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	Oilseed rape tolerant to glufosinate herbicide and male sterile (*bar, barnase, Brassica napus* L.) (MS8, OECD UI :ACS-BN005-8)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, 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 the concerning donor nucleic acid

1) Composition and origins of component elements

Composition of the donor nucleic acid that was used for the production of glufosinate tolerant and male sterile oilseed rape (*bar, barnases*, *Brassica napus* L., MS8, OECD UI : ACS-BN005-8) (hereinafter referred to as “the recombinant oilseed rape MS8”) and the origins of component elements are shown in Table 1.

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 to the translated amino acid, methionine remains unchanged in the modification from GTG to ATG, but serine changes to asparatic acid in the modification from AGC to GAC. However, it is confirmed that the function of PAT protein produced by modified *bar* gene remains unchanged in this modification (Reference 100).

The nucleotide sequences of modified *bar* gene and *barnase* gene are shown in Figure 1 and Figure 2. The nucleotide sequence of entire donor nucleic acid is shown in Annex 3.

Table 1 Origin and function of component elements

Component elements	Size (kbp)	Position in vector (bp)	Origin and function
barnase gene expression cassette			
PTA29	1.5	4877-3368	It is the promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces specific expression in the tapetum cell layer of the anther (Reference 82).
barnase	0.3	3367-3032	It is derived from *Bacillus amyloliquefaciencs*, and encodes RNA-degrading enzyme (BARNASE protein). It expresses in the tapetum cell layer of the anther under the control of PTA29, and confers male sterility (Reference 31).
3'nos	0.3	2919-2659	It is the 3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 18).
Modified *bar* gene expression cassette			
PSsuAra	1.7	2608-883	It is derived from *Arabidopsis thaliana*, and the promoter of rubisco small subunit gene. It induces expression selectively in the chlorenchyma (Reference 48).
Modified *bar*	0.5	882-331	It encodes phosphinothricin acetyl transferase (PAT protein) derived from *Streptomyces hygroscopicus* and confers tolerance to glufosinate herbicide (Reference 95). The two codons in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC, respectively.
3'g7	0.2	309-98	It is the 3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (Reference 20, Reference 98).
Others			
RB	0.02	1-25	It is the right border of the T-DNA derived from pTiB6S3 (Reference 28).
LB	0.02	4922-4946	It is the left border of the T-DNA derived from pTiB6S3 (Reference 28).
Sm/Sp	1.0	6515-5505	It encodes *aminoglycoside adenyltransferase* (*aadA*) which confers streptomycin/spectinomycin tolerance, derived from *Escherichia coli* (Reference 25).
barstar	0.3	7140-6868	It encodes ribo-nuclease inhibitor (BARSTAR protein), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to BARNASE protein specifically, and inhibits its activity (Reference 31).
pVS1ori	3.8	7484-11258	It contains the replication origin of the plasmid pVS1 derived from *Pseudomonas sp*. (Reference 40).
pBRori	1.1	11260-12423	It contains the replication origin of the plasmid pBR322 derived from *Escherichia coli* (Reference 7).

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Figure 1 Nucleotide sequence of modified *bar* gene

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Figure 2 Nucleotide sequence of *barnase* gene

2) Function of component elements

(a) Functions of individual component elements of donor nucleic acid, including target gene, expression regulatory region, localization signal, and selective marker

Functions of component elements of donor nucleic acid which were used for the production of the recombinant oilseed rape MS8 are shown in Table 1.

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

[Function of 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 die.

On the other hand, in the plant body to which the modified *bar* gene is transferred, phosphinothricin acetyl transferase (PAT) is produced, and this enzyme acetylates the glufosinate to make N-acetylglufosinate. This action prevents the inhibition of glutamine-synthetase by the glufosinate, ammonia is not accumulated in the plant body, and the crop does not die even if it is sprayed with glufosinate (Figure 3).

The PAT protein exhibits a high affinity to glufosinate 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 95). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by PAT protein was never inhibited (Reference 100). As a result, it is considered that the PAT protein possesses high substrate specificity to glufosinate.

[Function of BARNASE protein]

The 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 the ribose hydroxyl (2'-OH group) 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 77).

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 production and during the growth of pollens. Therefore, it is considered that the absence of tapetum cells of the pollen is the major factor of resulting male sterility (Reference 44).

