

Corporation obtaining approval, the name of its representative, and the address of its main office

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Glufosinate herbicide tolerant, male sterile and fertility restored oilseed rape (Modified <i>bar</i> , <i>barnase</i> , <i>barstar</i> , <i>Brassica napus</i> L.) (MS1RF1, OECD UI :ACS-BN004-7×ACS-BN001-4)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Glufosinate herbicide tolerant and male sterile and fertility restored oilseed rape (modified *bar*, *barnase*, *barstar*, *Brassica napus* L., MS1RF1, OECD UI: ACS-BNØØ4-7 x ACS-BNØØ 1-4) (hereinafter referred to as “MS1RF1”) is a hybrid obtained by a cross between glufosinate herbicide tolerant and male sterile oilseed rape (modified *bar*, *barnase*, *Brassica napus* L., MS1, OECD UI: ACS-BNØØ4-7) (hereinafter referred to as “MS1”) and glufosinate herbicide tolerant and fertility restored oilseed rape (modified *bar*, *barstar*, *Brassica napus* L., RF1, OECD UI: ACS-BNØØ1-4) (hereinafter referred to as “RF1”). Composition of the donor nucleic acid that was used for the production of MS1 and RF1, the parent plants of MS1RF1, and the origins of component elements are shown in Table 1-1 and Table 1-2 respectively.

For the wild-type *bar* gene obtained from *Streptomyces hygroscopicus*, GTG was modified to ATG to conform to frequently-used codons in plant, and AGC was modified to GAC to improve efficiency of translation. Regarding the translated amino acid, methionine remains unchanged in the modification from GTG to ATG, though serine changes to asparatic acid in the modification from AGC to GAC. However, it is confirmed that the function of modified PAT protein produced by the modified *bar* gene (hereinafter referred to as “the modified PAT protein”) remains unchanged in this modification (Reference 98).

The nucleotide sequences of modified *bar* gene, *barnase* gene and *barstar* gene are shown in Figures 1-1, 1-2 and 1-3 respectively.

Table 1-1 Origin and function of component elements (MS1)

Component elements	Size (kbp)	Origin and function
<i>Modified bar gene expression cassette</i>		
3'g7	0.2	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 21 and 96).
Modified <i>bar</i>	0.6	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from <i>Streptomyces hygroscopicus</i> and conferring tolerance to glufosinate herbicide (Reference 93). The two codons, GTG and AGC, in the N-terminal of wild-type <i>bar</i> gene are replaced for ATG and GAC respectively.
PSsuAra	1.9	Composed of the promoter SsuAra of RuBisCo small subunit gene which is derived from <i>Arabidopsis thaliana</i> and induces expression selectively in the chlorenchyma (Reference 47) and the transit peptide (TP) sequence targeted at the chloroplast.
<i>barnase gene expression cassette</i>		
3'nos	0.3	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 20).
<i>barnase</i>	0.3	Derived from <i>Bacillus amyloliquefaciens</i> . It expresses RNA-degrading enzyme (ribo-nuclease) in the tapetum cell to confer male sterility (Reference 30).
PTA29	1.5	A promoter of anther-specific gene TA29 derived from <i>Nicotiana tabacum</i> . It induces specific expression in the tapetum cell (Reference 80).
<i>neo gene expression cassette</i>		
Pnos	0.4	A promoter of nopaline synthase gene derived from pTiT37 of <i>Agrobacterium tumefaciens</i> . It initiates transcription of <i>neo</i> gene in plants (Reference 20).
<i>neo</i>	1.0	Derived from transposon Tn5 of <i>Escherichia coli</i> , encoding neomycin phosphotransferase II (NPT II) and conferring resistance to aminoglycoside derivative antibiotics (Reference 4). The ATG initiation codon has been replaced for linker sequence (Reference 72).
3'ocs	0.9	3'-terminal regulated region of octopine synthase gene derived from <i>Agrobacterium tumefaciens</i> , terminating transcription and inducing polyadenylation of transcripts (Reference 19).
Additional information		
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

Table 1-2 Origin and function of component elements (RF1)

Component elements	Size (kbp)	Origin and function
<i>Modified bar gene expression cassette</i>		
3'g7	0.2	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 21 and 96).
Modified <i>bar</i>	0.6	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from <i>Streptomyces hygroscopicus</i> and conferring tolerance to glufosinate herbicide (Reference 93). The two codons, GTG and AGC, in the N-terminal of wild-type <i>bar</i> gene are replaced for ATG and GAC respectively.
PSsuAra	1.9	Composed of the promoter SsuAra of RuBisCo small subunit gene which is derived from <i>Arabidopsis thaliana</i> and induces expression selectively in the chlorenchyma (Reference 47) and the transit peptide (TP) sequence targeted at the chloroplast.
<i>barstar gene expression cassette</i>		
3'nos	0.3	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 20).
<i>barstar</i>	0.3	It encodes ribo-nuclease inhibitor (BARSTAR protein), derived from <i>Bacillus amyloliquefaciens</i> . BARSTAR protein binds to ribo-nuclease, the product of <i>barnase</i> gene specifically, and inhibits its activity to restore male sterility (Reference 30).
PTA29	1.5	A promoter of anther-specific gene TA29 derived from <i>Nicotiana tabacum</i> . It induces specific expression in the tapetum cell (Reference 80).
<i>neo gene expression cassette</i>		
Pnos	0.4	A promoter of nopaline synthase gene derived from pTiT37 of <i>Agrobacterium tumefaciens</i> . It initiates transcription of <i>neo</i> gene in plants (Reference 20).
<i>neo</i>	1.0	Derived from transposon Tn5 of <i>Escherichia coli</i> , encoding neomycin phosphotransferase II (NPT II) and conferring resistance to aminoglycoside derivative antibiotics (Reference 4). The ATG initiation codon has been replaced for linker sequence (Reference 72).
3'ocs	0.9	3'-terminal regulated region of octopine synthase gene derived from <i>Agrobacterium tumefaciens</i> . It terminates transcription and induces polyadenylation of transcripts (Reference 19).
Additional information		
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.

