OIL DERIVED FROM GLUFOSINATE-AMMONIUM TOLERANT AND POLLINATION CONTROLLED CANOLA

A Safety Assessment

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SUMMARY

Canola has been genetically modified (GM) to provide growers with a range of hybrid production and breeding lines that are tolerant to the herbicide glufosinate-ammonium. The lines include two open pollinated lines (known as Topas 19/2 and T45) and five lines, denoted as either Ms or Rf, that have been specifically developed for use in a plant breeding system for the purpose of generating hybrids with increased vigour.

Nature of the genetic modifications

The herbicide tolerance trait has been introduced into all seven lines by the addition of a bacterial gene, either *bar* or *pat*, to enable the canola plants to produce an enzyme, phosphinothricin acetyl transferase (PAT), which chemically inactivates the herbicide, phosphinothricin (also known as glufosinate-ammonium). Plants expressing the PAT protein are able to function normally in the presence of the herbicide.

In addition to the herbicide tolerance trait, five of the genetically modified lines (Ms1, Ms8, Rf1, Rf2 and Rf3) contain one or both of the bacterial genes, *barnase* and *barstar*. Expression of *barnase* in specific parts of the flower at a particular developmental stage gives rise to plants that are male sterile (Ms). In the Ms plants, the presence of the *barnase* gene product, a non-specific ribonuclease, destroys the pollen-producing cells during development of the flower. The ribonuclease activity is, however, specifically inactivated by the presence of the *barstar* gene product. Plant lines expressing *barstar* at the same time and in the same floral tissue are referred to as fertility restorer (Rf) lines because, when crossed with a male sterile line, the production of the barstar protein counteracts the effects of the barnase protein, thereby restoring male fertility. The Rf plants are phenotypically normal.

Thus, the hybrid system consists of crossing a Ms line (female parent) with a specific Rf line, giving rise to progeny that are fully fertile. The primary objective of these modifications is the production of a range of parental lines with superior agronomic performance that are to be used in a breeding system for producing hybrids yielding significantly more seed.

The transferred genes appear to be stably integrated into the plant genome and all introduced traits are stably maintained over multiple generations.

History of use

Traditional rapeseed is considered unsuitable as a source of food for either humans or animals due to the presence of two naturally occurring toxicants, erucic acid and glucosinolates. Following intensive conventional plant breeding over a period of twenty years, *canola* is now confined to those cultivars (*Brassica rapa* and *B. napus*) that contain low levels of erucic acid and glucosinolates, so called “double low” varieties. Quality control measures also stipulate that no protein is present in canola oil suitable for human consumption.

Since its development, use of canola oil has become widespread in the food industry as a vegetable oil in table spreads and cooking, and as an ingredient in a range of mixed foods.

Antibiotic resistance genes

Four of the GM canola lines (Ms1, Rf1, Rf2 and Topas 19/2) also contain a bacterial antibiotic resistance marker gene, *nptII*, under the control of a plant promoter. The *nptII* gene is used for the selection of transformed plants in the laboratory as well as for identification purposes in the field. Apart from its use as a marker in the field, the gene serves no agronomic purpose in the crop.

Some concerns are expressed in relation to the use of antibiotic resistance genes in GM foods due to the potential for transfer of the novel genetic material to cells in the human digestive tract. In this assessment it was concluded that the *nptII* gene would be most unlikely to transfer to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. Furthermore, if transfer could occur, the impact on human health would be negligible because bacteria carrying such resistance are already widespread in nature or are found to naturally inhabit the human digestive tract. Moreover, the antibiotics kanamycin or neomycin are rarely, if ever, used for clinical purposes because of unwanted side effects.

In this particular case, where the food under assessment is an oil, the risks of horizontal DNA transfer are even further reduced. Experimental evidence demonstrated conclusively that there is no novel DNA present in canola oil.

General safety issues

There are potentially four novel proteins, PAT, NPTII, barnase and barstar, expressed in the genetically modified canola lines. The enzyme responsible for herbicide tolerance, PAT, is expressed in all tissues of the plant, but at such low levels that specific enzyme activity was not detectable. The NPTII marker protein expressed in four of the seven lines was detected at very low levels in the leaves, but not in the seeds. Expression of the barnase and barstar proteins is tightly controlled in the plant and both of these proteins occur only in a non-edible part of the plant. For this reason, these proteins are not considered to be of major significance with respect to allergenicity, nutritional properties or overall food safety. The patterns and levels of gene expression conformed to those predicted and intended by the modification process.

In addition, data were presented to demonstrate that the processing involved in the production of canola oil effectively removes all traces of protein. Consequently, consumers will not be exposed to plant proteins, including the novel proteins, through consumption of canola oil. Notwithstanding the absence of protein in the oil, there is no evidence to indicate that either PAT or NPTII are likely to be allergenic or toxic to humans. Neither of these proteins shows any sequence similarity with known allergens or toxins using data obtained from public genetic and protein databases, and both proteins were readily degraded in simulated digestive systems.

Comparative analyses

A comprehensive set of analytical data has been evaluated for the safety assessment of food derived from glufosinate-ammonium tolerant and pollination-controlled canola. The results of extensive compositional analyses of the seeds from both herbicide-treated and untreated plants demonstrate that the oil composition and fatty acid profile of the GM lines are similar to those of control cultivars, and to an extensive published range for commercial varieties of canola. The analyses were conducted on test material grown over multiple growing seasons and at different geographical locations and thus demonstrate that the genetic modifications have not resulted in any significant variation in composition or agricultural performance in the transformed lines when compared to the non-transformed control lines grown under the same conditions.

Detailed compositional analyses on the transformed seeds also showed no differences in the levels of natural toxicants. In particular, the level of erucic acid in the oil (and glucosinolates in the meal) conformed to the compliance requirements for certification as canola. The transformed lines were tested in a range of environmental situations and following treatment with commercial levels of glufosinate-ammonium.

The nutritional value of the transformed seeds was evaluated in two animal feeding studies using rabbits and broiler chickens. In both instances, where the transformed canola seeds were included in the diets of the animals over a defined period, no adverse effects attributable to the test material were observed in the animals, and all animals displayed normal patterns of growth.

Conclusion

On the basis of the available evidence, oil derived from the genetically modified canola lines (T45, Topas 19/2, Ms1, Ms8, Rf1, Rf2, Rf3 and their crosses), is equivalent to oil from non-GM canola in terms of its safety and nutritional properties.

OIL DERIVED FROM GLUFOSINATE-AMMONIUM TOLERANT AND POLLINATION CONTROLLED CANOLA:

A SAFETY ASSESSMENT

INTRODUCTION

Oil derived from glufosinate-ammonium tolerant and pollination-controlled canola has been the subject of an evidence-based, scientific safety assessment. The lines are known commercially in Australia and New Zealand as LibertyLink® open pollinated and InVigor® hybrid canola.

Seven lines of canola (*Brassica napus*, *B. rapa* and crosses) have been genetically modified (GM) to confer tolerance to the broad spectrum herbicide, glufosinate-ammonium. Five of these lines have been generated primarily for use in a hybrid seed production system by expressing one of two genes that enable control of pollen production, in conjunction with the herbicide tolerance trait. Two lines of open pollinated canola have been genetically modified with the herbicide tolerance trait only. Up to three new traits may be expressed in the GM canola, however not all lines contain all the traits. The new traits are conferred by the presence of the bacterial genes *bar* (or *pat*), *barnase* and *barstar*. In addition, some lines contain the *nptII* gene, a bacterial marker gene that confers resistance to certain antibiotics.

The bacterial genes, *bar* and *pat*, both produce an enzyme, phosphinothricin acetyl transferase (PAT), that metabolises the herbicide phosphinothricin (PPT) into an inactive form. Phosphinothricin is the active ingredient of the commercial herbicide glufosinate-ammonium (OECD, 1999). Glufosinate-ammonium is currently registered in Australia under the commercial name of Basta® for non-selective uses, or Finale® for turf and home garden uses, and as Buster® in New Zealand.

The mode of action of glufosinate-ammonium (or phosphinothricin) is to inhibit the plant enzyme glutamine synthetase (GS), an essential enzyme in nitrogen metabolism and amino acid biosynthesis in plants. The result of GS inhibition is the over accumulation of inorganic ammonia leading to the death of plant cells.

In addition to the herbicide tolerance gene, five of the GM canola lines for use in hybrid production contain one or both of the genes, *barnase* and *barstar*. Expression of the *barnase* gene in specific plant cells induces male sterility (Ms) and when these plants are crossed with fertility restorer (Rf) canola plants expressing the *barstar* gene, fertility is restored in the hybrid offspring. Hybrids produced from crosses between the Ms and Rf lines are reported to have significantly higher yields of oil-bearing seeds.

Canola oil and meal are the two major products produced from oilseed rape plants. Canola oil is used extensively in the food industry as vegetable oil and in products such as margarine, salad dressings, bakery products, low-fat foods and confectionery. It is also used in pharmaceuticals and nutritional supplements. Canola meal is primarily used as a protein supplement in feed for livestock, but it is also used in

poultry and fish feed, pet foods and fertilisers. In Australia, canola plant stubble may be grazed by livestock following harvest.

HISTORY OF USE

Host organism

The plant species *Brassica napus* L. oleifera Metzg. is more commonly known as oilseed rape, rape or rapeseed, with some cultivars referred to as canola. The two significant modifications introduced by classical breeding techniques that have stimulated the development of this species as a commercial crop are the lowering of the erucic acid and glucosinolate content of the seeds. Presently, oilseed rape is grown primarily for its seeds which yield about 40% oil and a high protein animal feed.

Since being developed as a vegetable oil suitable for human consumption, canola oil has not been associated with any food safety concerns. World production of oilseed rape in 1996-1997, was the third most important of oilseed crops behind soybean and cottonseed, but above peanut, sunflower and palm. The main producers of the crop are China, India, Canada and countries of the European Union.

By using traditional plant breeding methods, *Brassica napus* can be crossed with the closely related species, *Brassica rapa*, to produce hybrids capable of producing canola quality oil. *B. rapa* has a similar life history to *B. napus*, but with a shorter growing season allowing the crop to be planted later in the canola season. Oil produced from *B. rapa* is required to exhibit the same qualities as that from *B. napus*, namely low erucic acid and glucosinolate content, for marketing as canola.

Gene donor organisms

The introduced genes are derived from several species of bacteria.

The *bar* gene is derived from *Streptomyces hygroscopicus*, while the *pat* gene is derived from *Streptomyces viridochromogenes*, both common soil microorganisms which may also exist in water. These bacterial species are not used in the food industry and therefore do not have a history of use associated with food.