The *barnase* gene expresses the ribonuclease (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 RNA in the tapetum cells and thus the plants are unable to produce pollens (References 23, 32, 51). It is also shown that the *barnase* gene, under the control of the promoter PTA29, expresses stably even in the high-temperature condition (35-37℃) (Reference 3). It is not reported that the promoter PTA29 induces the expression of a temperature-dependent property.

In general, the first cross cultivar (F1 cultivar) possesses stronger and higher productivity compared to the fixed cultivar and excellent uniformity (Reference 47). 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 seed without fail by crossing the female strain (the recombinant oilseed rape MS8 which possesses *barnase* gene and not produces pollens) with the male strain [the oilseed rape which possesses *barstar* gene (*bar, barstar, Brassica napus* L., RF3, OECD UI: ACS-BNØØ3-6) (hereinafter referred to as "the recombinant oilseed rape RF3")]. In the F1 generation, pollen fertility is restored by the function of BARSTAR protein which inhibits the BARNASE protein (Reference 52), therefore, the seed production in high-yield by self-pollination becomes available.

[Allergenicity of PAT protein and BARNASE protein]

For the amino acid sequence of PAT protein and BARNASE protein, comprehensive homology search with known allergens (Swiss-Prot, PIR and HIV-AA), and allergen epitope search (short sequences with 8 amino acids) were conducted. Consequently, no homology with known toxins and allergens was observed.

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

[PAT protein]

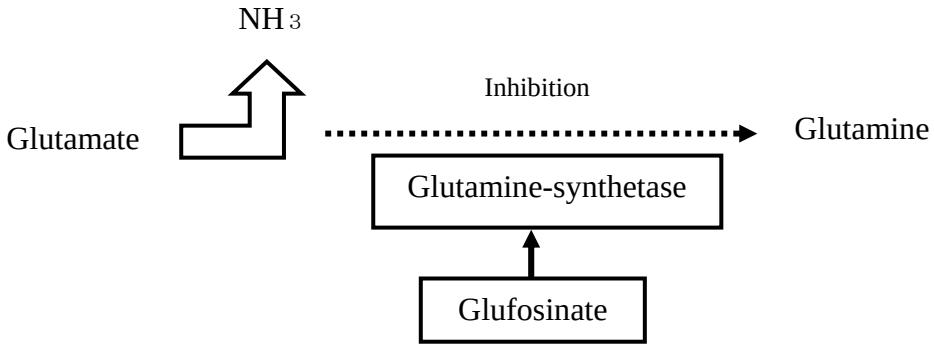
Since the PAT protein possesses high substrate specificity (Reference 95), it is considered that the PAT protein never causes any acetyl group transfer reaction to the compounds other than L-glufosinate. Therefore, it is considered that the PAT protein does not affect the metabolic system of the recipient organism.

[BARNASE protein]

The expression of the *barnase* gene is limited to the tapetum cells under the control of the promoter PTA29, and the *barnase* gene is not expressed in the other tissue (Reference 52). The tapetum cell mostly develops at the 4-tetrad stage of pollen production, and degrades/breaks along with the development of pollens (Reference 90). As a result of Northern blotting analysis conducted in the other countries, any products of the *barnase* gene were not detected in leaves, flower buds and seeds (detection limit: 0.5pg) (Annex 3). The result shows that the expression of the *barnase* gene is under the control of the promoter PTA29. 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 system of the plant body is extremely low.

A) Normal Plant

Since glufosinate herbicide inhibits glutamine synthetase, ammominia accumulates in the plant body, causing the plant to die.



B) Recombinant Plant

Glufosinate herbicide is acetylated and becomes N-acetylglufosinate by action of the PAT protein, and the inhibition of the glutamine-synthetase does not occur. Therefore, ammonia does not accumulate in the plant body, and the plant can grow.