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Figure 1-1 Nucleotide sequence of the modified *bar* gene

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Figure 1-2 Nucleotide sequences of the *barnase* gene

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Figure 1-3 Nucleotide sequence of the *barstar* gene

2) Functions of component elements

- (a) Functions of target genes, expression-regulating regions, localization signals, selectable markers and other component elements of donor nucleic acid

Functions of component elements of donor nucleic acid which were used for the production of MS1 and RF1 are shown in Table 1-1 and Table 1-2 respectively.

- (b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

[Modified PAT protein]

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. Glutamine synthetase plays an important role in detoxification of the ammonia produced, though the glutamine synthetase is inhibited if crops are sprayed with glufosinate herbicide, ammonia accumulates, and the crops wither and die.

The modified PAT protein produced by the transferred modified *bar* gene acetylates the glufosinate to make N-acetylglufosinate, which inactivates the inhibition of glutamine synthetase by the glufosinate. Then ammonia is not accumulated in the plant body, and the crop does not die even if it is sprayed with glufosinate herbicide.

The modified PAT protein exhibits a high affinity to glufosinate. Glufosinate is classified into L-amino acid, though it does not cause any acetyl group transfer reaction to the other various amino acids and it has little affinity for the glutamic acid which has specifically high structural similarity to glufosinate and it causes

virtually no transfer reaction *in vivo* (Reference 93). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by modified PAT protein was never inhibited (Reference 98). As a result, it is considered that the modified PAT protein possesses high substrate specificity to glufosinate.

[BARNASE protein]

BARNASE protein is a single stranded protein consisting of 110 amino acids, and it degrades RNA by two-stage reaction mode. It breaks the 3',5'-phosphodiester bond of the polyribonucleotide strand, transfers the phosphate group to 2'-OH group of the ribose and produces the 2',3'-cyclic nucleotide as an intermediate (the first-stage phosphotransfer reaction). Then, the BARNASE protein hydrolyzes the intermediate and produces the 3'-nucleotide specifically (the second-stage hydrolysis reaction) (Reference 33). It possesses high specificity for breaking the 3'-site of guanine, but it also breaks the other site, therefore only mono-nucleotide and di-nucleotide are detected from the complete degradation products (Reference 76).

Pollens are produced during the highly controlled process in an anther. Tapetum cell, one of the tissues of the anther, plays an important role such as providing nutrition at the time of pollen formation and during the growth of pollens. Therefore, it is considered that the absence of tapetum cells in the pollen production is the major factor of resulting male sterility (Reference 43).

The *barnase* gene expresses the ribo-nuclease (BARNASE protein) which hydrolyzes the single stranded RNA molecule in the tapetum cell layer of the anther under the control of the promoter PTA29. The BARNASE protein degrades the RNA in the tapetum cells and thus the plants are unable to produce pollens (References 23, 31 and 50). It is also shown that the *barnase* gene, under the control of the promoter PTA29, expresses stably even in the high-temperature condition (37 °C during the daytime) (Reference 2). It is not reported that the promoter PTA29 induces temperature-dependent expression.

[BARSTAR protein]

BARSTAR protein is an intracellular inhibitor for BARNASE protein (References 28 and 31). BARSTAR protein forms non-covalently bonded complex specifically with BARNASE protein in one-to-one correspondence and inhibits the ribo-nuclease activity of BARNASE protein completely (References 29, 31 and 85).

In general, the first cross cultivar (F1 cultivar) possesses stronger and higher productivity and excellent uniformity compared to the fixed cultivar (Reference 46). However, it is hard to obtain the F1 cultivar without fail for self-fertile crops such as oilseed rape. It becomes possible to obtain the F1 seeds without fail by crossing the female strain (the recombinant oilseed rape MS1 which possesses *barnase* gene and inhibits the production of pollens) with the male strain (the recombinant oilseed rape RF1 which possesses *barstar* gene). In the F1 generation, pollen fertility is restored by the function of BARSTAR protein which

inhibits the BARNASE protein (Reference 51), therefore, the seed production in high-yield by self-pollination becomes available.

[Toxicity and allergenicity of individual proteins]

In order to identify the allergenicity of modified *bar* gene product, *barnase* gene product and *barstar* gene product, homology search with known allergens in several databases was conducted for the respective amino acid sequences. As a result, no homology with any known toxins and allergens was observed for all the gene products examined.

(c) Contents of any change caused to the metabolic system of recipient organism

[Modified PAT protein]

Since the modified PAT protein possesses high substrate specificity (Reference 93), it is considered that it does not cause any acetyl group transfer reaction to the compounds other than glufosinate. Therefore, it is considered that the modified PAT protein does not affect the metabolic pathway of the recipient organism.

[BARNASE protein]

The expression of the *barnase* gene is limited to the tapetum cells under the control of the promoter PTA29 (Reference 50), and the *barnase* gene is unlikely to be expressed in any other tissue. The tapetum cell mostly develops at the 4-tetrad stage of pollen formation, and degrades/breaks along with the development of pollens (Reference 88). Therefore, it is considered that the possibility of the *barnase* gene to express in the tissues other than tapetum cells and to affect the metabolic pathway of the plant body is extremely low.

[BARSTAR protein]

The *barstar* gene is under the control of the promoter PTA29 and then it is unlikely to be expressed in any tissue other than tapetum cell. In addition, BARSTAR protein forms non-covalently bonded complex specifically with BARNASE protein in one-to-one correspondence, and the stability of the complex is high (References 49 and 52). Furthermore, the ribo-nuclease of bacteria and filamentous fungi is found considerably homologous in the structure and sequence and thus, it is expected that these enzymes contain some inhibitors homologous with BARNASE protein. However, such inhibitors are known only in the ribo-nuclease BINASE protein produced by the *Bacillus intermedius*. The BINASE protein possesses high homology (85%) with the BARNASE protein, and it is inhibited by the BARSTAR protein (Reference 101). , There is a report that the extra-cellular ribo-nuclease of *Streptomyces*, which has the similar protein structure although its homology with the amino acid sequence of BARNASE protein is only 20 to 25% (Reference 35), is also inhibited by the BARSTAR protein (Reference 32). However, it is not reported that the BARSTAR protein exhibits the inhibiting activity against the ribo-nuclease in plants. The BARSTAR protein is reported not to bind to any ribo-nuclease of human or animals (References 30, 31, 35 and 85). Based on the above understanding, it is considered that BARSTAR protein would

not affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

The vector used for the production of MS1 and RF1 is pTTM8RE and pTVE74RE respectively, which were both constructed based on the vector pGV825 (Reference 17).

