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Plant Health and Production Division,
Plant Biosafety Office

Decision Document DD95-04: Determination of Environmental Safety of Plant Genetic Systems Inc. (PGS) Novel Hybridization System for Canola (*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 proposed guidelines [Pro94-04 Guidelines for the Assessment of Plants with Novel Traits as Livestock Feed](#).

Agriculture and Agri-Food Canada (AAFC), specifically the Plant Biotechnology Office and the Feed Section of the Plant Health and Production Division, has evaluated information submitted by Plant Genetic Systems Inc. (PGS). This information is in regard to a novel canola hybridization system comprising two transgenic parental lines, MS1 and RF1, and their hybrid MS1x RF1. AAFC has determined that those plants with novel traits do not present altered environmental interactions when compared to currently commercialized canola varieties in Canada and are considered substantially equivalent to canola currently approved as livestock feed.

Unconfined release into the environment of MS1, RF1, MS1xRF1, and other *B. napus* lines derived from them, but without the introduction of any other novel traits, is therefore considered safe.

Feed use of varieties produced using this hybridization system is approved provided that the feed ingredients produced conform to the *Feeds Regulations*.

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The development of the MS1 and RF1 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. Another gene, conferring resistance to kanamycin, was also inserted. This gene is of no agronomic interest but was used to select modified plants from those that remained unmodified in the development stage.

These materials have been field tested in Canada under confined conditions in Saskatchewan (1992-94), Alberta (1992, 94), Manitoba (1992, 94) and Ontario (1991-94).

PGS has submitted data to AAFC on the identity of each of MS1, RF1 and MS1xRF1: detailed descriptions of the modification methods, data and information on the stability of the gene insertions, 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 livestock and non-target organisms, potential for allergenicity, and levels of expression in the plant. A number of detailed scientific publications providing further data were also supplied.

Agronomic characteristics such as seed production, time to maturity, flowering period and male and female fertility were compared to those of unmodified *B. napus* counterparts. PGS has also provided data and information on their materials survival adaptations: siliques shattering potential, seed dormancy, seed dispersal mechanisms, vegetative vigor, reproductive characteristics, and overwintering capacity. Stress adaptation was evaluated, including susceptibilities to various *B. napus* pests and pathogens, to abiotic stresses such as temperature, drought and soil moisture and to herbicides other than glufosinate ammonium that are normally used on canola crops. Invasiveness and competition studies under unmanaged and arable environments were discussed.

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 above information in light of the assessment criteria for determining safety and efficacy of livestock feed, as described in Pro94-04:

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

III. Description of the Novel Traits

1. Nuclear Male Sterility

- The male sterility gene codes for 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 only detected in the tapetum cell layer of anthers, only in early stages of their development. It was not detected in other plant tissues.
- 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 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, other than with ribonucleases from other bacilli. No resemblance with potential toxins or allergens was observed.
- 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.

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 production of the enzyme only occurs at a specific stage during anther development in the tapetum cell layer of the anther.
- 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.

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 MS1 and RF1 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.
- The gene was isolated from *Streptomyces hygrosopicus*, 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.

4. Kanamycin Resistance

- Kanamycin is an aminoglycosidic antibiotic that binds to bacterial ribosomes thus disrupting normal protein synthesis and killing the bacterial cell.
- The kanamycin resistance gene codes for the enzyme neomycin phosphotransferase (NPTII) that prevents kanamycin from binding to ribosomes, thereby rendering the cells resistant. The gene was isolated from *Escherichia coli*, a facultative anaerobic member of the Enterobacteriaceae usually found in the lower part of the intestinal tract of warm blooded animals. The full nucleotide sequence of the gene was provided.
- The gene is linked to a weak constitutive promoter. The enzyme was not detected in unprocessed honey or pollen samples.
- The expressed enzyme was compared to the bacterial protein: molecular weights were similar, indicating that the protein had not been glycosylated nor had it undergone post-transcriptional modifications.
- The nucleotide sequence showed no significant homology with any known toxins or allergens. 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, other than with other peptides that have a similar function (aminoglycoside 3'-phosphotransferases, kanamycin kinases, streptomycin 3'-kinases). No resemblance with potential toxins or allergens was observed.

5. Development Method

- *Brassica napus* cultivar Drakkar[®] was transformed using a disarmed non-pathogenic *Agrobacterium tumefaciens* vector. The vector contained the 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 MS1 and RF1.