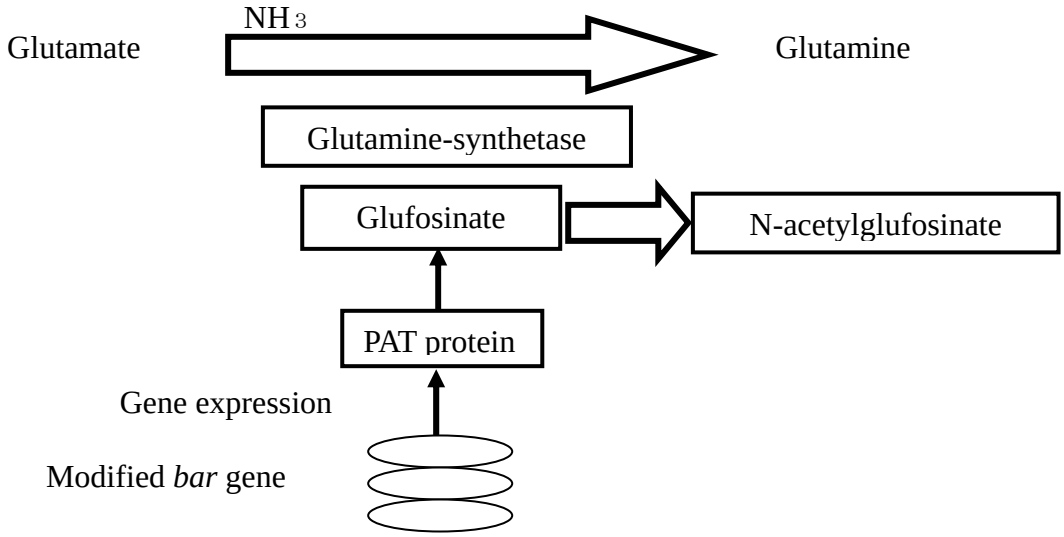


Figure 3 Mechanism of tolerance to glufosinate herbicide by the product of modified *bar* gene

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

(2) Information concerning vectors

1) Name and origin

The vector used for the production of the recombinant oilseed rape MS8 is the binary Ti plasmid vector pTHW107, which is produced from the vector pGSV1 derived from *Escherichia coli* (Reference 16).

2) Properties

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

The total number of base pairs of the plasmid pTHW107 is 12,622bp. Figure 4 shows the map of the plasmid. In addition, the entire nucleotide sequence is shown in Annex 4.

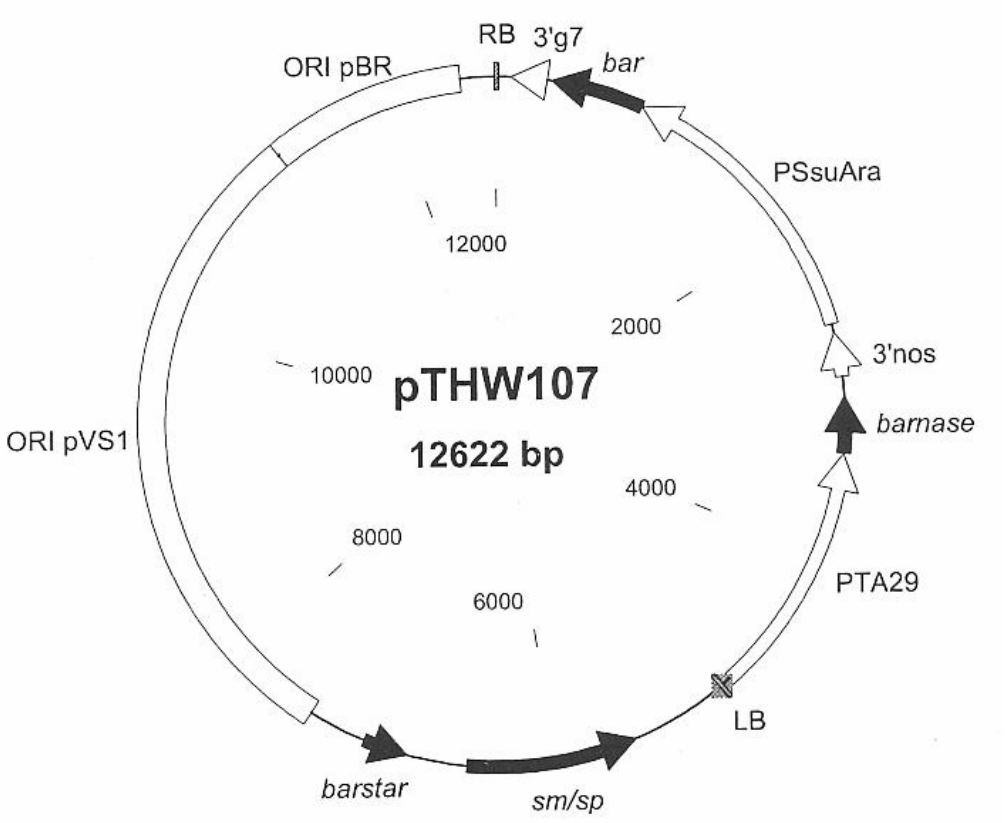


Figure 4 Physical Map of plasmid pTHW107

The "bar" refers to the modified *bar* gene.