2) Properties

(a) The numbers of base pairs and nucleotide sequence of vector

The total number of base pairs of the vectors pTTM8RE and pTVE74RE is 15,339bp and 15,225bp respectively. The entire nucleotide sequences of the both vectors are shown in Annex 1-1 and 1-2. In addition, the physical map of vector and the restriction enzyme cleavage site are shown in Figure 2 and Figure 3 respectively.

(b) Presence or absence of nucleotide sequence having specific functions, and the functions

The plasmids pTTM8RE and pTVE74RE possess the streptomycin/spectinomycin tolerance gene (*Sm/Sp*), kanamycin tolerance gene (*KanR*), ORI_{pBR}, and *barstar* gene outside the T-DNA region. The *Sm/Sp* and *KanR* were used as selectable markers for the vectors. In addition, ORI_{pBR} is the replication origin, which functions to cause autonomous replication in the *E.coli*. Furthermore, the *barstar* gene has been present in the basic plasmid used for constructing the both plasmids. At the time of inserting the *barnase* gene to the plasmid by using *E.coli* for constructing the pTTM8RE, a small amount of BARNASE protein would express even though using the promoter for plants and the *E.coli* would die. Therefore, the *barstar* gene was used for inhibiting the enzyme activity of the BARNASE protein. These sequences locate outside the T-DNA region, and they are considered not to be transferred into the oilseed rape genomes (Annex 2, Figure L03b).

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

It is known that the range of recipient organisms for the autonomous replication of plasmids pTTM8RE and pTVE74RE is limited to *Agrobacterium tumefaciens*, *E.coli* and gram-negative bacteria, and the plasmids do not possess the infectious characteristics in plants.

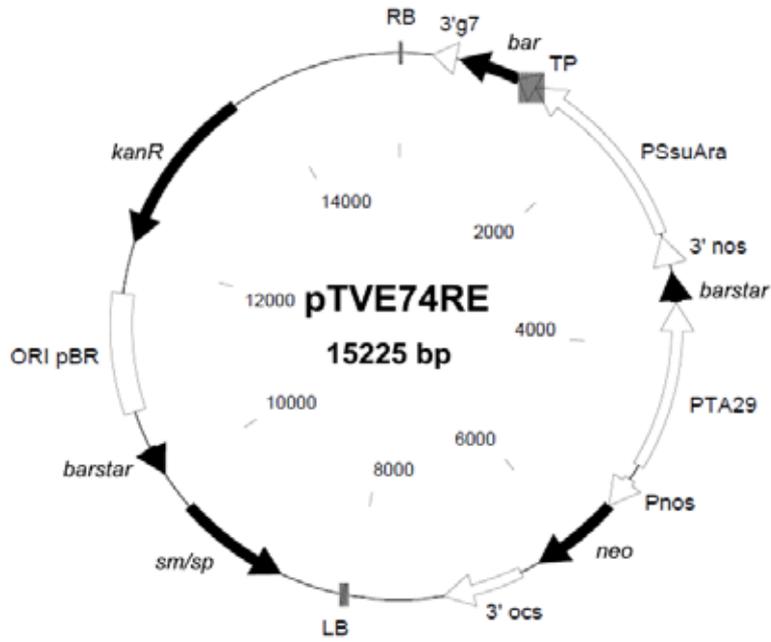


Figure 3-1 Physical Map of plasmid pTVE74RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon the applicant.)

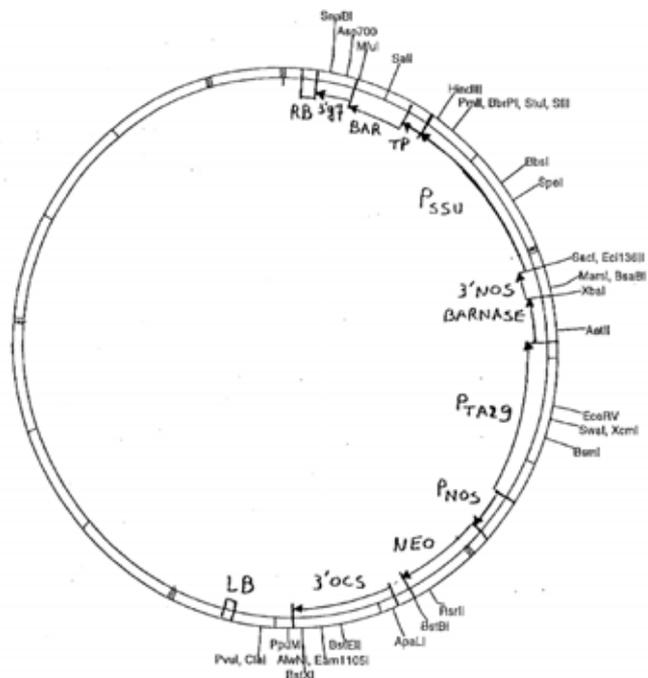


Figure 3-2 The restriction enzyme cleavage site of pTVE74RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon the applicant.)