The source of the *barnase* and *barstar* genes is *Bacillus amyloliquefaciens* which are aerobic, spore forming bacteria commonly found in the soil. *B. amyloliquefaciens* is used widely in the food industry as a source of enzymes.

The *nptII* gene is derived from transposon Tn5 from the bacterium *Escherichia coli* (Beck *et al.* 1982). Particular strains of *E. coli* are used in the food industry, also in the production of enzymes.

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

The new genes were introduced into canola plants (*Brassica napus*, AC Excel and Drakkar lines) by transformation with one of several plasmid vectors, using

Agrobacterium mediated transformation (Zambryski, 1992). Six separate plasmids carrying the required genes were used to generate the seven new lines.

Agrobacterium mediated transformation involves incubation of the bacteria carrying the particular plasmid with plant cells for a few hours to days, during which time T-DNA transfer takes place. The cells were then washed and cultured in the presence of the selection agent, and transformed shoots were regenerated. In the case of one of the plasmids, two independent lines were derived from the original transformation event. As usually occurs, only one plant line was derived from transformation with each of the remaining plasmids.

Function and regulation of the novel genes

Genes conferring herbicide tolerance

Both *bar* (*S. hygroscopicus*) and *pat* (*S. viridochromogenes*) genes encode the enzyme phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin (PPT), the active constituent of the non-selective herbicide glufosinate-ammonium. Either the *bar* or *pat* gene was transferred to canola plants as a marker for use during *in vitro* selection of transformed plants, and as a breeding selection tool in seed production.

Phosphinothricin was initially characterised as an antibiotic (bialaphos) which is produced naturally by both species of bacteria, but was later shown to be effective as a broad spectrum herbicide. The PAT enzyme prevents autotoxicity in the bacteria by acetylation of the free amino group of PPT. When expressed in plants, the enzyme generates complete resistance towards high doses of PPT, bialaphos or the synthetically produced glufosinate-ammonium.

The *pat* and *bar* genes are very similar, sharing 87% homology at the nucleotide sequence level (Wohlleben *et al*., 1988, 1992). The respective PAT enzymes encoded by these genes are also very similar, and share 85% homology at the amino acid level (Wohlleben *et al*., 1988, 1992). Further biochemical characterisation of the two enzymes found that they are so similar as to be functionally equivalent for the purpose of conferring tolerance to PPT (Wehrmann *et al*., 1996).

The native *pat* gene has been resynthesised to modify codon usage for improved protein expression in plant cells (Strauch *et al*., 1993). At the nucleotide sequence level, the synthetic gene demonstrates 70% homology with the native *pat* gene from *S. viridochromogenes*. The amino acid sequence of the PAT enzyme encoded by both the native and synthetic genes is identical.

Either the *bar* or the *pat* gene is present in all of the canola lines to confer tolerance to the herbicide. The *bar* gene is under the control of a plant promoter (Pssu-Ara) which generates expression of PAT predominantly in the green tissues (leaves, stems) of the canola plant. Alternatively, in constructs involving the synthetic *pat* gene, a plant viral promoter (P35S) has been used for constitutive expression of the PAT protein in all tissues of the plant.

Genes for control of pollination

The *barstar* and *barnase* genes from *B. amyloliquefaciens* each encode a different small, single-chain protein. Both have been studied extensively as models for protein folding because of their small size, and there is an abundance of published scientific information relating to research work conducted since the early 1960s (Smeaton *et al*., 1967, Hartley, 1968, Mauguen *et al*., 1982).

The *barnase* gene encodes a ribonuclease that is naturally secreted by the bacterium. Ribonucleases are enzymes that degrade and digest ribonucleic acid (RNA), the biochemical intermediate between a gene (DNA) and its encoded protein. Ribonucleases are ubiquitous in nature, and serve many biological functions. In this case, the secreted ribonuclease serves to protect the environment of the bacteria (Hartley *et al*. 1989).

The *barstar* gene encodes a specific protein inhibitor of the ribonuclease encoded by *barnase*. In the *Bacillus* species from which the two proteins are derived, the function of the *barstar* protein is to protect the organism from the otherwise toxic effects of its own *barnase* activity. This naturally occurring system is well studied (Hartley *et al*., 1988 & 1989) and the interaction of the two proteins is known to be highly specific.

In the GM canola lines, both genes have been placed under the control of a highly tissue-specific plant promoter, TA29, that restricts their expression exclusively to the tapetal cell layer and only during anther development. The specificity of the interaction between the barnase and barstar bacterial proteins has lead to their use in transgenic canola to allow development of a breeding system to generate high yielding hybrid plants (Mariani *et al*. 1990 & 1992).

Hybrid design

One of the major goals of plant breeders is to create higher yielding varieties. Compared to the best open-pollinated varieties of canola, yields of seeds from F1 (first generation) hybrids can be increased by as much as 20-25%. In addition, the F1 hybrid seed is more uniform which facilitates both harvesting and marketing. Since canola is capable of both self-pollination (approximately 70%) and cross-pollination (30%), an effective pollination control system is required to enable production of high yielding 100% F1 hybrid seeds, containing all of the desired characteristics of both parental varieties.

One method of control that has been used widely in breeding programs of many different crops to ensure cross-pollination is the use of male sterile plants featuring abnormal pollen production. These plants are incapable of self-fertilisation but can be crossed with other plants to produce seed. Although naturally occurring male sterile canola plants have been used to a certain extent to develop hybrids, they have lacked appropriate features to allow commercial production.

The novel system described here involves the generation of high yielding canola hybrids by crossing two different genetically modified parental lines. The basis of the system is that a male sterile (Ms) line, unable to undergo self-pollination, when

crossed only with a specific fertility restorer (Rf) line, produces 100% true hybrid seed.

The male sterile parental lines (Ms1, Ms8) contain the genes *bar* (herbicide tolerance) and *barnase* (with or without *nptII*), and the fertility restorer parental lines (Rf1, Rf2, Rf3) contain the genes *bar* and *barstar* (with or without *nptII*). Due to the presence of the *bar* gene, all parental lines and the subsequent hybrids exhibit tolerance to the herbicide glufosinate-ammonium.

The Ms lines do not produce pollen but are otherwise phenotypically unaffected by the genetic modification. The use of a plant promoter from an anther specific gene results in expression of the *barnase* gene only during flowering in the developing anthers or male tissue of the flower. Consequently, plants containing this gene have an altered anther shape and reduced pollen production (Mariani *et al*., 1990).

The Rf lines contain the *barstar* gene under the control of the same plant promoter. Expression of the gene is therefore also restricted to the tapetum cells of the pollen sac, and only when flowering during anther development. In contrast to the Ms lines however, the Rf lines produce normal amounts of pollen, are fully fertile and in all respects are phenotypically normal.

The effects of the *barstar* gene activity are only apparent after crossing a male sterile line (Ms) with a fertility restorer line (Rf). When both introduced genes are expressed in combination in the same part of the flower, as occurs in a cross between Ms and Rf plants, the fertility of the resulting hybrid progeny is restored due to the inactivation of the barnase enzyme by the barstar protein, thereby ensuring full seed development. In this system, hybrid canola plants therefore contain the *bar*, *barnase* and *barstar* genes and some may also contain the *nptII* gene.

The nptII gene

The bacterial *nptII* gene is derived from *Escherichia coli* and codes for the enzyme neomycin phosphotransferase II (NPTII). Expression of this protein confers resistance to the aminoglycoside antibiotics kanamycin, geneticin (G418) and neomycin. The presence and expression of this gene, linked to the other genes of interest, allows for the early selection in tissue culture of transformed plant cells carrying the required genetic traits.

Gene constructs

A range of plasmids was constructed to deliver a specific number of gene expression cassettes to the plants. The expression cassettes consist of a promoter sequence for initiation of transcription in plants, sometimes in specific tissues only, the coding sequence of the gene of interest, followed by a 3' untranslated region providing the signals for termination of transcription and polyadenylation. The genetic elements are all well described in the published literature in terms of their molecular size and their function in plants. In addition, the complete nucleotide sequence between the left and right borders of each plasmid has been determined.

The plant or bacterial genetic components used in the cassettes are described as follows:

- the plant promoter from the atS1A ribulose-1,5-bisphosphate carboxylase small subunit gene (*ssu*), known as PssuAra, from *Arabidopsis thaliana*. The PSsuAra element comprises the 1.7 kilobase (kb) fragment upstream of the atS1A ATG codon and the transit peptide (tp) encoding sequence, for targeting to the chloroplasts (Krebbers *et al*. 1988). This promoter allows for expression predominantly in the green tissues of the plant;

- the promoter fragment from the anther specific gene TA 29 (PTA29) from the tobacco plant (*Nicotiana tabacum*);

- the promoter sequence (PNos) from the T-DNA nopaline synthase (nos) gene from *Agrobacterium tumefaciens*;

- the CaMV 35S promoter from the cauliflower mosaic virus. This promoter, denoted as P35S, gives rise to constitutive expression throughout the plant;

- the coding region of the *barstar* gene from *Bacillus amyloliquefaciens*;

- the coding sequence of the bialaphos resistance gene (*bar*), from *Streptomyces hygroscopicus* (Thompson *et al*. 1987);

- the synthetic *pat* gene, derived from *Streptomyces viridochromogenes* (Strauch *et al*. 1993);

- the coding region of the *neo* gene encoding neomycin phosphotransferase II from *Tn*5 of *Escherichia coli* (Beck *et al*., 1982);

- the coding region of the barnase gene from *Bacillus amyloliquefaciens* (Hartley, 1988);

- the 3' untranslated region of the TL-DNA gene 7 (3'g7) derived from the octopine Ti plasmid of *Agrobacterium tumefaciens* (Dhaese *et al*., 1983);

- the 3' untranslated region of the octopine synthase (3'ocs) gene from *Agrobacterium tumefaciens*;

- the 3' untranslated region of the CaMV 35S transcript; and

- the 3' untranslated region of the nopaline synthase gene (3'nos) from *Agrobacterium tumefaciens*, containing plant polyadenylation signals.

The two open pollinated lines of canola, T45 and Topas 19/2, were generated using a separate plasmid for each line. Open pollinated lines derived from these events do not contain the pollination control genes (*barnase*/*barstar*) used in the hybrid system.

Four separate plasmids were used to generate multiple parental lines (Ms and Rf) for the production of hybrid canola seed. Both the open pollinated and pollination controlled lines are summarised in Table 1.