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

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

The plasmid pTHW107 possesses the streptomycin/spectinomycin tolerance gene (Sm/Sp), the *barstar* gene, the pBRori and the pVS1ori. The Sm/Sp gene was used as a selectable marker for the vector. At the time of introducing the *barnase* gene to the plasmid by using *E. coli*, 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 existing in the basic plasmid used for constructing the plasmid pTHW107 was used for inhibiting the above mentioned *barnase* activity. The pBRori and the pVS1ori are replication origins, which they function to causeautonomous replication in the *E. coli* and the *Pseudomonas aeruginosa*, respectively. The pBRori and the pVS1ori locate outside the T-DNA region, and they are not transferred into the oilseed rape genomes. These facts are confirmed by the Southern blotting analysis using three proves which cover the region containing each sequence (Annex 5).

(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 plasmid pTHW107 is limited to *Agrobacterium tumefaciens*, *Escherichia coli* and a few gram-negative bacteria, and the plasmid pTHW107 does not possess the infectious characteristics.

(3) Method of preparing living modified organisms

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

For the production of the recombinant oilseed rape MS8, the plasmid pTHW107 was used, in which the *barnase* gene expression cassette and the modified *bar* gene expression cassette ([PTA29]-[*barnase*]-[3'nos]-[PSsuAra]-[modified *bar*]-[3'g7]) was located between the LB and the RB on the vector.

The position and direction of component elements of the nucleic acid in the vector is shown in Figure 4. In addition, the restriction enzyme cleavage site is shown in Figure 5.

