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Plant Health and Production Division,
Plant Biosafety Office

Decision Document 96-17: Determination of Environmental Safety of Plant Genetic Systems Inc.'s (PGS) Novel Hybridization System for Rapeseed (*Brassica napus* L.)

This Decision Document has been prepared to explain the regulatory decision reached under the guidelines [Dir94-08 Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits](#) and its companion document [Dir94-09 The Biology of *Brassica napus* L. \(Canola/Rapeseed\)](#) and the guidelines [Dir95-03 Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits](#).

Agriculture and Agri-Food Canada (AAFC), specifically the Plant Biotechnology Office of the Plant Health and Production Division, with input from the Plant Health Risk Assessment Unit, has evaluated information submitted by Plant Genetic Systems Inc. (PGS). This information is in regard to a rapeseed hybridization system comprising two transgenic parental lines, MS8 and RF3, and their hybrid MS8 x RF3. AAFC has determined that these plants with novel traits do not present altered environmental interactions or pose concerns for the safety of livestock consuming feed derived from the PNT when compared to currently commercialized rapeseed varieties in Canada.

Unconfined release into the environment and use as livestock feed of MS8, RF3 and MS8xRF3 is therefore authorized. Any other *B. napus* lines and intra-specific hybrids resulting from the same transformation events, and all their descendants, may also be released, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known following thorough characterization, that these plants do not display any additional novel traits and provided that the resulting lines can be shown to be substantially equivalent to currently grown rapeseed, in terms of their potential environmental impact and livestock safety.

Please note that, while determining the environmental and livestock feed safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as the evaluation of food safety (Health Canada) and Variety Registration (AAFC).

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I. Brief Identification of Plants with Novel Traits (PNT's)

<i>Designation(s) of the PNT:</i>	<i>Male sterile line:</i> MS8 (DBN230-0028) <i>Fertility restorer:</i> RF3 (DBN212-0005) <i>Hybrid line:</i> MS8 x RF3
<i>Applicant:</i>	Plant Genetic Systems (Canada) Inc. (PGS)
<i>Plant Species:</i>	<i>Brassica napus L.</i>
<i>Novel Traits:</i>	MS8: male sterility; glufosinate ammonium (herbicide) tolerance RF3: fertility restoration; glufosinate ammonium (herbicide) tolerance
<i>Trait Introduction Method:</i>	<i>Agrobacterium tumefaciens</i> -mediated transformation
<i>Proposed Use of PNT's:</i>	Production of <i>B. napus</i> for seed oil for human consumption and seed oil and meal for livestock feed. These materials will not be grown outside the normal production area for canola in Canada.

II. Background Information

Plant Genetic Systems Inc. has developed a novel *B. napus* oilseed rape hybridization system. This system, derived from the *B. napus* variety "Drakkar," involves the use of two parental lines. The first parental line (MS8) is male sterile; does not produce viable pollen grains; and cannot self-pollinate. The second parental line (RF3) codes for specific restoration of the male sterility coded by the first parental line. When the two lines are crossed, the progeny is one hundred per cent true hybrid, and since fertility is restored, the hybrid plants are fully fertile and produce seed. These lines are similar to the lines MS1 and RF1 authorized for unconfined release by AAFC on April 28 1995, as explained in Decision Document [DD95-04](#).

To date, attempts to develop hybridization systems in oilseed rape by traditional methods have not been completely successful for commercial applications. Potential benefits are that F1 hybrids of oilseed rape are estimated to potentially yield 20-25% more than open-pollinated varieties, and their uniformity facilitates harvesting and marketing.

The development of the MS8 and RF3 lines was based on recombinant DNA technology by the introduction of bacterial genes into the *B. napus* variety "Drakkar." Nuclear male sterility of the first

parental line results from the localized production of an RNase (*barnase*) in a specific anther cell layer, and at a specific stage in anther development. Fertility restoration in the hybrid line is obtained through insertion, in the second parental line of a gene coding for *barstar*, a specific inhibitor of the enzyme *barnase*. A gene conferring tolerance to the herbicide glufosinate ammonium was inserted in both lines, coding for phosphinothricin acetyltransferase, an enzyme that inactivates glufosinate ammonium through acetylation. Herbicide tolerance was introduced as a field selection trait to obtain 100% hybrid seed.