Table 1 Summary of transformed lines relevant to *Brassica napus*, open-pollinated canola and pollination controlled canola.

Line	Number of gene expression cassettes	Glufosinate-ammonium tolerance	Pollination control genes	*nptII* gene
T45	1	pat	N/A	-
Topas 19/2	2	pat	N/A	yes
Ms1	3	bar	barnase	yes
Ms8	2	bar	barnase	-
Rf1, Rf2	3	bar	barstar	yes
Rf3	2	bar	barstar	-

As described above, the Ms and Rf lines refer to plants that carry either the *barnase* or the *barstar* gene respectively, in conjunction with the *bar* gene. In addition, the *nptII* gene is present in the following lines only: Ms1, Rf1, Rf2 and Topas 19/2.

Characterisation of the genes in the plant

All of the lines were characterised using testing material from untransformed plants of the same cultivar, Drakkar, as a control. The transformed plants were characterised at the molecular and biochemical level using a range of laboratory techniques and procedures outlined below in Table 2.

Table 2 Outline of molecular and biochemical methods used for identification of glufosinate-ammonium tolerant male sterile and fertility restorer lines, with and without the *nptII* gene.

Molecular or biochemical Methodology	Purpose
Southern Hybridisation analysis	- Detection of the gene cassettes in the canola plant genome - Quantification of the insertions in the plant genome - Verification of the physical linkage of the introduced genes - Verification that inserted DNA corresponds with plasmid DNA - Investigation of T-DNA borders - Identification of transgenic lines by their hybridisation pattern.
Polymerase Chain Reaction (PCR)	- Verification of the presence of the introduced genes - Characterisation of plant DNA sequence flanking the inserted DNA - Determination of target site deletion sequences - Development of primers to fingerprint specific male sterile or restorer alleles

Northern Blotting	- Analysis of the expression of the transgenes in different plant tissues (seeds, leaves, pollen)
NPTII assay	- Quantification of enzymatically active NPTII enzyme
PAT assay	- Quantification of enzymatically active PAT enzyme

Following transformation, shoots were regenerated on selective medium under tissue culture conditions. From these, all suitable plantlets identified for transfer to the glasshouse were first analysed by Southern blot hybridisation for the presence of the inserted genes and the number of insertions, using molecular probes specific for each gene expression cassette.

Open pollinated lines

Using the *pat* gene as a probe, Southern blot analysis on the open pollinated line T45 showed that a single copy of the T-DNA was stably incorporated at a single locus in the plant genome. Further Southern blot analyses, using several probes to detect regions outside of the T-DNA border, indicated that there was no incorporation of any coding regions beyond the T-DNA border. This was confirmed by PCR analysis of T45 to verify integration of the *pat* gene and the absence of any unintended vector sequences.

Pollination controlled lines

The genetic analysis of each line selected for hybrid production (Ms1, Ms8, Rf1, Rf2 and Rf3) indicated that a single insertion event had occurred. Further analysis of lines Ms1, Ms8, Rf1 and Rf2 using gene specific primers in a range of PCR based detection methods revealed that, as intended, only DNA sequences within the T-DNA borders were transferred to the plant.

In the Rf3 line, detailed analysis of the site of integration of the introduced DNA revealed that one full copy and one truncated copy of the T-DNA gene expression cassette were present as one segment. The complete nucleotide sequence of the segment of introduced DNA, together with approximately 800 base pairs of flanking plant DNA, revealed the presence of only a partial promoter sequence within the truncated gene cassette. The partial promoter lacks essential sequences necessary for it to function in the plant.

Phenotypic testing

In addition to the above techniques, other test procedures were available to identify and detect the inserted selectable marker genes and their gene products. For example, to investigate the expression of the inserted *bar* gene in the transformed canola plants on a larger scale, glufosinate-ammonium dot or spray assays were performed at different stages of development. This involved applications of a commercial formulation of the herbicide either directly to the surface of a young leaf or by aerial spraying. Since the *bar* gene is genetically closely linked to the *barnase* or *barstar* gene in the plasmid constructs, this technique indirectly allowed the selection of

plants carrying the male sterile (*barnase*) or fertility restorer (*barstar*) genes in larger populations.

Gene expression

In the lines created for hybrid production, RNA analyses were also carried out to further characterise the levels of expression of the transferred genes in specific plant tissues. The results obtained from these experiments were consistent with gene expression patterns expected from the specific plant promoters used in each case.

Bar/pat

For the Ms1, Ms8, Rf1, Rf2 and Rf3 transformants, messenger RNA (mRNA) corresponding to either the *bar* or *pat* gene could be detected at extremely low levels in the leaves and flower buds, but not in the seeds of the plants.

Barnase/ barstar

For the Rf1, Rf2 and Rf3 transformants, *barstar* mRNA was barely detected in flower buds only, but not in any other plant tissues, including the seeds. As expected, because expression of *barnase* results in cell death, *barnase* mRNA could not be detected in any tissues from the Ms1 and Ms8 transformants.

NptII

Using a hybridisation system that could detect 0.1 pg/µg of total RNA, there was no detectable *nptII* mRNA in any tissues from the Rf1 and Ms1 transformants. This result indicates that the level of expression of this gene is extremely low in all parts of the plant.

Stability of the genetic changes

The stability of the transferred genes was investigated for all lines to ascertain plant characteristics over multiple generations. For example, the open pollinated lines T45 and Topas 19/2 were monitored extensively in field trials in Canada during the 1994, 1995, 1996 and 1997 growing seasons. Mendelian analysis was applied to at least four generations derived from the original T45 transformant and the stability of the inheritance pattern was demonstrated. Overall, the segregation patterns observed on analysis of the progeny of the original transformants, including hybrids, indicated the stable physical integration of the genes.

Genetic and agronomic performance of the Ms and Rf lines

The expected expression of the traits and the absence of unintended changes to agronomic characteristics were evaluated in a wide range of field conditions. Multiple crosses and backcrosses in more than 40 different spring or winter varieties were performed in field experiments across Europe and Canada over a three year period (1991-1993) to generate segregation data on the glufosinate-ammonium trait as well as the hybrid production traits. These data indicated no loss of any of the new traits either by observation of the phenotype or in the molecular definition of the plants.

The extensive field experiments were carried out in a broad range of countries, including Canada, Sweden, UK, France, Belgium, Denmark, Spain, USA and Chile. Normal agricultural breeding practices were adopted in conducting these experiments to monitor the genetic and agronomic performance characteristics of the Ms and Rf lines in comparison with non-transformed canola, and to demonstrate the stability of gene expression in terms of the sustainability of the phenotype under different environmental conditions. Factors such as germination, crop establishment, plant vigour, flowering characteristics, seed yield and glufosinate-ammonium tolerance levels were monitored.

Under field conditions, transformed and non-transformed seedlings germinated at about the same time after sowing. Thereafter, both types developed evenly and uniform plant stands were established. Plant height and plant vigour of the Ms and Rf plants and their restored hybrid combinations were comparable to the control plants. No difference in susceptibility to variables such as temperature, humidity, dessication, light or other environmental stress factor from those of other non-transformed canola cultivars was observed from planting to harvest.

Similarly, evaluations of the flowering characteristics of the Ms and Rf lines and their progeny, as well as their hybrid combinations, in the different environments revealed no major differences. Flower morphology was normal at all sites, nectaries in male sterile canola flowers developed normally and insect activity was also normal for both groups.

Spraying of mixed populations of plants (transformed and non-transformed) with variable rates of glufosinate-ammonium was carried out to determine field tolerance levels. Some non-transformed plant development was observed at sub-agronomic doses of the herbicide. The non-transformed plants did not survive a treatment at or above a rate of 750 g active ingredient due to competition with the glufosinate-ammonium tolerant plants. However, glufosinate-ammonium applications performed before planting and shortly after seeding showed no selectivity for the transformed plants.

Gene expression was scored from observation of the phenotype and subsequently confirmed by Northern blots and NPTII and PAT enzyme assays. These experiments demonstrate that the expression of the *nptII*, *bar*, *barnase* and *barstar* genes, when incorporated into the plant genome of the male sterile, the fertility restorer and subsequent hybrid lines, was stable throughout the growing season under varying conditions.

The data therefore support the conclusion that once integrated into the different genetic backgrounds, the transferred genes were inherited as a single locus in a predicted manner according to standard Mendelian patterns of inheritance.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the consumption in food of the antibiotic

resistance gene DNA *per se* (WHO 1993). There have been concerns expressed however that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of some antibiotics.

Under the control of a plant promoter, the antibiotic resistance gene *nptII* is present in four of the transgenic canola lines - Ms1, Rf1, Rf2 and Topas19/2. The *nptII* gene confers resistance to the aminoglycoside antibiotics, neomycin, kanamycin and geneticin (G418). These antibiotics only have very limited clinical use. Neomycin is not used orally because of its toxicity, but is still used topically in certain circumstances (Davies *et al* 1986).

In relation to the presence of the *nptII* gene in the transgenic canola, the probability of transfer to, and expression of the gene in, microorganisms present in the human digestive tract must be considered. The following steps are necessary for this to occur:

1. a fragment of DNA, containing the coding region of each gene, would have to be released, probably as a linear fragment, from the DNA in the GM food;

2. the DNA fragment would then have to survive exposure to various nuclease enzymes excreted by the salivary glands, the pancreas and the intestine;

3. the DNA fragment would have to compete for uptake with other dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;

4. the recipient bacteria would have to be competent for transformation;

5. the DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;

6. the *nptII* gene would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium; and

7. the antibiotic resistance gene would have to be stably maintained by the bacterial population.

The transfer of the *nptII* gene to microorganisms in the human digestive tract is therefore considered to be highly unlikely because of the number and complexity of the steps that would need to take place consecutively. In the unlikely event that successful transfer of a functional antibiotic resistance gene to microorganisms in the human digestive tract did occur, the potential impact on human health has also been considered.

In the case of transfer of the *nptII* gene, the human health impacts are considered to be negligible. This gene occurs naturally in bacteria inhabiting the human digestive tract and therefore the additive effect of an *nptII* gene entering the human gastrointestinal

flora from a GM plant would be insignificant compared to the population of kanamycin resistant microorganisms already naturally present. Natural populations of antibiotic resistant bacteria are far more likely to be sources of transferred antibiotic resistance than ingested plant material.

Canola oil is a highly processed product. The process of refining and purification is considered to remove traces of natural seed components including proteins and residual plant DNA, to the extent that these are no longer detectable in the oil. Dietary exposure to any DNA derived from the canola seeds is therefore considered to be virtually zero. The results from various methods of analysis demonstrated the absence of novel DNA in oil from the transformed canola plants.