6. Stable Integration into the Plant's 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.
- Deletion studies at the insertion site showed no evidence that a native plant gene was interrupted. The insertion site was found to be located in the *B. rapa* portion of the amphidiploid *B. rapa*/*B. oleracea* genome of *B. napus*.
- Segregation was predictable over all generations observed and showed that transformation resulted in integration at one single dominant locus.
- MS1 and RF1 are several generations removed from the original transformants. Comparisons between the original transgenic plant and these lines 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 MS1, RF1 and the resulting hybrid. It was determined that vegetative vigor, overwintering capacity, flowering period, seed production of both transgenic lines, seed dissemination, germination and establishment, and seed dormancy were within the normal range of expression of characteristics in unmodified *B. napus* counterparts. Seed production of the hybrid line was greater than that of counterparts. These lines have no specific added genes for cold tolerance or winter survival. Flowers of the MS1 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 MS1, RF1 and MS1xRF1 did not show any stress adaptation other than their resistance to glufosinate ammonium. Its resistance or susceptibility to major *B. napus* pests and pathogens (e.g., blackleg, sclerotinia, flea beetles, diamondback moth larvae) fall within the ranges currently displayed by commercial varieties. The lines were tested under various environmental conditions, 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. According to the information provided by PGS, MS1, RF1 and MS1xRF1 were determined not to be different from their counterparts in this respect. Published data showed that seed survival of transgenic *B. napus* seeds expressing kanamycin resistance and glufosinate ammonium tolerance was significantly lower than seed survival of unmodified counterparts, when seeded under a variety of wild conditions. A competition study was also carried out, where the transgenic lines and barley were sown together at various ratios, and showed that introduction of the novel genes into the plants did not confer novel competitive advantages. 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 MS1, RF1 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 MS1 line is male sterile and will therefore not outcross with any plant. Although they can act as pollen recipients, the progeny will also be male sterile and will not produce pollen. The RF1 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 or fertility restoration do not confer any ecological advantage to potential hybrid offspring of MS1 or RF1 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, stress adaptation, and qualitative and quantitative composition of MS1, RF1 and MS1xRF1 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. In addition, detailed studies of pollination behavior in the field and in greenhouses showed no effects on honeybees. An avian dietary test was performed with the seed eating canary bird (*Serinus canaria domestica*), and a feeding study was performed with the domesticated rabbit (*Oryctolagus cuniculus*); these studies showed no differences in food consumption, behavior and body weight between birds or rabbits fed with the transgenics or counterparts.

Microorganisms living in the rhizosphere surrounding the roots were compared quantitatively and qualitatively in transgenic versus counterpart fields, and were shown to be no different.

Based on the above, AAFC has determined that the unconfined release of the MS1, RF1 and MS1xRF1 lines 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 hybrid have no novel phenotypic characteristics which would extend their use beyond the current geographic range of canola/rapeseed production in Canada. Since outcross species are only found in disturbed habitats, transfer of novel traits would not have an impact on unmanaged environments. Studies have shown AAFC that

these lines are not invasive of natural habitats and that they are no more competitive than the unmodified counterparts, both in natural and managed ecosystems.

AAFC has therefore concluded that the potential impact on biodiversity of MS1, RF1 and MS1xRF1 is equivalent to that of currently commercialized canola lines.

V. Assessment Criteria for Use as Livestock Feed

1. Anti-Nutritional Factors

Data on the glucosinolate and erucic acid content of meal and oil content of a range of *B. napus* strains produced using this PGS novel hybridization system for canola (MS1, RF1 and MS1xRF1) in breeding programs was submitted. No statistical differences in the content of these anti-nutritional factors were observed between the parental and modified strains. Therefore, this PGS novel hybridization system for canola, does not in itself, modify the production of these anti-nutritional factors by the PNT's.

2. Nutritional composition of PNT's

Data on the nutritional composition (i.e., crude protein, crude fat, crude fibre, ash and gross energy content) of the whole seed, processed meal or oil from a range of *B. napus* strains produced using this PGS novel hybridization system for canola (MS1, RF1 and MS1xRF1) in breeding programs was submitted. No statistical differences in nutritional composition were noted between the parental and modified strains. Therefore, the introduction of DNA into *B. napus* via this PGS novel hybridization system for canola, did not likely result in any secondary effects impacting on the composition or nutritional quality of the PNT's.

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 MS1, RF1 or MS1xRF1. Should these traits be transferred through outcrossing to relative plants, these also would result in no ecological advantage.

Based on the review of submitted data and information, the Feed Section of the Plant Products Division has concluded that the novel genes and their corresponding traits, introduced by this PGS novel hybridization system for canola, do not in themselves raise any concerns regarding the safety or nutritional composition of lines derived from this system. As this PGS hybridization system has been assessed and found to produce lines which are substantially equivalent to traditional varieties, all varieties produced using this PGS hybridization system are approved for use as livestock feed ingredients in Canada provided that the feed ingredients produced therefrom conform to the definitions for canola seed, canola meal or canola oil as listed in Schedule IV of the *Feeds Regulations*.

Unconfined release into the environment of MS1, RF1, MS1xRF1, and other *B. napus* lines derived from them, but without the introduction of any other novel traits, is therefore considered safe.

Feed use of varieties produced using this hybridization system is approved provided that the feed ingredients produced conform to the Feeds Regulations.

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