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid transferred in the recipient organism

In the MS1, *neo* gene expression cassette, *barnase* gene expression cassette and modified *bar* gene expression cassette ($3'ocs-neo-Pnos-PTA29-barnase-3'nos-PSsuAra-modified\ bar-3'g7$) in the T-DNA region were transferred which are located between LB and RB on the pTTM8RE (Figure 2-1). In addition, in the RF1, *neo* gene expression cassette, *barstar* gene expression cassette and modified *bar* gene expression cassette ($3'ocs-neo-Pnos-PTA29-barstar-3'nos-PSsuAra-modified\ bar-3'g7$) in the T-DNA region were transferred which are located between LB and RB on the pTVE74RE (Figure 3-1).

2) Method of transferring nucleic acid transferred to the recipient organism

The *Agrobacterium*-mediated transformation method was used for transferring nucleic acid to both MS1 and RF1 (Reference 18).

The *E.coli* MC1061 strain which possesses either pTTM8RE or pTVE74RE, the *E.coli* HB101 strain which possesses the transferable (helper) plasmid pRK2013, and the non-oncogenic *A.tumefaciens* C58C1Rif^R strain were coexistent. After the *A.tumefaciens* C58C1Rif^R strain which possesses pTTM8RE or pTVE74RE was produced, a piece of hypocotyls of the recipient organism was infected with it, and the T-DNA region between the RB and the LB was transferred into the oilseed rape genomes.

3) Processes of rearing of living modified organisms

(a) Mode of selecting the cells containing the transferred nucleic acid

After transformation, a piece of hypocotyls was grown in a solid medium containing 50 mg/L kanamycin-SO₄ or 20 mg/L phosphinothricin for selection. Then, selected clones were moved to a hormone-free medium and regenerated to the plant body.

(b) Presence or absence of remaining *Agrobacterium* in case of using *Agrobacterium* method for transferring nucleic acid

After transformation by the *Agrobacterium*, 500 mg/L of Carbenicillin was added to the medium and the remaining *Agrobacterium* cells were removed (Reference 18), therefore, the plant body of MS1 and RF1 contains no *Agrobacterium* cell. (Annex 2, Figure 2).

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line with which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

After transformation, the regenerated plant body of each of MS1 and RF1 was comprehensively examined for the traits expressed and agricultural characteristics. The MS1RF1 was produced by crossing the lines of BC2F1 or later generations of MS1 with the lines of T3, BC2F1 or later generations of RF1. The process of rearing of MS1RF1 is shown in Figure 4. The approvals of the MS1RF1 received from organizations in Japan are as follows.

[Food safety]

Based on the “Guideline for food safety assessment of food and food additives derived from recombinant-DNA technology”, the conformity to the Guideline for safety of use as food regarding PGS1 in September 3, 1996 and regarding PHY14 and PHY35 in May 26, 1997 was confirmed by the Ministry of Health and Welfare (the Ministry of Health, Labour and Welfare, currently). In addition, along with legislating, passing through the “Procedures for food safety assessment of food and food additives derived from recombinant-DNA technology”, safety of use for food was approved by the Ministry of Health, Labour and Welfare in March 30, 2001.

[Feed safety]

Based on the “Guideline for feed safety assessment of recombinant feed”, the compatibility to the Guideline regarding glufosinate herbicide tolerant canola PGS1 in September 26, 1996 and regarding glufosinate herbicide tolerant canola PHY14 and glufosinate herbicide tolerant canola PHY35 in December 24, 1997 was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, along with legislating, passing through the “Procedures for feed safety assessment of feed and feed additives derived from recombinant-DNA technology”, safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in March 27, 2003.

[Environmental safety]

In 1995, based on the “Guideline for the use of recombinant in agriculture, forestry and fisheries,” the compatibility to the guideline regarding the isolated field test on the recombinant oilseed rape was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, in May 8, 1996, the compatibility to the guideline regarding the recombinant oilseed rape being imported to Japan (used for processing and feed) was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

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Figure 4 Pedigree tree of MS1RF1

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

1) Place where the replication product of transferred nucleic acid exists

It is expected that the genetic locus of both MS1 and RF1 (the original gene transformation) is heterozygote for the transferred gene locus. In addition, MS1, which is conferred the male sterile trait, is maintained by a cross with the non-recombinant oilseed rape and then, it is expected that a segregation ratio of 1:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals under the control of single gene locus. Furthermore, it is expected that in the T1 generation raised by self-pollination of RF1 (the original gene transformation), a segregation ratio of 3:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals and that in the population of the T2 generation strains estimated as homozygote among multiple individuals exhibiting the tolerance to glufosinate herbicide, all the individuals would exhibit glufosinate tolerance.

As a result of examination on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals in various generations of MS1 and RF1, the BC1F1 and BC2F1 generations of MS1 exhibited the segregation ratio in good agreement with the theoretical segregation ratio (Annex 6, Table GBN092 and Table FBN9301₂). In addition, in the T1 generation of RF1, about three-fourth of individuals exhibited the tolerance to glufosinate herbicide (Annex 6, Table GBN030₁), and in the T2 generation raised by self-pollination of T1 generation individuals estimated as homozygote, all the individuals exhibited glufosinate tolerance (Annex 6, Table GBN030₂). Consequently, as all the cases examined resulted in the segregation ratio as expected, the replication products of transferred nucleic acid in MS1 and RF1 are all considered to exist on the genome of oilseed rape at one site.

2) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations

In order to identify the number of copies of transferred nucleic acid, the genome DNA of MS1 (BC2F1 generation) and RF1 (T3 generation) was cleaved by the restriction enzymes, and the Southern blotting analysis was conducted using the modified *bar* probe, PSuAra probe, PTA29 probe and *neo* probe. As a result, in each analysis, the band equivalent in size to the fragment derived from the vector was observed, and it was confirmed that one copy of T-DNA region was transferred in each of MS1 and RF1 (Annex 2). In addition, as a result of determination of nucleotide sequence (sequence analysis) in MS1 and RF1, it was confirmed that the identical sequence as in the T-DNA region on each plasmid was transferred (Annex 3-1 and Annex 3-2). The T-DNA region transferred in MS1 and RF1 is shown in Figure 5-1 and Figure 5-2.