PCR analysis of oil from hybrid canola seeds

To determine whether recombinant DNA could be present in the oil or meal fractions of canola seeds, PCR analysis was performed on processed fractions of hybrid seed produced from the Ms8/Rf3 cross. Four different samples of processed canola material were subjected to DNA extraction and PCR analysis to test whether the introduced *bar* gene was detectable. The results showed that whereas the seed meal contains DNA detectable by the PCR method, no DNA could be detected in the bleached oil samples. This negative result was confirmed even when three additional different extraction protocols were applied. The negative PCR analysis on the oil fractions confirms that commercial processing of canola oil results in a product that is free of DNA, using the most sensitive analytical method available.

DNA digestibility study

Schneider, R., 1993. Fate of introduced DNA in gut: Degradation of phosphinotrhicin acetyl transferase gene from transgenic rape HCN 92 (*Brassica napus*) in stomach fluids from pig, chicken and cow. Hoechst AG Agricultural Division, Frankfurt am Main, Germany. Study No. BR 93/06

A study was conducted to determine whether the introduced DNA present in transformed canola line Topas 19/2 (containing *pat* and *nptII*) is sensitive to degradation by mammalian and avian digestive fluids. The study consisted of two separate experiments using leaf material from transformed plants incubated in digestive stomach fluids extracted from pig, chicken and cow.

In the first experiment, leaf samples were incubated at 37°C in pH step gradients of the digestive fluids over a range of time points up to 1 hour. DNA was extracted and analysed by PCR using primers specific for the detection of the *pat* gene and a labelled molecular probe. The PCR analysis indicated that the *pat* gene was readily degraded after *in vitro* incubation in any of the digestive fluids tested. Degradation was somewhat pH dependent, being most efficient at low pH which more closely mimics physiological conditions. The degradation was less complete at higher pH, well above the normal acidic environment of the human stomach.

The aim of the second experiment was to test whether the introduced DNA in the plant material could transfer to competent *E. coli* bacteria in a laboratory situation, using the *nptII* gene as a marker for transformation. The *E. coli* strain was converted

from a disabled laboratory strain to a competent living strain for this experiment. Transformed bacteria were recovered by selection on medium containing the antibiotic kanamycin. Both plasmid DNA and leaf-extracted DNA from the transformed canola plants were exposed to the same range of digestive fluids, or to water as a control, prior to use in the transformation process.

The results obtained showed that, as expected, antibiotic resistant bacteria were recoverable at the beginning of the experiment, prior to incubation in digestive fluids, using the proprietary plasmid as the gene source. However, no colonies were recovered after the plasmid was incubated for 60 minutes in the various stomach fluid preparations. More significantly, when the transformed plant material itself was used as the gene source, no transformed colonies could be recovered either initially or after 1 hour incubation in the stomach fluids from any of the test animal species.

These results confirm that the transfer to intestinal bacteria of introduced DNA present in transformed plants, including the antibiotic resistance gene *nptII,* is extremely unlikely to occur.

Conclusions

Both the open pollinated and pollination control (Ms and Rf) canola lines, contain a bacterial gene conferring tolerance to the herbicide glufosinate-ammonium. In addition, the Ms and Rf lines contain up to two bacterial genes to generate plants that either (a) do not produce pollen (male sterile) or (b) are phenotypically normal, but contain a fertility restorer gene. A bacterial gene conferring resistance to kanamycin is present as a selectable marker in four lines only, that is Ms1, Rf1, Rf2 and Topas 19/2.

All lines were characterised at the molecular level and the analyses indicate that the genes of interest were transferred in a single insertion event. Full nucleotide sequence information at the integration site was generated. In one of the fertility restorer lines, Rf3, an additional truncated gene expression cassette was shown to be non-functional in the plants.

The conclusion from the numerous greenhouse and field experiments on the genetic stability of the traits is that the transferred genes remain structurally stable through meiosis and are transmitted in the seed. The organisation of the transferred DNA (as defined by Southern hybridisation), as well as the timing, tissue specificity and levels of gene expression in the original transformant is preserved in all progeny under all environmental circumstances. The incorporated genes (*nptII*, *bar*/*pat*, *barnase*, *barstar*) are 100% linked and are inherited as a single locus according to Mendelian patterns of inheritance and are expressed as dominant markers.

It is extremely unlikely that the *nptII* gene could transfer from canola oil to bacteria in the human digestive tract. There is virtually no dietary exposure to plant DNA in the consumption of canola oil. Even if present, an intact gene is unlikely to survive digestion or to be taken up by intestinal bacteria because of the number and complexity of steps that would need to take place consecutively. If these events did occur, the human health impacts would be negligible because antibiotic resistance

genes are already commonly found in bacteria in the environment and the human digestive tract.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function

PAT

The herbicide tolerant trait is conferred by the expression of either the introduced *bar* gene or the synthetic *pat* gene, as both code for the phosphinothricin-acetyl-transferase (PAT) protein that detoxifies phosphinothricin (PPT). The mode of action of PPT is to inhibit the endogenous plant enzyme glutamine synthetase, an enzyme involved in amino acid biosynthesis. By inhibiting this enzyme, PPT causes rapid accumulation of ammonia in the plant cell, leading to plant death. In transformed canola plants, the introduced PAT enzyme chemically inactivates the PPT by acetylation of the free ammonia group, giving rise to herbicide tolerance in the whole plant.

The PAT protein consists of 183 amino acids, has a molecular weight of 22 kDa, and exhibits a high degree of enzyme specificity, recognising only the one substrate L-glufosinate in the acetylation reaction. This high substrate specificity was tested in the presence of each of 21 L-amino acids at substrate concentrations exceeding 50 times the K_M value for L-glufosinate. None of the tested amino acids substituted as an alternative substrate in the PAT catalysed reaction, but the enzyme reaction with L-glufosinate was not inhibited (Schulz, A., 1993. L-Phosphinothricin-N-Acetyltransferase – Biochemical Characterisation. Hoechst Biol. Research C., Company File No: A51230).

Barnase and barstar proteins

The *barnase* gene, used to produce the male sterility trait in canola, encodes a ribonuclease which degrades RNA in the tapetum at early stages of pollen formation. The eventual complete loss of RNA in the restricted cell layer leads to the death of these cells expressing the ribonuclease enzyme. In turn, this leads to the deposition of wound callose which prevents nutrients reaching the tissues of the anther filament, thereby leading to wilting of the anther. Consequently, plants containing the *barnase* gene are phenotypically normal except that, during flowering, the shape of the anther is altered and pollen production is significantly reduced.

In contrast, transformed plants expressing the *barstar* gene are phenotypically normal and are fertile. The effects of the *barstar* gene activity in the hybrid canola are only visual after crossing with the male sterile line. Microscopic analysis of the anthers and the pollen grains of the restored plants show a complete resemblance to those of non-transformed plants.

The barnase enzyme is a small protein consisting of a single chain of 110 amino acids. The enzyme is characterised by no disulphide bonds, metal ion cofactors or other non-peptide components. The barstar enzyme is a small protein consisting of a single chain of 89 amino acids and includes some disulphide bonds.

Neomycin phosphotransferase II

The enzyme neomycin phosphotransferase II (NPTII) is a commonly used marker protein that allows the selection of transformed plant cells early in the regeneration phase, and can also be used for monitoring gene expression and genetic stability during later development of the plants (Kärenlampi 1996).

NPT II is a bacterially-derived enzyme with a molecular weight of 29 kDa that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, including neomycin, kanamycin and gentamicin A and B, thereby inactivating the antibiotics (Davies *et al.* 1986).

Protein expression analyses

Generally, protein is considered to be a contaminant of processed canola oil, and causes cloudiness in the final product. The extraction process includes the use of high temperatures and solvent extraction, which denatures and removes plant protein from the initial sample. Therefore, due to the extensive processing methods applied during canola oil extraction and refinement, no protein, including any of the novel proteins, would be expected to be present at detectable levels. Extensive protein expression data for PAT confirmed the absence of protein in the oil.

Studies evaluated:

Determination of Phosphinothricin Acetyltransferase (PAT) and NPTII content in glufosinate resistant canola (*Brassica napus*) cultivars HCN-10 and Innovator. B. Dang, Xenos Laboratories Inc., Ontario, Canada. Study Number 97AC26, 1997.

PAT enzyme content in glufosinate-tolerant canola seed and processed fractions. B. Dang, Analytical Testing Facility: Xenos Laboratories Inc., Ontario, Canada. Project Number XEN98-15, 1998.

Benchtop Processing of Oilseed Rape (SWO2631 Sprayed and Unsprayed). 1998 Technical Research Report to MacDonald, B., AgrEvo. POS Pilot Plant Corp., Saskatchewan, Canada. Project No. 98-690.

Measurement of PAT activity in leaves and seeds of the male sterile Ms8 transformant and the fertility restorer Rf3 transformant. A. van Vliet, Plant Genetic Systems (PGS), Belgium.

PAT ELISA on different oil fractions derived from Ms8/Rf3 *Brassica napus* seeds. A.van Vliet, Plant Genetic Systems, Belgium. Report ID PAT-ELISA oil Ms8/Rf3, June 1999.

PAT protein

Open-pollinated lines

Levels of PAT protein were measured in the seed harvested from a conventional cross between two glufosinate-ammonium tolerant lines, T45 and Topas 19/2. The processing and compositional characteristics of two seed lots from this resultant line (SWO2631) were compared, harvested from (a) plots treated with the herbicide and (b) from untreated plots.

Protein levels were analysed in whole raw seed, toasted meal and refined bleached and deodorised (RBD) oil. The processing of both seed lots (357 grams of unsprayed and 383 grams of sprayed seeds) was performed by a contracted company using methods which emulate normal industrial practice in the preparation of oilseed rape fractions. There were no differences in processing characteristics of the seed from either the sprayed or unsprayed plants.

Data were provided on the amount of PAT enzyme present as determined by enzyme-linked immunosorbent assay (ELISA) with a detection sensitivity limit of 2 ng/g in seed or meal, and 0.4 ng/g in oil. The reference substance for the assay system was purified PAT protein. Negative controls fortified with PAT protein at 2.0 ng/g and 4.0 ng/g were included. The recoveries were 81.8% and 107% respectively, indicating that the assay system was optimised to detect PAT in the samples.

The results of the ELISA analyses indicated that PAT protein was found in the treated and untreated whole seed samples at approximately the same levels. There was no PAT protein detectable in the toasted meal or refined bleached deodorised oil samples. A summary of the results is presented in Table 3.