These materials have been field tested in Canada under confined conditions in Saskatchewan (1994-96), Alberta (1996), Manitoba (1995, 96) and Ontario (1996).

PGS has submitted data to AAFC on the identity of each of MS8, RF3 and MS8xRF3: detailed descriptions of the modification method, data and information on the inserted DNA and the gene insertion site, the role of the inserted genes in donor organisms, the role of regulatory sequences in donor organisms, their molecular characterization and full nucleotide sequences.

The novel proteins were identified and characterized, including their potential toxicity to non-target organisms, potential for allergenicity, and levels of expression in the plant. A number of relevant scientific publications were referenced.

Agronomic characteristics such as seed dormancy, vegetative vigour, seed production, time to maturity, flowering period, male and female fertility, and disease and insect susceptibilities were compared to those of unmodified *B. napus* counterparts.

The Plant Biotechnology Office of the Plant Health and Production Division, AAFC, has reviewed the above information, in light of the assessment criteria for determining environmental safety of plants with novel traits, as described in the regulatory directive [Dir94-08](#):

- potential of the PNT's to become weeds of agriculture or be invasive of natural habitats,
- potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential for the PNT's to become plant pests,
- potential impact of the PNT's or their gene products on non-target species, including humans, and
- potential impact on biodiversity.

The feed section of the Plant Health and Production Division, AAFC, has also reviewed the information submitted by PGS in light of the assessment criteria for determining safety and efficacy of livestock feed, as described in the regulatory directive [Dir95-03 Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits](#):

- potential impact on livestock, and
- potential impact on livestock nutrition.

III. Description of the Novel Traits

1. Nuclear Male Sterility

- The male sterility gene encodes the barnase ribonuclease (RNase). Male sterility is caused by the production of this enzyme at a specific stage during anther development in the tapetum cell layer of the anther. The RNase affects RNA production, disrupting normal cell functioning and arresting early anther development.
- The gene is linked to an anther-specific promoter, and the enzyme was detected only in early stages of development of the tapetum cell layer of anthers. It was not detected in other plant tissues.
- The *barnase* gene was isolated from *Bacillus amyloliquefaciens*, a common soil bacterium frequently used as a source for industrial enzymes. The enzyme is therefore naturally occurring in the soil. More generally, ribonucleases are very commonly found in various organisms including bacteria and plants.

- The full nucleotide sequence of the gene was provided. *Barnase* is a small single-domain protein, containing no disulfide bonds, metalion cofactors or other non-peptide components. It unfolds completely into an inactive form when heated. When subjected to comparative analyses using a database of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the database other than bacilli ribonucleases. No resemblance with potential toxins or allergens was observed.
- The gene and its associated regulatory sequences are the same as those of the line MS1 that was authorized for unconfined release by AAFC on April 28, 1995 (please see [DD95-04](#)).

2. Fertility Restoration

- The fertility restoration gene codes for the *barstar* enzyme. This enzyme is a ribonuclease inhibitor and specifically inhibits the barnase RNase. Barnase and its inhibitor barstar form a one-to-one complex, in which the RNase is inactivated. The barnase-barstar complex is very stable in the absence of a denaturant and the inhibition is very specific.
- The *barstar* gene was isolated from *Bacillus amyloliquefaciens*, a common soil bacterium frequently used as a source for industrial enzymes. The enzyme is therefore naturally occurring in the soil. More generally, ribonuclease inhibitors are very commonly found in various organisms including bacteria and plants.
- The gene is linked to an anther-specific promoter, and the enzyme is only produced at a specific stage during anther development in the tapetum cell layer of the anther. It was not detected in other plant tissues.
- The full nucleotide sequence of the gene was provided. *Barstar* is a small single-domain protein that unfolds completely into an inactive form when heated. When subjected to comparative analyses using the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the databases. No resemblance with potential toxins or allergens was observed.
- The gene and its associated regulatory sequences are identical to those of the line RF1 that was authorized for unconfined release by AAFC on April 28, 1995 (please see [DD95-04](#)).