Additionally, the genome DNA of MS1 of the generations F1, BC2F1 and BC4F1 (obtained by the backcrossing with Cultivar A) and BC5F1 (obtained by the backcrossing with Cultivar B), and of RF1 of the generations T1, T3, BC3F1 (obtained by the backcrossing with Cultivar A) and BC3F1 (obtained by the

backcrossing with Cultivar B) was cleaved by the restriction enzymes, and the Southern blotting analysis was conducted using the PTA29 probe. As a result, the identical band pattern was observed in all the generations of MS1 and RF1, and it was confirmed that the replication products of the transferred nucleic acid are inherited stably through the multiple generations (Annex 2, Figures 3 and 4).

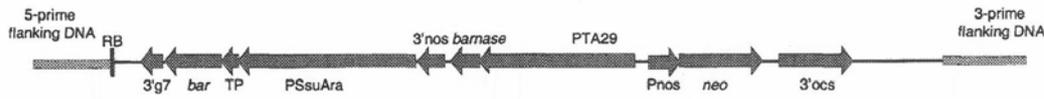


Figure 5-1 Map of the entire T-DNA region transferred to MS1

In the diagram, *bar* refers to the modified *bar* gene and TP refers to the transport peptide. (Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)



Figure 5-2 Map of the entire T-DNA region transferred to RF1

In the diagram, *bar* refers to the modified *bar* gene and TP refers to the transport peptide. (Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

3) The position relationship in the case of multiple copies existing in chromosome

As mentioned above, MS1RF1 contains one copy of modified *bar* gene on the chromosome derived from the parent plants and then, possesses two copies of modified *bar* gene.

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

[Modified *bar* gene]

Since the genetic locus of MS1 is heterozygote for the modified *bar* gene locus, about 50% of MS1 individuals are expected in theory to exhibit the tolerance to glufosinate herbicide, while regarding RF1 and MS1RF1, 100% of individuals are expected to exhibit glufosinate tolerance.

As a result of field tests conducted in foreign countries on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals, about 50% of MS1 individuals of the BC1F1 generation and 100% of RF1 individuals of RF1 of the T2 generation exhibited the tolerance to glufosinate herbicide (Annex 6, Table GBN114), and also in the following generations of each line { BC2F1 generation of MS1 and T3 generation of RF1 }, about 50% of MS1 individuals and 100% of RF1 individuals exhibited the glufosinate tolerance (Annex 6, Table FBN9301₂). Consequently, it was confirmed that the tolerance to glufosinate herbicide is stably expressed across the multiple generations.

[*barnase* gene and *barstar* gene]

MS1 is maintained by a cross with the non-recombinant oilseed rape and then, it is expected that MS1 would contain male-sterile individuals caused by expression of the *barnase* gene and male-fertile individuals at a segregation ratio of 1:1 in theory. On the other hand, it is expected that for RF1, all the individuals would exhibit male fertility and for MS1RF1, all the individuals would exhibit male fertility due to expression of the *barstar* gene.

In the test conducted in foreign countries at several areas, for MS1 (BC2F1 generation), RF1 (T3 generation) and MS1RF1 {F1 generation: MS1(BC2F1) maintained by Drakkar} × RF1(T3), the number of male-sterile individuals and male-fertile individuals was measured and as a result, in all the areas investigated, the segregation ratio for male sterility and fertility was observed as expected independently of generation (Annex 6, Table FBN9301₆). Consequently, it is considered that the *barnase* gene and the *barstar* gene are stably inherited under a natural environment.

Moreover, in order to examine the expression of the modified *bar* gene, *barnase* gene (only for MS1), *barstar* gene (only for RF1) and *neo* gene transferred in the MS1 and RF1, Northern blotting analysis was conducted for the leaves, flower buds, seeds and pollens of the MS1 (BC2F1 generation) and RF1 (T3 generation).

[MS1]

The transcripts of the modified *bar* gene were detected only in leaves (detection limit: 0.1 pg/μg the entire RNA). In addition, the transcripts of the *barnase* gene were not detected in any tissue (detection limit: 0.4 pg/μg entire RNA). Furthermore, the transcripts of the *neo* gene were not detected in any tissue (detection limit: 0.1 pg/μg total RNAs) (Annex 2).

[RF1]

The transcripts of the modified *bar* gene were detected in leaves and flower buds, but not detected in any other tissues (detection limit: 0.1 pg/μg entire RNA). In addition, the transcripts of the *barstar* gene were detected only in flower buds and not detected in any other tissues (detection limit: 0.4 pg/μg total RNAs). Moreover, the transcripts of the *neo* gene were not detected in any tissue (detection limit: 0.2 pg/μg total RNAs) (Annex 2).

In addition, as a result of examination to identify the expression of the modified PAT protein in leaves of MS1 in the individual generations of F1, BC2F1, BC4F1 (obtained by the backcrossing with Cultivar A) and BC4F1 (obtained by the backcrossing with Cultivar B) and RF1 in the generations T1, T3, BC3F1 (obtained by the backcrossing with Cultivar A) and BC3F1 (obtained by the backcrossing with Cultivar B), it was confirmed that the modified PAT protein is expressed in all the generations examined (Annex 2). Moreover, as a result of similar examination to identify the expression of the NPT II protein in the individual generations of MS1 and RF1, it was confirmed that the NPT II protein is stably expressed in all the generations of MS1 and RF1 examined (Annex 2, Figure L11₁).

- 5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

MS1 and RF1 contain no DNA sequence which possesses transferring factor and therefore, there is no possibility of transmission of nucleic acid transferred to wild animals and wild plants under a natural environment.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

Detection of MS1RF1 is available by PCR method using the flanking sequences of DNA transferred in MS1 and RF1 respectively for each plant body (seed). This PCR method is utilized effectively for cultivation management of individual events (Annex 7-1 and Annex 7-2).