Table 3: PAT content in canola seed and processed fractions from T45/Topas 19/2 cross.

Sample	PAT content (ng/g)
Raw seed – untreated	563
Raw seed – treated	669
Toasted canola meal – untreated	Not detected*
Toasted canola meal – treated	Not detected*
RBD oil – untreated	Not detected*
RBD oil – treated	Not detected*

* Below the limit of quantitation (2ng/g for seed and meal, 0.4 ng/g for oil).

The ELISA data support the conclusion that although the PAT protein is expressed in the seed at levels that are readily detectable, approximately 0.6µg/g for the T45/Topas 19/2 hybrid, the extensive processing which is required to produce the oil fractions effectively removes all traces of PAT protein from the oil.

The PAT protein is present in the seed meal at approximately 0.005% of total protein (as determined by ELISA analysis of Topas 19/2). Processing affected the activity of the enzyme and the protein levels such that the levels of PAT in the toasted meal were approximately one-tenth of the levels in untoasted meal. The toasting process uses temperatures in excess of 90°C which denature the enzyme.

Using the same ELISA system, measurement of PAT protein in the seeds (pooled sample) of the T45 line was determined to be 295 ng/g, approximately half that of the hybrid. This result is consistent with the number of *bar* genes present in the plants – one copy of the gene in each parental line, and therefore two copies at different loci in the hybrid. As expected, there was no PAT protein (below the limit of quantitation) found in the negative control sample (Excel).

Ms and Rf lines

Data were also provided on the amount of PAT protein in seeds obtained from a number of the Ms and Rf lines (and their crosses). In this instance, the amount of introduced PAT protein was calculated from a measurement of PAT enzyme activity detectable in a seed extract, and was not a direct measurement of the protein. These results are presented in Table 4 and show that the introduced PAT enzyme does not result in specific PAT activity above background acetyl-transferase activity in seeds.

Table 4 **PAT content in seeds from Ms1, Rf1, Rf2 and crosses (Ms1xRf1, Ms1xRf2) and untransformed control variety (1995)**

Sample	Protein extract mg/ml	PAT protein U/ml	PAT protein in seed µg/g	PAT protein µg/mg protein
Ms1xRf1	3.6	0.08 ± 0.04	4.6 ± 2.3	0.02 ±0.01
Rf1	3.4	0.14 ± 0.03	4.8 ± 1.9	0.04 ±0.01
Ms1xRf2	3.7	0.12 ± 0.03	7.4 ± 1.8	0.03 ±0.01
Rf2	3.5	0.19 ± 0.02	11.3 ± 1.2	0.05 ±0.01
Ms1	3.7	0.22 ± 0.02	13.2 ± 0.9	0.06 ±0.01
Drakkar (control)	3.2	0.22 ± 0.04	13.0 ± 2.2	0.06 ±0.01

- PAT U measured in seed extract concentrated 8.5 times, U refers to enzyme units (amount of enzyme to produce one micromole per minute).
- Protein concentration measured using Biorad assay (Lowry method) with BSA as standard.
- µg PAT is based on an estimated specific activity of 170 U/mg PAT.

Analysis of the seeds and leaves from the Ms8 and Rf3 lines confirm a similar pattern of expression of the PAT protein in these lines. Triplicate seed and six replicate leaf samples were assayed for PAT activity using a spectrophotometric assay system. Five replicate samples of leaves and seeds from a non-transformed control cultivar were also analysed. When expressed as a fraction of total protein, the levels of PAT protein in the seeds of the Ms8 and Rf3 lines were only marginally higher than in the seeds from the control cultivar. As expected with a herbicide tolerance trait where expression of the introduced gene is directed to the green tissues of the plant, the levels of PAT found in the leaves of the transformed lines were above those detected in the leaves from the non-transformed control cultivar.

In other experiments, the biochemical methods available for detecting the PAT enzyme in various plant tissues were applied to various oil fractions obtained from hybrid seeds produced from crossing the Ms8 and Rf3 lines. Ten kilograms each of non-transformed and transformed hybrid seeds were processed under simulated industrial processing conditions to produce crude oil, degummed oil, refined oil, washed oil and bleached oil. In addition, oil samples derived from crude seed pressing were obtained (POS Pilot Plant Corporation, Canada, 1998) for testing.

An ELISA system was used to determine the PAT content of the different oil fractions. The limit of detection of this assay system in crude oil and seed press oil was estimated to be 1µg/ml, while the limit of detection of the PAT protein in degummed, refined, washed and bleached oil fractions was estimated to be 3µg

PAT/ml. As an additional measure, processed fractions from non-transformed seeds were fortified with purified PAT protein prior to assay in order to validate recovery of known, added amounts of PAT protein in the samples.

The PAT protein was not detected in any of the oil fractions tested, including the crude seed pressing, from either the transformed or the non-transformed samples. The validation analyses demonstrated that the PAT protein could be recovered using this assay system and therefore showed that the industrial processing effectively removes protein from the canola oil, with none detected after just the first stage of processing.

NPTII protein

Study evaluated:

Determination of Neomycin Phosphotransferase II (NPTII) Levels by ELISA in Seeds of *Brassica napus* Hybrid Varieties PGS1, PHY14 and PHY35 (based on Ms1/Rf1), PGS2 and PHY23 (based on Ms1/Rf2). Xenos Laboratories Inc., Ottawa, Ontario, Canada, 1997.

Seed samples were collected from field trials conducted in 1995 in Canada. The seeds were shipped to Xenos Laboratories Inc. for determination of neomycin phosphotransferase II (NPTII) enzyme content using enzyme-linked immunosorbent assay (ELISA). Protein content was measured using the Bradford assay (Analytical Biochemistry, vol.72, pp248-254, 1976). Multiple samples of several hybrid varieties derived from the lines Ms1/Rf1 and Ms1/Rf2 were tested as well as a non-transformed control cultivar.

The results obtained showed that there was no detectable NPTII protein in the seeds derived from any of the hybrid lines tested. The limit of detection of this assay system was 350 pg/g seed, using this highly sensitive method of analysis. The results from additional control samples using laboratory fortified NPTII canola seeds indicated that the assay system was able to recover almost all of the NPTII spikes over a ten fold variation in concentration.

Barnase and barstar proteins

From previously published scientific studies, it is known that expression of the *barnase* gene generates ribonuclease activity which is lethal to the cells in which it occurs. In the Ms lines, the expression of the *barnase* gene coupled to the plant promoter (PTA29), has been demonstrated to be specifically confined to the developing anthers where the enzyme causes the degeneration of a specific layer of cells known as the tapetal cell layer, resulting in a characteristic wilting of the anthers (Mariani *et al*., 1990).

A detailed description of the anther and floral tissue development of male sterile canola plants has been obtained by histochemical analysis. These studies revealed that no cytological nor histochemical differences between transformed and non-transformed plants could be detected in other floral tissues for example, ovarium, style, sepals and the bottom of the developing flowers. The male sterile anther is therefore an observable characteristic (De Block *et al*., 1993).

Similarly, the plant promoter (PTA29) used in the fertility restorer lines limits expression of the *barstar* gene to the same specific sites within the plant (tapetum cells of the pollen sac) and to the same specific developmental stages (only when flowering, during anther development). Therefore, these proteins are coordinately expressed in the same specific cell types early in the flowering stage. They are not expressed in the parts of the plant that are used for human food.

Potential toxicity of novel proteins

As canola oil from the various transformed lines has been shown to contain no traces of protein (see above), consumers are not expected to be exposed to the novel proteins through consumption of the food derived from these lines. Nevertheless, an evaluation of the potential toxicity of both the PAT and NPTII enzymes was conducted. The barnase and barstar proteins were not included in the toxicity assessment of the novel proteins because of the nature of their biochemical function and the specific localisation to non-edible parts of the flower.

PAT

The lack of toxicity of PAT (see OECD, 1999) is well documented in the scientific literature. In addition, the enzyme has been evaluated previously in relation to its use in a glufosinate-ammonium tolerant corn. For that assessment, an acute oral limit test was performed using microbially-produced PAT protein administered by gavage in a single dose of 2500 mg/kg body weight to CD-1 mice. The animals were observed daily for the duration of the 14 day study and weighed weekly. There were no adverse clinical signs for the duration of the study and no gross internal findings were observed at necropsy on day 14 (Merriman, 1996). Under the conditions of the test, the acute oral LD_{50} of PAT protein was concluded to be greater than 2500 mg/kg body weight in the mouse.

In accordance with these results and other available evidence, an exemption from the requirement to establish a maximum permissible level for residues of PAT, and the genetic material necessary for its production, was granted by the United States Environment Protection Agency in April 1997 (US EPA, 1997).

The metabolite, N-acetyl–L-glufosinate, that results from detoxification of the herbicide in glufosinate-ammonium tolerant canola, is also considered to be non-toxic to both plants and mammals, including humans (OECD, 2001).

NPTII

The potential toxicity of NPTII has been previously evaluated in acute oral toxicity studies using mice as the test animals. There were no significant adverse findings in the animals when administered by gavage doses up to 5000 mg/kg body weight. On the basis of these findings, NPTII was considered to have low acute oral toxicity. In addition, the safety of this protein has also been considered on numerous occasions in the peer reviewed scientific literature (Flavell *et al.* 1992, Nap *et al.* 1992, Fuchs *et al.* 1993a, Fuchs *et al.* 1993b). These studies are relevant to this evaluation as the NPTII enzyme expressed in lines Ms1, Rf1, Rf2 and Topas 19/2 is identical to the NPTII

previously assessed for toxicity. In all instances it has been concluded that NPTII is non-toxic to humans.

Potential allergenicity of novel proteins

Studies evaluated:

Van den Bulcke, M., 1997. Phosphinothricin acetyl transferase, neomycin phosphotransferase II, barnase, barstar allergenicity assessment: a common approach. Plant Genetic Systems Internal report 000463/ALLERMVDB/01.

Bremmer, J.N. & Leist, H. 1996. Statement on the lack of allergenic potential of PAT-protein and Glufosinate Tolerant crops containing PAT-protein. Report No. 96.0351.,

Many foods have been reported to cause allergies in some people, and it is well established that this is primarily due to an idiosyncratic immune reaction to a particular protein component of the food. Other components of foods such as fats (oils) are not generally associated with such reactions. In the case of canola, the seed meal, containing the proteins, is used primarily only for animal feed. The quality requirements of commercial canola oil production dictate the absence of protein in the final product. Consequently, humans are not generally exposed through the diet to any plant proteins (including the novel proteins) from consumption of canola oil.