3. Glufosinate Ammonium Tolerance

- Phosphinothricin (PPT), the active ingredient of glufosinate ammonium, inhibits glutamine synthetase, which results in the accumulation of lethal levels of ammonia in susceptible plants within hours of application.
- The phosphinothricin tolerance gene engineered into MS8 and RF3 codes for PPT-acetyltransferase (PAT). This enzyme detoxifies phosphinothricin by acetylation into an inactive compound. PAT has extremely high substrate specificity for L-PPT and dimethylphosphinothricin (DMTT), but cannot acetylate L-PPTs analog L-glutamic acid, D-PPT, nor any protein amino acid. Expression levels of PAT varied from 0.04 mg/g (f.w.) of protein in seeds, to 1.80 mg/g (f.w.) of protein in leaves.
- The gene was isolated from *Streptomyces hygroscopicus*, an aerobic soil actinomycete. The PAT enzyme is therefore naturally occurring in the soil. More generally, acetyltransferases are ubiquitous in nature.
- A plant derived coding sequence expressing a chloroplast transit peptide was co-introduced with the gene. This peptide facilitates the import of the newly translated enzyme into chloroplasts. The PAT enzyme was detected in leaves, but not in flower buds or seeds.
- The nucleotide sequence of the gene was provided. When subjected to comparative analyses using the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the databases, except with other phosphinothricin acetyltransferases originating from different organisms. No resemblance with potential toxins or allergens was observed.
- The gene and its associated regulatory sequences were identical to those of the lines MS1 and RF1 that were authorized for unconfined release by AAFC on April 28, 1995 (please see [DD95-04](#)).

4. Development Method

- *Brassica napus* cultivar "Drakkar" was transformed using a disabled non-pathogenic *Agrobacterium tumefaciens* vector. The vector contained the transfer DNA (T-DNA) region of an *Agrobacterium* plasmid from which disease-causing genes were removed and replaced with the genes of interest. The T-DNA portion of the plasmid is known to insert randomly into the plant's genome and the insertion is usually stable, as was shown to be the case in MS8 and RF3.

5. Stable Integration into the Plants' Genomes

- The data provided clearly showed that there was no incorporation of any coding region from outside the T-DNA borders and that only one copy was integrated at a single insertion site.
- The insertion site was very well characterized and determined to be located in the *B. oleracea* portion of the amphidiploid *B. rapa/B. oleracea* genome of *B. napus* for RF3, and in the *B. rapa* genome of *B. napus* for MS8.
- Segregation was predictable over all generations observed and showed that transformation resulted in integration at one single dominant locus.
- Comparisons between the original transformants and derived lines several generations away from these transformants show no difference in the presence and expression of the genes nor in the insertion site.

IV. Assessment Criteria for Environmental Safety

1. Potential of the PNT's to Become Weeds of Agriculture or be Invasive of Natural Habitats

AAFC has evaluated data submitted by PGS on the reproductive and survival biology of MS8, RF3 and resulting hybrids. It was determined that germination, vegetative vigour, flowering period, time to maturity and seed production of both transgenic lines were within the normal range of expression of characteristics in unmodified *B. napus* counterparts. These lines have no specific added genes for cold tolerance or winter survival. Flowers of the MS8 line have undeveloped anthers, slightly smaller petals and do not produce fertile pollen, but nectar production remains unchanged and normal insect pollination was observed. Seed morphology, size, and average seed weight did not change, indicating that seed dispersal potential was not altered.