(6) Difference from the recipient organism or the taxonomic species to which the recipient organism belongs

- 1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of replication products of transferred nucleic acid

MS1 is given the traits of tolerance to glufosinate herbicide and male sterility, and RF1 is given the traits of tolerance to glufosinate herbicide and fertility restoration. Crossing between MS1 and RF1 allows restoration of fertility in the F1 generation since the BARNASE protein forms non-covalently bonded complex specifically with BARSTAR protein in one-to-one correspondence in the anther tapetum cells and inhibits the activity of BARNASE protein.

- 2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between modified plant and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

In FY 1995, the isolated field tests were conducted at the Hokkaido Agricultural Research Center to compare morphological and growth characteristics, summer survival of matured plant, and production, shedding habit and germination rate of the seed between MS1RF1 {F1 generation: MS1(BC6F1) × RF1(T5) maintained by Westar; corresponding to “PGS1” in Annex 4}, the recipient organism cultivar Drakkar (hereinafter referred to as “the non-recombinant oilseed rape”) and

Karafuto, which has been cultivated in Hokkaido as a spring sowing variety (hereinafter referred to as “Karafuto”). In addition, crossability was examined based on the comparison between the non-recombinant oilseed rape, Karafuto, and the *B.juncea* (Cutlass and leaf mustard) in the above mentioned isolated field tests (Annex 4). Moreover, in 2006, in the special screened greenhouse in Japan, examination was made regarding the heat-tolerance at the early stage of growth, fertility and size of the pollen, dormancy of the seed, and productivity of harmful substances (Annex 5).

(a) Morphological and growth characteristics

Comparison was made between MS1RF1, the non-recombinant oilseed rape and Karafuto for the plant height, the number of primary branches, dry weight of aerial parts (stems and leaves), plant shape, color of leaves, time of bolting, flowering period, maturation period, rate of pods formation, length of pod, the number of seed setting (seeds/pod), property of open pods, color of seed, seed yield (g/plant) and 1000-seed weight.

As a result, for the time of bolting, flowering period and maturation period, MS1RF1 was found equivalent to the non-recombinant oilseed rape and Karafuto. The plant height in average was found shorter for the MS1RF1 by 14 cm and 4 cm compared to the non-recombinant oilseed rape and Karafuto respectively. For the number of primary branches, plant shape, property of open pods and seed yield, no significant difference was observed between the plants examined. The 1000-seed weight was found less for the MS1RF1 by about 0.5 g compared to Karafuto, though equivalent to the non-recombinant oilseed rape. The weight of stems and leaves for the MS1RF1 was found 51 g smaller compared to the non-recombinant oilseed rape and 52 g larger compared to Karafuto, thus being between the both control plants. MS1RF1 showed lower rate of pods formation and smaller length of pod compared to the non-recombinant oilseed rape and Karafuto, though the number of seed setting per pod was 25.9 for the MS1RF1, 20.3 for the non-recombinant oilseed rape, and 15.7 for Karafuto, showing the largest number of seed setting per pod for the MS1RF1. On the other hand, based on the calculation of the number of seeds per plant from the seed yield and 1000-seed weight, MS1RF1 and the non-recombinant oilseed rape both exhibited the equivalent result of 2.02×10^4 seeds. The color of leaves was found slightly lighter for the MS1RF1 than the non-recombinant oilseed rape but equivalent to Karafuto, and the color of seed was found black for the MS1RF1 and dark brown for the non-recombinant oilseed rape (Annex 4, Tables 1 and 2).

(b) Cold-tolerance or heat-tolerance at the early stage of growth

Seedlings of MS1RF1 and the non-recombinant oilseed rape were raised under the conditions (35°C and 12-hours day length and 12-hours night length) and as a result, it was confirmed at one month later observation that all the individuals had died (Annex 5, Table 23). Therefore, it is considered that the heat-tolerance of MS1RF1 at the early stage of growth is as low as that of the non-recombinant oilseed rape.

The oilseed rape varieties sown in autumn in Japan are generally known to grow even in winter in both warm and cold districts, though the rate of growth varies (Reference 82).

(c) Wintering ability or summer survival of the matured plant

As a result of observation for the summer survival in the isolated fields, no difference was observed between the recombinant oilseed rape MS1RF1 and the other varieties examined (Annex 4).

It is generally known that oilseed rape shows high cold tolerance and high snow endurance (Reference 82).

(d) Fertility and size of the pollen

Pollens were collected from the MS1RF1 and the non-recombinant oilseed rape cultivated in the special screened greenhouse, and stained with acetocarmine solution and observed under a microscope. As a result, 100% of the pollens from the MS1RF1 and 99% of the pollens from the non-recombinant oilseed rape were found stained, showing a high fertility of the pollens (Annex 5). In addition, as a result of comparison of size of pollen, no statistically significant difference was observed between the recombinant oilseed rape MS1RF1 and the non-recombinant control oilseed rape (Annex 5, Table 21).

(e) Production, shedding habit, dormancy, and germination rate of the seed

MS1RF1 showed no difference in seed yield per plant (g/plant) and 1000-seed weight from the non-recombinant oilseed rape (Annex 4, Table 2).

Regarding the shedding habit, comparison was made for the rate of open pods formed and as a result, MS1RF1 and the non-recombinant oilseed rape were both found easy to open pods similarly to each other without any difference (Annex 4, Table 2).

To evaluate the germination rate, 20 seeds each obtained from self-fertilization of the MS1RF1 and the non-recombinant oilseed rape in the special screened greenhouse were sown. As a result, one week after sowing, the germination rate was found 90.0% (18/20 grains) for the MS1RF1 and 85.0% (17/20 grains) for the non-recombinant oilseed rape (Annex 5, Table 24). In addition, the seeds that were found not germinated were evaluated for life or dead based on the tetrazolium method after removal from the soil and they were confirmed to be dead. Based on the understanding that the seeds found surviving one week after sowing all germinated successfully, the MS1RF1 and the non-recombinant oilseed rape are considered not to possess any dormancy.