Notwithstanding the absence of protein in the final food, the safety assessment must include an evaluation of the potential for allergenicity of the novel proteins in the GM canola. Potential allergenicity can be evaluated by comparing a range of certain molecular and biochemical properties of the proteins with those of known allergens. The comparison uses information available on food allergens already known and identified. Known allergens generally have certain physical characteristics in common, including poor digestibility. Other important factors include whether a novel protein is derived from a known allergenic source and whether it exhibits amino acid sequence similarity to any known allergens. The relative abundance of the novel protein in the food is also a consideration relevant to an evaluation of potential allergenicity.

The protein expression analyses demonstrated that the introduced PAT protein is present in the leaves, stems and seed of all of the transformed lines. However, the NPTII protein is below the limit of detection in the seed of the Ms1, Rf1, Rf2 and Topas 19/2 lines, using the most sensitive methods available to date. Furthermore, expression of the barnase and barstar proteins is restricted to particular floral tissues only in the Ms and Rf lines (and hybrid crosses of these lines). The novel proteins in this case are therefore either absent, or are in very low abundance in the canola seeds from which the oil is derived.

The data showed that the molecular weight of the introduced proteins PAT (approx. 22kD) and NPTII (approx. 29kD) are within the molecular weight range exhibited by known allergens, while the barnase and barstar proteins are below this range (12kD and 10kD respectively). As determined by ELISA, the levels of both PAT and NPTII proteins are <0.002% of total extractable protein in the seeds[1].

[1] The study reports are based on pooled data from the lines Ms1 and Rf1/Rf2.

In addition, the amino acid sequence of the introduced proteins PAT, barnase and barstar was compared with amino acid sequences of known allergens (inhalation and food allergens) from both plant and animal origin available on three public protein databases (AA HIV, PIR and SwissProt). This comparison revealed that the novel sequences do not exhibit any significant amino acid homology with published sequences of toxins or allergens. The additional study by Van den Bulcke (1997, PGS Internal report), which included the NPTII protein, confirmed this finding.

Further evidence is available to indicate that the PAT protein in particular lacks any of the characteristics of known allergens. Common plant food allergens are usually glycosylated proteins and most are tolerant to heat denaturation, remaining stable during the high temperatures involved in cooking or processing (Taylor, 1995). However, the PAT protein lacks glycosylation sites and studies have determined that the enzyme is heat labile and is completely inactivated by temperatures above 75°C. Using Western blot analysis, experiments conducted by Shulz *et al*. in 1997 (Internal reports listed below) found that although the purified protein was not degraded by an experimental heat treatment at temperatures up to 100°C, a centrifugation experiment demonstrated that the protein is denatured at temperatures above 40°C.

Digestibility of PAT

Studies submitted:
Schulz, A. (1993). L-Phosphinothricin -N-Acetyltransferase, Inactivation by pig and cattle gastric juice. Biologische Forschung C, Biochemie der Pflanzen, Hoechst Aktiengesellschaft, Frankfurt. Hoechst Report 93.02.

Schulz, A. (1994). Digestion of the Phosphinothricin Acetyltransferase Enzyme in Human Gastric Fluid (Simulated). Hoechst Schering AgrEvo Ltd., Research Biochemistry, Frankfurt, Germany. Company Report No. AS 94.12E.

Schulz, A., Lutge, K. and Taggeselle, P. (1997). Stability of the Phosphinothricin Acetyltransferase Enzyme: Heat stability and digestion in Simulated Gastric Fluid and Simulated Intestinal Fluid. Hoechst AgrEvo, Frankfurt, Germany. Company File No. A58686.

Typically, most food allergens are resistant to digestion, proteolysis and other forms of hydrolysis (Bargman *et al*., 1992). Studies were conducted to test whether the PAT protein, which is expressed in all transformed lines, is susceptible to proteolytic degradation.

When tested in simulated human digestive fluids, the results of studies using Western blot analysis showed that PAT protein (purified from over-expressing *E. coli*) was readily degraded within seconds. The degradation of the protein was dependent on the presence of proteases, pepsin in simulated gastric fluid (SGF) and pancreatin in simulated intestinal fluid (SIF). The protein was also rapidly inactivated (within one minute) by acidic conditions in dog and pig gastric fluid and with bovine rennet-bag fluid (pH 1.3). Inactivation of PAT protein in bovine paunch fluid, which has a neutral pH (7.1), was slower but occurred within 30 minutes.

Digestibility of NPTII

The NPTII protein has been comprehensively assessed with respect to potential allergenicity in previously published studies (for example, Fuchs *et al*., 1993 a,b) as part of the assessment of other GM foods. In these studies, large quantities of recombinant protein were generated for extensive physical and biochemical analyses, and to provide sufficient material for a rodent feeding study. The results of the analyses support the food safety aspects of the NPTII protein, by establishing that the protein is rapidly inactivated and degraded in simulated digestive conditions, and that it does not exhibit structural similarities with known food allergens.

Conclusion

Of the four novel proteins introduced into canola plants, only PAT and NPTII proteins are expressed in the seed. The scientific evidence indicates that both PAT and NPTII are non-toxic to humans and neither protein exhibits biochemical or physical characteristics in common with known food allergens. Furthermore, humans are extremely unlikely to be exposed to either of these proteins through the consumption of canola oil because of the stringency of the commercial processing in removing plant proteins from the final food product.

COMPARATIVE ANALYSES

The genetic modification could, in some circumstances, affect the overall nutritional composition of the food, or cause unintended changes that could adversely affect the safety of the product. Therefore a safety assessment of food produced from genetically modified plants must include analysis of the composition of the food, based on a comparison with other commercial varieties of the crop. Generally, comparisons are made not only with the parental line but also with other non-transformed lines. If the parameter for the transformed line is within the normal range for the non-transformed lines, the lines are considered to be equivalent (Hammond and Fuchs 1998).

Key nutrients

Studies submitted:
MacDonald, R. (1997) A Comparison of Moisture, Oil, Protein, Ash, Carbohydrate, Gross Energy and Amino Acid Levels of Harvested Seed From Transgenic *Brassica napus* Line HCN-19 and a Standard Commercial Variety AC Excel. Analysis performed at: Smith Laboratory, NOVAMANN International, Toronto, Ontario, Canada. Report No. AC197-42.

MacDonald, R. (1997) Effect of Glufosinate Ammonium Treatment on the Composition of Glufosinate Tolerant Canola Meal and Oil. Report No. AC 197-07.

MacDonald, R. (1998) Seed Composition Characteristics of the Line SW02631 (T45/Topas 19/2). Report No: AC198-19.

Belyk, M. (1999) Comparison of HCN28 (pHoe4/AcII) Glufosinate Resistant Canola Fatty Acid Profile and Glucosinolate Content with Innovator (pOCA/Ac) Glufosinate Resistant Canola and Three Standard Commercial Varieties in 1994 and 1995. Report No: AC196-02/01.

Beriault, J.N. (1999) The Effect of Glufosinate Ammonium on the Seed Composition of T45 Glufosinate Tolerant Canola, POS Pilot Plant Corporation, Analytical Services Divisions, SK, Canada. Study Number: 98AC13.

Canola oil is a relatively recent inclusion in the human diet brought about through intensive modification of oilseed rape during the past thirty years. This systematic modification by conventional plant breeders to improve the nutritional and functional characteristics of the food is supported by extensive research relating to seed composition, oil and meal quality and seed processing performance. This information in turn provides a sound basis for the analysis of properties of new varieties of canola, including those generated using gene technology.

The key nutrients in canola have been evaluated in order to compare equivalent data from the transformed lines, the non-transformed counterpart and published literature ranges obtained for conventional varieties of canola. This process includes a study of the major constituents that are characteristic of canola seeds, with particular reference to the oil as a human food. The process also may take into account natural variation in composition due to genetic variability and environmental factors, which are known to be major variables in determining the measured range obtained for most constituents.

The term canola has been registered and adopted in Canada to describe the oil (seeds and plants) obtained from the cultivars *B. napus* and *B. campestris*. In 1986 the definition of canola was amended to refer to *B. napus* and *B. campestris* lines containing <2% erucic acid in the oil and <30 μmol/g glucosinolates in the air-dried, oil-free meal (Codex, Downey, 1995). These varieties are referred to as double low (00) varieties. All of the GM canola lines in this assessment, by definition, must comply with the above specifications to be permitted for use in commercial production of canola products.

The concerted breeding program to reduce or remove the presence of the natural toxicants in rapeseed oil has resulted in more extensive investigations, in both animal and chemical studies, than for most other edible vegetable oils. Canola oil is characterised by a low level of saturated fatty acids, a relatively high level of monounsaturated fatty acids (oleic acid) and an intermediate level of polyunsaturated fatty acids (linoleic and linolenic acid).

Detailed compositional analyses were conducted on the seeds from transformed lines Ms1, Ms8, Rf1, Rf2, Rf3, T45 and Topas19/2. The analyses included measurements of glucosinolates, protein and oil content of the seeds and the fatty acid profile of the oil.

The data presented in Table 5 are a compilation of data showing that the percentage of oil in canola seeds harvested from transformed varieties is comparable to both the non-transformed counterpart and to commercial control varieties. These data demonstrate that the presence of the *bar* or *pat* genes, *barnase*, *barstar* and *nptII* (in some lines only) has not resulted in any change to the constituent levels of oil in the transformed seeds. The data were collected over a number of seasons from 1991 to 1995 and in a number (up to 9) of different locations in Canada.

Table 5: Oil content as a percentage of the seed from varieties of canola plants grown and tested in Canada. The values are the maximum and minimum measurements recorded over a number of seasons and at a number of different locations for any particular line. The groupings within the bolded lines represent concurrent analyses.