Based on the submitted information, AAFC has determined that MS8, RF3 and MS8xRF3 did not show any change in resistance or susceptibility to major *B. napus* pests and pathogens (e.g., blackleg, sclerotinia, flea beetles, diamondback moth larvae). The lines were tested in several countries, and showed no differences in agronomic performance when compared to unmodified counterparts under the same conditions.

The biology of *B. napus*, described in [Dir94-09](#), shows that unmodified plants of this species are not invasive of unmanaged habitats in Canada. Information provided by PGS shows that MS8, RF3 and their hybrids were not different from their counterparts in this respect. Published data showed that seed survival of similar transgenic *B. napus* seeds expressing kanamycin resistance and glufosinate ammonium tolerance was significantly lower than seed survival of unmodified counterparts, when seeded at a variety of unmanaged locations. Glufosinate ammonium is not used in normal crop rotation cycles, and resistance is therefore not an issue of concern in weed management control. Glufosinate ammonium resistant *B. napus* volunteer plants can easily be managed by mechanical means and other available chemicals used to control *B. napus*.

The above considerations, together with the fact that the novel traits have no intended effect on weediness or invasiveness, led AAFC to conclude that MS8, RF3 and their hybrid progeny have no altered weed or invasiveness potential compared to currently commercialized *B. napus* varieties.

Note: A longer term concern, if there is general adoption of several different crop and specific herbicide weed management systems, is the potential development of crop volunteers with a combination of novel resistances to different herbicides. This could result in the loss of the use of these herbicides and any of their potential benefits. Therefore, agricultural extension personnel, in both the

private and public sectors, should promote careful management practices for growers who use these herbicide tolerant crops to minimize the development of multiple resistance.

2. Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive

The MS8 line is male sterile and will therefore not pollinate any other plants. Although these plants can act as pollen recipients, their progeny will also be male sterile and will not produce pollen. The RF3 and hybrid plants displayed normal reproductive characteristics. *Brassica napus* plants are known to outcross up to 30% with other plants of the same species, and potentially with plants of the species *B. rapa*, *B. juncea*, *B. carinata*, *B. nigra*, *Diplotaxis muralis*, *Raphanus raphanistrum*, and *Erucastrum gallicum* (Dir 94-09). Studies show that gene flow is most likely to occur with *B. rapa*, the other major canola species, and an occasional weed of cultivated land especially in the eastern provinces of Canada.

The genes coding for male sterility and fertility restoration do not confer any ecological advantage to potential hybrid offspring of MS8 or RF3 plants. If glufosinate ammonium tolerant individuals arose through interspecific or intergeneric hybridization, the novel traits would confer no competitive advantage to these plants unless challenged by glufosinate ammonium. This would only occur in managed ecosystems where glufosinate ammonium is used for broad spectrum weed control, e.g., in the cultivation of plant cultivars developed to exhibit glufosinate ammonium tolerance and in which glufosinate ammonium is used to control weeds. As with glufosinate ammonium tolerant *B. napus*, these herbicide-tolerant individuals, should they arise, would be easily controlled using mechanical and other available chemical means. Hybrids, if they developed, could potentially result in the loss of glufosinate ammonium as a tool to control these species. This, however, can be minimized by the use of sound crop management practices.

The above considerations led AAFC to conclude that gene flow from the transgenic lines or their hybrids to canola relatives is possible, but would not result in increased weediness or invasiveness of these relatives.

3. Altered Plant Pest Potential

The intended effects of the novel traits are unrelated to plant pest potential and *Brassica napus* is not a plant pest in Canada (Dir94-09). In addition, agronomic characteristics, insect and disease susceptibilities, and qualitative and quantitative composition of MS8, RF3 and MS8xRF3 were shown to be within the range of values displayed by currently commercialized *B. napus* varieties, leading to the conclusion that plant pest potential was not inadvertently altered. AAFC therefore concurs with the conclusion that plant pest potential of these plants has not been inadvertently altered.