(f) Crossability

In the isolated field, the non-recombinant oilseed rape, Karafuto, and two varieties of *B.juncea* (Cutlass and leaf mustard) were cultivated adjacent to the MS1RF1 at distances of 1 to 2 m (Annex 4, row planting plan). The test plots

were entirely shielded with insect screening frames and honeybees were released. The seeds harvested from the individual varieties were sown and then, the seedlings germinated were sprayed with glufosinate herbicide. As a result, the glufosinate tolerance was observed in 7.4% of the non-recombinant oilseed rape, 2.5% of Karafuto, and 0.1% of Cutlass and leaf mustard (Annex 4, Table 4).

It is reported that the out-crossing rate of oilseed rape through wind or insect pollination is 5 to 30% (References 37 and 68) and then, the crossability with the non-recombinant oilseed rape and Karafuto was found not exceeding the existing findings. Also the crossability with Cutlass and leaf mustard was found not exceeding the known crossability of 0.3 to 1.1% between *B.juncea* and oilseed rape (planting rate 1:1) (Reference 6).

(g) Productivity of harmful substances

In order to check whether the substances are excreted from the roots of MS1RF1 which can affect other plants, exists in the plant body which can affect other plants after dying, and are excreted from the roots which can affect microorganisms in soil, the succeeding crop test, plow-in test and soil microflora test were carried out respectively in the special screened greenhouse.

Succeeding crop test : After cultivating the recombinant oilseed rape MS1RF1 and the non-recombinant control oilseed rape for about two months, radishes were cultivated as test plants in the remaining soil respectively, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed (Annex 5, Tables 1, 3, 4, 6, 7). Therefore, it is considered that the recombinant oilseed rape MS1RF1 has not acquired any productivity of the substances excreted from the roots which can affect other plants.

Plow-in test : The dried powder of plant body of the MS1RF1 and the non-recombinant oilseed rape cultivated about three months after sowing was mixed with soil (1%), respectively, and seeds of radish were sowed in the soil and cultivated. Then the comparison was made for germination rate, plant height, root length, fresh weight and dry weight. As a result, there was no statistically significant difference observed in any items (Annex 5, Tables 11, 13, 14, 16, and 17). Therefore, it is considered that MS1RF1 has not newly acquired any productivity of the substances which can affect other plants after dying.

Soil microflora test : The soil was obtained after cultivating the recombinant oilseed rape MS1RF1 and the non-recombinant control oilseed rape for about two months, and was diluted by adding sterilized phosphate buffer solution. Bacteria and Actinomyces were incubated in PTYG medium, and filamentous fungi were incubated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, there was no statistically

significant difference observed in the number of actinomycete and filamentous fungi between MS1RF1 and the non-recombinant oilseed rape. On the other hand, in terms of the number of bacteria, MS1RF1 exhibited higher values than the non-recombinant oilseed rape, showing a statistically significant difference (Annex 5, Table 19). Based on the above results, it is considered that MS1RF1 has not newly acquired any productivity of the substances excreted from the roots which can affect the decrease in viable cell count in microorganisms in soil.

II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the “Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms.” Results of the review are listed below.

(Note 1) The underlined description in the following sections is intended to alert the readers to differences between three varieties of stack line oilseed rape.

MS1RF1 is produced by a cross between the glufosinate herbicide tolerant and male sterile oilseed rape (MS1) and the glufosinate herbicide tolerant and fertility restored oilseed rape (RF1). MS1RF1 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS1, and the BARSTAR protein derived from RF1.

In the MS1RF1, the BARNASE protein and the BARSTAR protein interact with each other in the anther tapetum cell ^(Note 2) (non-covalently bonding specifically to each other in one-to-one correspondence) and as a result, the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein and the male fertility is restored. As mentioned above, MS1RF1 exhibits the interaction of the traits derived from the both parent lines and then, evaluation has been focused on the stack line oilseed rape rather than the both parent lines.

The modified PAT protein possesses high substrate specificity and thus, it is considered not to interact with the BARNASE protein and the BARSTAR protein.

(Note 2) By a cross between the female strain (male-sterile MS1) and the male strain (fertility restored RF1), seeds of the first cross cultivar (F1) is obtained which possesses the male fertility.

1. Item-by-item assessment of Adverse Effect on Biological Diversity

(1) Competitiveness

Oilseed rape (*Brassica napus* L.) to which the recipient organism belongs was introduced to Japan in early Meiji period, and it is reportedly growing on river banks, along roadsides, in the surroundings of seed off-loading harbors, and in other such areas. It is generally known that oilseed rape would be eventually replaced with

perennial plants and shrub in the environmental conditions without any regular disturbance such as roadsides, cliffs and riverside areas.

In the isolated fields and special screened greenhouses in Japan, a total of 22 traits relating to the competitiveness of MS1RF1 were examined based on the comparison with the control plants (Drakkar and Karafuto).

As a result, the plant height was found shorter on average for the MS1RF1 compared to Drakkar and Karafuto. For the weight of stems and leaves, MS1RF1 was found lighter than Drakkar and heavier than Karafuto, thus being between the both control plants. For the rate of pods formation and the length of pod, MS1RF1 exhibited lower values compared to Drakkar and Karafuto, and the number of seed setting per pod was found larger for the MS1RF1 than for Drakkar and Karafuto. However, these differences are considered not to cause MS1RF1 to become competitive.

MS1RF1 is given;

- (i) a trait to be tolerant to glufosinate herbicide, though it is generally considered that the glufosinate does not exert selective pressure under a natural environment.
- (ii) MS1RF1 produces pollens due to the interaction between the BARNASE protein derived from MS1 and the BARSTAR protein derived from RF1, though the pollens of MS1RF1 are found to have equivalent fertility as those of Drakkar, and for the size of the pollens, it is confirmed that there is no significant difference from the control plants.
- (iii) The BARNASE protein degrades RNA in the tapetum cells of the recipient organism, though there is no report that it possesses activity against any other substrates.
- (iv) There is no report that the BARSTAR protein possesses any function other than that which inhibits the ribo-nuclease of the BARNASE protein.