Canola varieties	Oil content (% in seed)
Topas 19/2	40.1 – 48.0 (1991-1993)
Non-transformed (8 lines)	36.2 – 48.3 (1991-1993)
Male sterile (Ms1)	35.2 – 47.8 (1991-1993)
Fertility restorer (Rf1)	36.3 – 48.6 (1992-1993)
Cross (Ms1xRf1)	35.6 – 47.4 (1992-1993)
Drakkar control	35.1 – 49.0 (1991-1993)
Rf1	38.2 – 51.9 (1993-1994)
Rf2	38.7 – 51.7 (1993-1994)
Ms1xRf1	38.2 –51.4 (1993-1994)
Ms1xRf2	37.5 – 52.3 (1993-1994)
Drakkar control	39.0 – 53.0 (1993-1994)
Male sterile (Ms8)	37.5 – 44.1 (1995)
Fertility restorer (Rf3)	36.8 – 47.5 (1995)
Ms8 x Rf3	39.1 – 48.1 (1995)
Non-transformed counterpart	37.7 – 48.5 (1995)
Commercial varieties	37.0 – 45.6 (1995)

Fatty acid composition

The fatty acid composition of the oil derived from a number of transformed and non-transformed lines was analysed in detail. The measurements included 11 different key fatty acids, including in particular, the erucic acid (C22:1) content of the oil. As well as control varieties (eg. Drakkar), different generations of the male sterile lines (Ms1 and Ms8) were tested together with different generations of the fertility restorer lines (Rf1, Rf2 and Rf3), multiple backcrosses of Ms and Rf lines in different canola varieties and unrestored (Ms/control) and restored (Ms/Rf) hybrids. The seed samples were collected from plants grown at locations in Belgium, France, Sweden, Canada and the United Kingdom, and following treatment with different application rates of phosphinothricin up to 40 l/ha[2]. Seed samples were generally analysed by external laboratories[3] to determine % humidity, % oil, % protein, glucosinolate content and composition, as well as fatty acid composition.

Due to the large amount of information provided, a representative set of data is presented in Table 6, which includes literature values for commercial non-transformed canola varieties. The profiling and quantification analyses clearly demonstrate that the 11 key fatty acid components are comparable in all of the oils tested from both a number of genetically modified canola varieties and a range of non-transformed control varieties. Variation across environmental conditions was greater than any variation between transformed and non-transformed canola plants.

[2] Within the hybrid canola program, standard selection level is determined at 5 l/ha.
[3] Laboratorium ECCA NV, Belgium; Plant Genetic Systems (PGS) Gent, Belgium; PGS, Canada; University of Guelph, Canada.

Table 6. **Minimum and maximum values of fatty acids (% of total) in canola oil (tested in Europe and North America in 1995).**

Entry	Oil composition (% of total)										
	C16:0 Palmitic acid	**C16:1 Palmitoleic acid**	**C18:0 Stearic acid**	**C18:1 Oleic acid**	**C18:2 Linoleic acid**	**C18:3 Linolenic acid**	**C20:0 Arachidic acid**	**C20:1 Gadoleic acid**	**C20:2 Eicosadienoic acid**	**C22:0 Behenic acid**	**C22:1 Erucic acid**
OSR literature	3 - 6	<0.5	1 - 3	50 - 66	18 - 28	6 - 14	<0.5	1	traces	<0.5	<1
Non-transgenic counterpart	3.9 – 5.2	0.0 - 0.4	1.6 - 2.1	60.8 - 68.4	16.3 -19.9	6.2 - 10.7	0.5 - 0.7	0.9 - 1.4	0.0 - 0.0	0.0 - 0.4	0.0 - 0.0
Ms8	3.9 - 4.8	0.3 - 0.4	1.5 - 1.8	60.1 - 67.6	16.4 -20.4	7.3 - 10.9	0.4 - 0.7	0.9 - 1.5	0.0 - 0.9	0.2 - 0.4	0.0 - 0.0
Rf3	3.9 - 5.1	0.3 - 0.4	1.5 - 1.7	58.2 - 67.4	17.4 -21.8	6.6 - 11.6	0.5 - 0.6	1.0 - 1.6	0.0 - 0.9	0.0 - 0.4	0.0 - 0.0
Ms8xRf3	3.9 - 4.5	0.2 - 0.3	1.6 - 1.8	60.9 – 67.4	17.4 -19.7	7.0 - 11.1	0.5 - 0.6	1.0 - 1.5	0.0 - 0.0	0.0 - 0.4	0.0 - 0.0
Other commercial control varieties	4.1 - 5.3	0.3 - 0.4	1.5 - 1.9	57.7 - 66.0	17.7 - 1.9	8.1 - 12.1	0.5 - 0.7	1.0 - 1.6	0.0 - 0.1	0.0 - 0.4	0.0 - 0.0
PGS hybrids based on Ms8 or Rf3	3.9 - 4.8	0.2 - 0.3	1.6 - 1.9	61.9 - 66.3	16.8 –19.2	7.9 - 10.6	0.3 - 0.7	1.0 - 1.5	0.0 - 0.3	0.0 - 0.4	0.0 - 0.0
PGS1 (Ms1xRf1)/ PGS2 (Ms1xRf2)	4.2 - 4.6	0.2 - 0.3	1.8 - 1.9	62.2 - 66.9	16.8 -17.8	7.4 - 10.5	0.5 - 0.7	1.1 - 1.9	0.0 - 0.0	0.0 - 0.3	0.0 - 0.0

Processing characteristics

Data were available on the detailed analytical evaluation of seeds, processed oil and meal from transformed and non-transformed (isogenic) canola plants extracted using benchtop processing designed to emulate commercial processing. The study was carried out by POS Pilot Plant Corporation (Canada) and was undertaken to compare the minor constituent composition of canola fractions at particular stages of processing (seed cleaning, seed tempering, flaking, cooking, pressing, solvent extraction, desolventising, blending, degumming, refining, washing, bleaching, hydrogenation and deodorisation) through to completion of the final product. During the study, the processing characteristics and sample-stage composition of the transformed material from the Ms1, Ms8, Rf1, Rf2, Rf3, T45 and Topas 19/2 lines were compared to the processing characteristics and sample-stage composition of non-transformed canola varieties presently grown.

The quality of the oil samples in this study was comprehensively analysed in terms of both compositional and physical parameters. The compositional parameters measured included fatty acid composition, free fatty acid content, phosphorus, sterol, chlorophyll and tocopherol levels. In addition, some physical properties exhibited by the oil were determined. These included specific gravity, viscosity, smoke point, and a cold test. Finally, the oxidative stability of the oil of the transformed and non-transformed samples was determined via a number of analytical tests (peroxide value, p-anisidine value, AOM) carried out at different stages of the refining process.

The results of the processing analyses do not show any significant differences between the transformed canola seeds containing the male sterility and fertility restorer gene constructs (Ms1, Ms8, Rf1, Rf2, Rf3), T45 and Topas19/2 and non-transformed canola, in any of the parameters tested. The processing characteristics and the quality of the oil derived from the transformed seed and control seed were essentially identical throughout the processing stages. Furthermore, there were no compositional differences between the transformed and non-transformed samples and all of the seedlots produced measurements that were within a typical range for canola oil.

Canola meal is a by-product of processing containing the seed proteins. Although it is not generally consumed by humans, the amounts of fibre, minerals and glucosinolates in the meal are nutritionally important in animal feed. In this case, these components also serve as additional biochemical indicators of any compositional differences brought about in the seed due to the genetic modification. As for most other commodity crops, the nutrient composition of canola seeds is known to vary considerably depending on environmental conditions and genetic factors and certain fluctuations in composition are considered to be normal. A detailed comparison of the components in seed meal harvested from transformed and non-transformed hybrid canola was subsequently conducted on material obtained during the simulated industrial processing.

The analyses were sufficiently detailed to measure a number of individual glucosinolates in the whole seed (alkenyls, indols) and in the desolventised meal. The results of these analyses indicate that the protein and glucosinolate content of the

transformed canola and/or the meal from lines Ms1, Ms8, Rf1, Rf2, Rf3, T45 and Topas19/2, were completely within the ranges observed for non-transformed canola varieties.

Proximate analysis following herbicide treatment

A study was conducted to directly compare the composition of seed derived from the open pollinated T45 line, untreated and treated with the herbicide glufosinate-ammonium. The plants were grown under normal agricultural conditions in field trials at two locations in Western Canada. Half of each plot was untreated and the remaining half was treated with Liberty® at a rate of 500 g active ingredient/ha, applied prior to bolting.

At harvest, a minimum of two 500 gram samples of canola seeds were taken from each treatment plot. In all cases, the untreated plots were sampled first, prior to sampling of the treated plots.

POS Pilot Plant Corporation was responsible for conducting a proximate analysis on the canola seed samples. The proximate analysis included moisture, oil, protein, ash and crude fibre expressed as a percentage of the seed. The analytical methods used were published, validated methods of the American Oil Chemists Society (5th Edition, 1998), and all results were statistically analysed. A summary of the results of these analyses is presented in Table 7, which represents the mean of 6 measurements for each treatment.

Table 7: Summary of Proximate Analysis on Canola Seed Comparing T45 Treated with T45 Untreated with Liberty®. Data from all sites combined.

VARIABLE	MEAN & STD T45 TREATED	MEAN & STD T45 UNTREATED	P-VALUE (T45 UNTREATED VS T45 TREATED)
% Moisture	5.09 ± 0.18	5.04 ± 0.19	0.646
% Oil	46.00 ± 1.50	46.80 ± 2.37	0.504
% Protein	22.54 ± 1.70	22.31 ± 2.28	0.851
% Ash	3.76 ± 0.15	3.59 ± 0.19	0.104
% Crude Fibre	10.99 ± 0.46	11.00 ± 0.29	0.953

The results demonstrate that there were no significant differences ($p>>0.05$) between the T45 canola seed samples from the untreated or treated plots for any of the proximate variables examined. In addition, the measured levels of protein and oil in both sets of seeds are consistent with similar proximate analyses for other canola varieties, including non-transformed varieties.

Key toxicants

Seeds from the original native oilseed rape plants naturally contain high levels of two toxins, erucic acid and glucosinolates, and prior to the mid 1950s, the extracted oil was used primarily for industrial purposes. Erucic acid, a long chain fatty acid, is a natural constituent of the seed oil, while glucosinolates are confined to the seed meal with the majority of the protein and other seed components.

In the early 1970s, the presence of erucic acid in rapeseed oil was reported to be associated with fat accumulation in the heart muscle of laboratory rats, resulting in cardiopathogenic effects. Glucosinolates were found to cause thymus enlargement and limited the nutritional value of the meal as feed for livestock.

In response to these findings, and subsequent detailed nutritional studies on erucic acid-free rapeseed oil, plant breeders systematically replaced the seedstock with varieties that were selected for a low erucic acid content (below 2%). As a result of this deliberate plant breeding program, the present cultivars, now referred to as *canola*, are low in both erucic acid and glucosinolates and are used extensively for use as animal feed and for the production of vegetable oil for human consumption.

Canola is therefore defined as seed, oil and meal specifically from *B. napus* or *B. rapa* cultivars, that must meet specific quality standards in relation to low levels of erucic acid and glucosinolates. These so-called double nought (00) varieties contain less than 2% of the total fatty acids as erucic acid, and less than 30 micromoles of aliphatic glucosinolates per gram of oil-free meal (Codex 1993, 1999; Downey, 1995). Only oil meeting these specifications, whether derived from a conventional or GM crop, is processed and permitted for use in the food industry.