4. Potential Impact on Non-Target Organisms

The detailed characterization of each novel gene and resulting protein, as summarized in Part III of the present document, led to the conclusion that they do not result in altered toxicity or allergenicity properties. The barnase and barstar proteins are only produced in the tapetum cell layer of anthers at a specific developmental stage. Potential toxicity of these proteins was previously evaluated (please see [DD95-04](#)).

Based on the above, AAFC has determined that the unconfined release of the MS8, RF3 and their hybrid progeny will not result in altered impacts on interacting organisms, including humans, compared with currently commercialized counterparts.

5. Potential Impact on Biodiversity

The transgenic lines and their hybrids have no novel phenotypic characteristics which would extend their use beyond the current geographic range of canola/rapeseed production in Canada. Since

potential outcross species are only found in disturbed habitats, transfer of novel traits would not have an impact on unmanaged environments.

AAFC has therefore concluded that the potential impact on biodiversity of MS8, RF3 and derived hybrids is equivalent to that of currently commercialized rapeseed lines.

V. Assessment Criteria for Use as Livestock Feed

1. Anti-nutritional Factors

Glucosinolate and erucic acid content of seed meal and oil of the transformed and hybrid lines was determined at several locations, representing a variety of conditions. The analysis revealed no differences in glucosinolate content in the meal, between the PNT's and the corresponding non-transformed line, at all sites. The reported values were also within the acceptable range for conventional canola, except at one location in Canada, where levels were elevated in both the PNT's and the corresponding control, as a result of drought induced stress. Erucic acid content of the oil was substantially equivalent to the non-transformed controls and within the acceptable range for conventional canola.

2. Nutritional Composition of PNT's

No statistical differences in the nutritional composition, i.e., crude protein, crude fat, crude fibre, ash and gross energy content, were noted between the whole seed, processed meal or oil, derived from MS8, RF3 and their resulting hybrids, when compared to the non-transformed controls or conventional canola cultivars. These results collectively demonstrate that the introduction of the novel traits into these lines and their presence in the resulting hybrids did not affect the composition or nutritional quality of the canola cultivar. Accordingly, MS8, RF3 and their hybrids are judged to be substantially equivalent to conventional canola varieties.

VI. Regulatory Decision

Based on the review of data and information submitted by PGS, and through comparisons of the transgenic lines with unmodified *B. napus* counterparts, AAFC has concluded that neither the novel genes nor their resulting gene products and associated novel traits confer any intended or unintended ecological advantage to either MS8, RF3 or MS8xRF3. Should these traits be transferred through outcrossing to related plants, these also would result in no ecological advantage.

Based on the review of data submitted to the Feed Section of the Plant Health and Production Division, AAFC concludes that the novel genes introduced into lines MS8 and RF3 and their corresponding traits do not raise any concerns regarding livestock safety or the nutritional composition of this line. Canola oil and meal are currently listed in Schedule IV of the *Feeds Regulations* and are, therefore, approved for use in livestock feeds in Canada. As lines RF3, MS8 and their resulting hybrid have been assessed and found to be substantially equivalent to traditional canola varieties, these lines and their byproducts are considered to meet the present feed definitions and are approved for use as livestock feed ingredients in Canada.

If at any time, PGS becomes aware of any information regarding risk to the environment, or risk to animal or human health, that could result from release of these materials in Canada, or elsewhere, PGS must immediately provide such information to AAFC. On the basis of such new information, AAFC may re-evaluate the potential impact of the release and re-evaluate its decision.

Unconfined release into the environment and feed use of MS8, RF3 and MS8xRF3 is therefore authorized. Any other *B. napus* lines and intra-specific hybrids resulting from the same transformation events, and all their descendants, may also be released, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known that these plants do not display any additional novel traits and provided that the resulting lines can

be shown to be substantially equivalent to currently grown rapeseed, in terms of their potential environmental impact and livestock feed safety.

Please note that, while determining the environmental and livestock feed safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as for the evaluation of food safety (Health Canada) and Variety Registration (AAFC).

This bulletin is published by the Plant Health and Production Division, Canadian Food Inspection Agency. For further information, please contact the Plant Biosafety Office at:

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