic acids between microorganisms is widespread, the pVS1-derived replication region from the GM plants is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

III.1.2.5. Agrobacteria used to generate the GM plants

To generate the GM oilseed rape plants, sterile explants were inoculated with *Agrobacteria* containing the genes intended for transfer between the border regions of the binary vector plasmid. In contrast to

the common wild-type *A. tumefaciens*, the *Agrobacterium* strains used are “disarmed”, i.e. they no longer have the capacity to induce tumours. After transformation had occurred, antibiotic treatment was carried out to eliminate the *Agrobacteria*. The plants used for the experimental release were propagated by seed over many generations. Any *Agrobacteria* that may have survived the antibiotic treatment will have been removed from the GM oilseed rape lines during these generative phases.