Data in relation to the content of these naturally occurring toxins present in canola seeds (seed meal and the oil) were submitted. Although data were presented in relation to the meal, the focus of the assessment was the oil component due to its significant use as a food. The genetic modification in this case does not change the usual pattern of consumption.

Erucic acid

Erucic acid is a mono-unsaturated 22 carbon fatty acid (C22:1). Due to its previously described adverse effects in animal studies, the applicant has provided detailed fatty acid analyses of the seeds from the transformed plants, noting in particular the erucic acid content.

A comprehensive analysis of the oil derived from the canola seeds is presented in the comparative analyses. Detailed analysis of the individual fatty acids showed that the level of erucic acid in the transformed lines Ms8 and Rf3, and the hybrid cross between these two lines, was equivalent to the commercial control varieties and the non-transformed counterpart (none detected in all lines tested). Furthermore, data on the fatty acid profile of several transformed lines, including the open pollinated Topas 19/2 line and Ms and Rf lines, showed that the levels of erucic acid were not above 1% and were generally less than 0.1%. These values were observed over different years of growth (1991-1995) in a number of different locations.

Because of considerable seasonal and locality variation, the transformed lines were compared to a significant number of non-transformed control varieties. The data indicate that the percentage of erucic acid in the transformed lines was always within the same narrow range as the control varieties, and that all lines tested (including non-transformed) were below the reported literature value for canola oil (below 1%).

Glucosinolates

Data were presented on the measured levels of glucosinolates in seeds and meal from transgenic lines T45, Topas 19/2, Ms1, Ms8, Rf1 and Rf3, together with a range of non-transformed varieties (at least fifteen control lines) when grown at locations in Canada, Belgium, Sweden and France over a number of seasons between 1991 and 1996. In addition, some lines were tested following spraying with glufosinate-ammonium at variable rates from 2.5L/ha to 10L/ha (data not presented in this report).

The data show no differences in the level of glucosinolates in any of the transformed lines when compared to the control varieties. These results support the conclusion that neither the presence of the introduced genes, nor the spraying of glufosinate-ammonium herbicide affected the levels of glucosinolates in the seed or meal of the transformed plants. The variation was greater between locations than between transformed and non-transformed lines.

As the meal is used predominantly as animal feed, these data mainly serve to illustrate that there were no unexpected changes in the level of glucosinolates in the seeds of the genetically modified canola, when compared to a large number of commercial control varieties and the non-transformed counterpart.

Key anti-nutrients

Consideration has been given to the use of canola meal in human nutrition as a source of food-grade protein. However, this has not been widely adopted so far due to the presence of components such as phytic acid and phenolic compounds. These compounds may not only add an astringent taste and flavour to the meal, but may also reduce the bioavailability of several minerals. In addition, the presence of glucosinolates has an effect on the quantity of digestible protein. As a consequence, broader use of canola meal as a food product for human consumption depends on improving the digestible utilization of the nutrients and limiting or destroying the anti-nutritional factors. The genetic modifications used in the Ms, Rf and open pollinated lines Topas 19/2 and T45 in this case do not alter the food uses of the seeds.

There are no compounds present in canola oil that are known to exhibit anti-nutritional properties.

NUTRITIONAL IMPACT

In assessing the safety of a genetically modified food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the

bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

Animal feeding studies

Animal feeding studies using the oil have not been conducted. The nutritional profile of the oil was determined by compositional analyses of the major components of the seed and these were found to be comparable to the conventional control lines. In addition, the level of dietary exposure to the novel proteins is expected to be zero, as all contaminating plant protein is removed in the production of canola oil.
Where the human food in question is an oil, animal feeding studies are generally not considered feasible as the oil itself is unsuitable as a complete food for animals and may cause nutritional and biochemical imbalances if included in the diet in large quantities. Instead, in this application the applicant has provided two animal feeding studies using whole seed in support of the nutritional adequacy of particular glufosinate-ammonium tolerant lines.

Feeding study in chickens

Leeson, S. (1999). The Effect of Glufosinate Resistant Canola (Topas 19/2) on the Appearance and Growth of Male Broiler Chickens. AgrEvo report No. B002184.

As whole canola seeds can be utilised as a major component in the diet of broiler chickens, a study was conducted to compare the performance of broiler chickens fed glufosinate-ammonium tolerant canola (Topas19/2) with a standard commercially available canola cultivar. The applicant claims that these animals represent a very sensitive test species for a nutrient feeding study as a 15 fold increase in body weight occurs during the first 18 days of life and therefore any differences in nutrient availability are readily detectable in terms of the development of the chickens.

The study involved the use of 280 commercial strain male broiler chickens obtained at one day of age. The birds were weighed and allocated at random to 1 or 2 treatment groups, replicated 4 times, with 35 birds per replicate. The birds were maintained at temperatures and in environments that were consistent with normal brooding practice. They were cared for by agriculture assistants at the Arkell Poultry Research Station and according to required guidelines of the Canadian Council on Animal Care and with the approval of the University of Guelph Animal Care Committee, Animal Utilisation Protocol #96R072.

Birds were fed starter diets to 18 days of age at which time feed intake was measured and all birds were weighed individually. Grower diets were fed between 18 and 32 days, feed intake measured as before and all birds were again weighed individually. The finisher diets were fed between 32 and 42 days of age and the same protocol was followed. During the course of the experiment, which reared the birds on one of two diet treatments and varied only with the type of canola used in each diet, all occurrences of mortality were submitted to the Ontario Veterinary College, Department of Pathology for post-mortem examination.

The variables considered were initial body weight, 18, 32 and 42 day body weight, body weight gain in the different diet periods, feed intake and feed intake:body weight

gain. The mortality rate was monitored and at the end of the study, various carcass characteristics were considered namely, chilled carcass weight and yield of deboned breast meat as a percent of carcass weight. For the statistical analysis, significance was accepted at $P<0.05$.

The results of this study showed that the source of the canola in the 3 diet types had no effect on body weight, feed intake, feed intake:body weight gain or percent mortality over the experimental period ($P>0.05$). The mortality rate was normal for this fast-growing strain of bird, where 5-8% is routinely expected. In all measured parameters, the birds were unaffected by the substitution of the genetically modified canola for the conventional form in the experimental diets.

Digestibility study in rabbits

Study submitted:
Maertens, L. and Van Eeckhoutte, A. (1993). Digestibility of Transformed Oilseed Rape for Rabbits, Government Agricultural Research Centre, Belgium.

A study was conducted in rabbits to investigate the nutritive value of transformed canola compared to the control line, Drakkar, also used in the compositional studies. Drakkar is the elite variety that was used to generate the hybrid parental transformed lines and is a double low variety, containing little erucic acid and low glucosinolates (<15 μmoles/g). The hybrid line tested in this study was a cross between the Ms1 and Rf1 parental lines, and represents plants that are direct sources of canola oil for human consumption, rather than the parental lines themselves used in the hybrid breeding program.

Seed from the original variety (Drakkar) and the Ms1/Rf1 cross were offered to growing rabbits in order to study the digestibility of protein, fat, crude fibre and to compare bioavailable gross energy. Thirty 7-week old rabbits of both sexes were randomly assigned (10 animals per diet) to either a basal diet containing no canola, or to one of two experimental diets containing either transformed canola or unmodified control canola seed to a level of 30% in the basal diet.

It was noted in the study that due to the high fat content of canola seeds, the experimental diets were both very fat-rich (>16%) and, as a result, the quality of the feeding pellets was poor. In order to avoid deblending of the feed, the experimental diets were pelleted several times until satisfactory pellet quality was obtained comparable to the basal diet. In addition, a preliminary adaptation period of one week was allowed with the diets before measurements were commenced. This was necessary to overcome differences in palatability noted with the experimental diets containing both the control and transformed canola seeds.

The rabbits were fed *ad libitum* and fecal output was measured and recorded daily for the duration of the 4 day study. The individual fecal samples were analysed for dry matter, ash, nitrogen, fat and crude fibre following AOAC methods (Association of Official Analytical Chemists, 1990). In addition, gross energy was measured by an adiabatic bomb calorimeter. Apparent whole tract digestibility coefficients (DC) and

digestible energy (DE) content of each diet were calculated from the respective dry matter intake and output, as well as their corresponding nutrient content.

Results and conclusion

Due to the high digestibility of both experimental canola seed diets, the DC was significantly higher than the basal diet ($p<0.01$). Furthermore, as both test diets containing the canola had higher energy content than the basal diet, some measurements were significantly higher for both test diets compared with the basal diet. For example, despite the allowed period of adaptation, the inclusion of 30% canola seed to the basal diet resulted in negative effects on the feed intake of the animals during the first days of the study. However, these effects diminished with time and the intake of feed was sufficient for the duration of the experiment, taking account of the increased dietary DE content of both experimental diets.

Of greater importance, the results demonstrate that there were no observed differences between the two experimental diets containing canola seeds, either transformed or non-transformed, indicating that the feeding value of the hybrid line (derived from transformed parental lines) is comparable to the original control variety. The conclusion therefore is that the seeds from the hybrid line (produced by a conventional cross between the Ms1 and Rf1 transformed lines) exhibited at least similar zootechnical performance as seeds from the original Drakkar variety.

CONCLUSION

The comparative analyses indicate that the genetic modifications in the transformed canola lines T45, Topas 19/2, Ms1, Ms8, Rf1, Rf2 and Rf3 have not produced any significant changes to the seeds with respect to processing characteristics, oil content, oil composition, oil quality (physical properties), protein content or glucosinolate content. The edible canola oil fraction derived from the transformed seeds is therefore indistinguishable from the oil fraction derived from unmodified seeds, when grown at a variety of locations representing different environments, and following agricultural usage of the herbicide glufosinate-ammonium. On the basis of the submitted data, canola oil from the open pollinated lines T45 and Topas 19/2 and the pollination control lines Ms1, Ms8, Rf1, Rf2 and Rf3 (and crosses) is considered substantially equivalent to the oil from non-transformed canola.

The extensive compositional data are supported by the two feeding studies which confirm that the introduced genes have not resulted in significant changes to the nutritional adequacy of the seeds derived from the transformed lines. Separate studies using the test animal species rabbits and chickens, showed that a diet containing the transformed canola seeds provided equivalent nutrition to control diets and adequately supported the growth of young animals. These studies do not raise any public health or safety concerns with respect to the overall nutritional characteristics of the oil derived from the transformed canola.

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