Therefore, it is considered unlikely that these traits could cause MS1RF1 to become competitive under a natural environment.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

It has been confirmed that the contents of erucic acid and glucosinolate in the MS1RF1, which are recognized as harmful substances to human and other mammals, fall within the ranges for the cultivars known as canola, in which the erucic acid and glucosinolate content was reduced by selective breeding.

MS1RF1 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS1, and the BARSTAR protein derived from RF1.

The modified PAT protein possesses high substrate specificity and then, it is considered unlikely to transfer the acetyl group to any substances other than the substrate glufosinate. In addition, in the MS1RF1, the BARNASE protein and the BARSTAR protein non-covalently bind specifically to each other in the anther tapetum cells in one-to-one correspondence, and the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein. Therefore, it is considered unlikely that these proteins affect any other metabolic systems in the plant body and newly produce any harmful substances.

In addition, for the amino acid sequences of those proteins, comprehensive homology search and allergen epitope homology search were conducted. Consequently, no homology with any known toxin and allergen was observed.

In the special screened greenhouses in Japan, the succeeding crop test, soil microflora test and plow-in test have been conducted to check the harmful substances productivity of this recombinant oilseed rape (the substances excreted from the roots which can affect other plants, the substances excreted from the roots which can affect microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying).

In the succeeding crop test and plow-in test, no statistically significant difference was observed between MS1RF1 and Drakkar. On the other hand, for the number of bacteria, actinomyces and filamentous fungi, examined in the soil microflora test, a statistically significant difference was observed in bacteria, though MS1RF1 showed higher values compared to Drakkar and then, it is considered that MS1RF1 has not newly acquired any productivity of the substances excreted from the roots which can affect the decrease in viable cell count in microorganisms in soil.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

In a natural environment in Japan, a number of plants of the family *Brassicaceae* are growing, though known species that can be crossed with oilseed rape (*Brassica napus* L.) include *B. rapa* L. (turnip, Komatsuna, conventional rapeseed, etc.) of the genus *Brassica*; *B. juncea* (L.) Czern (mustard, leaf mustard, etc.); *B. nigra* (L.) W.D.J.Koch (black mustard) and *Raphanus raphanistrum* L. (wild radish) in addition to oilseed rape itself.

Oilseed rape, *B. juncea*, *B. nigra*, and *R. raphanistrum* are regarded as all introduced species brought into Japan artificially after Meiji period. In addition, *B. rapa* is also a cultivar-derived introduced species though it was introduced to Japan in olden times. As such, these are not specified as wild species as to be possibly affected.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on

Biological Diversity attributable to crossability is reasonable.

(4) Additional information

The possible indirect Adverse Effect on Biological Diversity attributable to crossing of recombinant oilseed rape with non-recombinant oilseed rape and the related species described on the above was evaluated. The possible indirect Adverse Effect on Biological Diversity refers to that; i) hybrid produced by crossing would become competitive and exterminate species population of the other wild animals and wild plants, and ii) related species population would decrease due to the effect of transferred gene spread by crossing, and wild animals and wild plants such as insects which are dependent on the related species would be affected for maintenance of their population.

In concrete,

- (i) It was confirmed as a result of test on the crossability between MS1RF1 and the non-recombinant oilseed rape that the crossability of MS1RF1 with the non-recombinant oilseed rape does not exceed any existing findings on the crossability between oilseed rape varieties.
- (ii) Regarding the crossability with related species, it is reported that it would be hard to produce hybrid, and even if hybrid is produced, the progeny would possess low fertility.
- (iii) It is considered unlikely that MS1RF1 which possesses glufosinate tolerance and fertility restoration would become competitive under a natural environment.
- (iv) It is reported that the cross-progeny of plants which possess male sterility as dominant trait would decrease its population rapidly over generations.

Consequently, it is judged that the possibility that MS1RF1 would cross with related species to produce hybrid and the progeny would become competitive under a natural environment is as low as oilseed rape to which the recipient organism belongs.

In addition,

- (v) In the comparison between the individuals tolerant and not tolerant to glufosinate herbicide in the BC3 generation obtained by three-time repeated backcrossing of *B. rapa* with the hybrid between the recombinant oilseed rape, which contains both the modified *bar* gene and the *barstar* gene, and the *B. rapa*, through screening with glufosinate herbicide, it was reported that there was no difference in the fertility of pollen, survivability and the amount of seeds produced.
- (vi) Even if the *barnase* gene become out of control of the promoter PTA29 and acquire the promoter which would express constitutively or site-specifically in plant, the possibility that plant would grow properly is low, and it is considered unlikely that the gene would spread extensively in the related species population.

Consequently, it is considered that the possibility that the genes would affect maintenance of interspecies hybrid population in a shorter period of time is low.

Based on the above understanding, it was judged that the conclusion by the applicant that there is no risk of indirect Adverse Effect on Biological Diversity attributable to crossability is reasonable.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stack line recombinant oilseed rape (MS1RF1) in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Reference

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Annex

MS1 : Outline of the Biological Diversity Risk Assessment Report (MS1)

RF1 : Outline of the Biological Diversity Risk Assessment Report (RF1)

Annex 1-1 : Entire nucleotide sequence of plasmid pTTM8RE

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1-2 : Entire nucleotide sequence of plasmid pTVE74RE

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Annex 2 : Molecular analysis of male sterile oilseed rape MS1 and fertility restored oilseed rape RF1

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Annex 3-1 : Sequence analysis on the transferred genes in the male sterile oilseed rape MS1

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3-2 : Sequence analysis on the transferred genes in the fertility restored oilseed rape RF1

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Annex 4 : Isolated field test report

Confidential: Not made available or disclosed to unauthorized person

Annex 5 : Report on the tests in the special screened greenhouse

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Annex 6 : Report on the tests carried out in foreign countries

Confidential: Not made available or disclosed to unauthorized person

Annex 7-1 : Event Identifying Method (MS1)

Confidential: Not made available or disclosed to unauthorized person

7-2 : Event Identifying Method(RF1)

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