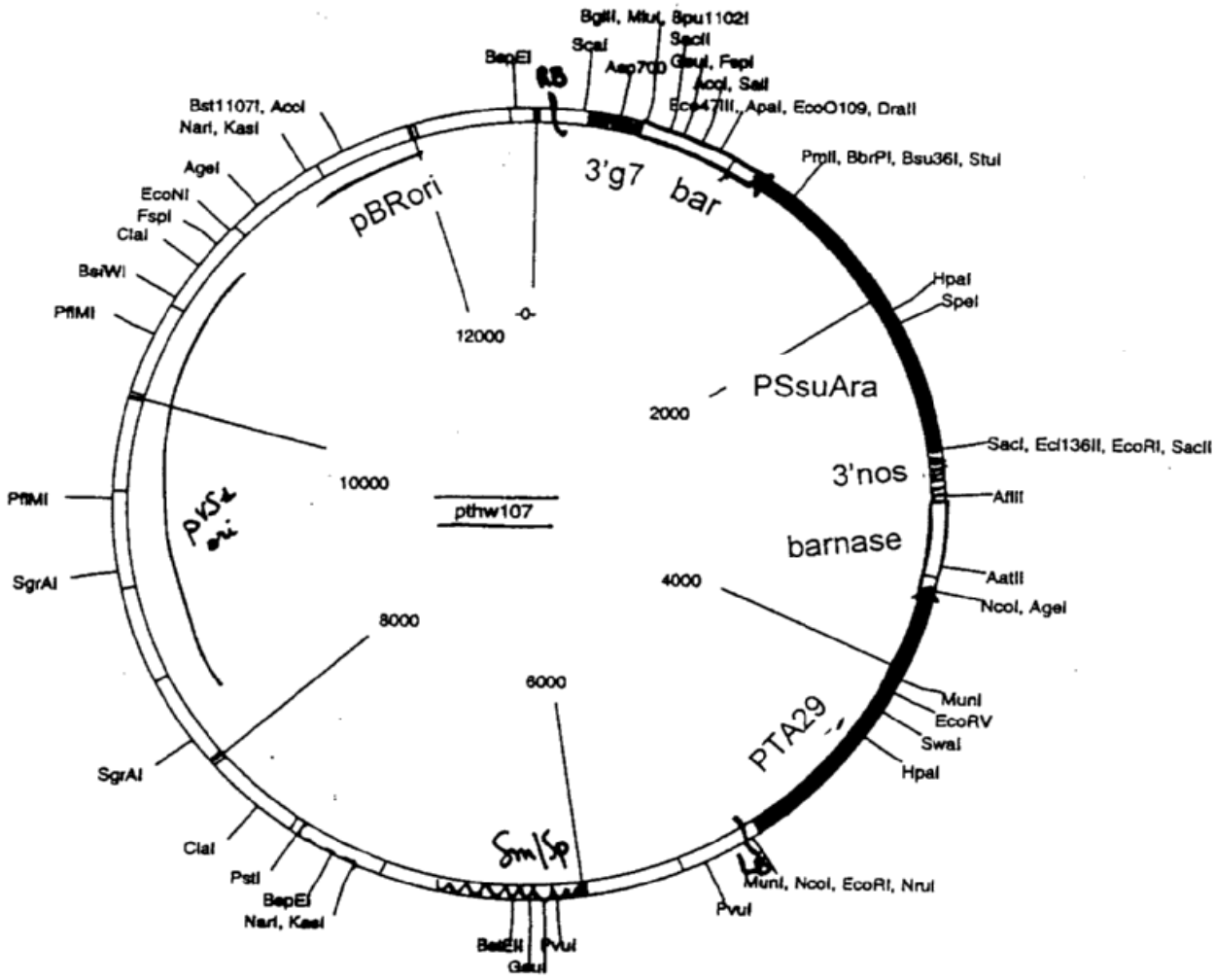


Figure 5 The restriction enzyme cleavage site of pTHW107

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

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

The *Agrobacterium*-mediated transformation method was used for transferring gene to the recipient organism (Reference 17). The *E.coli* MC1061 strain which possesses the plasmid pTHW107、the *E.coli* HB101 strain which possesses the transferable (helper) plasmid pRK2013, and the non-oncogenic *Agrobacterium tumefaciens* C58C1Rif^{R} strain were coexistent. After the *A.tumefaciens* C58C1Rif^{R} strain which possesses the plasmid pTHW107 was produced, a piece of hypocotyl cell of the recipient organism was infected with it, and the T-DNA region between the RB and the LB on the pTHW107 was transferred into the oilseed rape genomes (Reference 21).

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 cell was cultivated in a solid medium containing glufosinate herbicide. Then, the cell which showed the glufosinate-tolerance due to the expression of the modified *bar* gene was selected. In addition, it was moved to a hormone-free medium and regenerated to the plant body (Reference 17).

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

After transformation by the *Agrobacterium*, 500mg/L of Carbenicillin was added to the medium and the remaining *Agrobacterium* was removed (Reference 17).

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line to which confirmed the state of existence of replication products of transferred nucleic acid, 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 was comprehensively examined for the tolerance to glufosinate herbicide, male sterility, and other agricultural characteristics. Then, the recombinant oilseed rape MS8 was selected. The pedigree tree of the recombinant oilseed rape MS8 is shown in Figure 6.

The approvals of the recombinant oilseed rape MS8 received from organizations in Japan are as follows.

[Food safety]

In December 1999, based on the “Guideline 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 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]

In February 1999, based on the “Guideline for feed safety Assessment of recombinant feed”, the compatibility to the guideline 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 1999, based on the “Guideline for the use of recombinant in agriculture, forestry and fisheries”, conducting isolated field test was approved by the Ministry of Agriculture, Forestry and Fisheries. In addition, in November 2002, based on the “Guideline for the use of recombinant in agriculture, forestry and fisheries”, the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

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Figure 6 Pedigree tree of the recombinant oilseed rape MS8

(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 the recombinant oilseed rape MS8 (the original transformant) is heterozygotes for the transferred gene locus. Therefore, it is expected that in the BC1 generation raised by backcrossing of the non-recombinant control oilseed rape, a segregation ratio of 1:1 would be obtained between glufosinate-tolerant and glufosinate-sensitive individuals. In addition, in the recombinant oilseed rape MS8, pollens are not produced due to the expression of the *barnase* gene. Therefore, the seeds were produced by crossing with the non-recombinant control oilseed rape. A segregation ratio between the recombinant

body and the non-recombinant body in the seeds is 1:1, therefore it is expected that the segregation ratio of 1:1 would be obtained between glufosinate-tolerant and glufosinate-sensitive individuals in the F1, BC1F1 and BC2F1 generations. As a result of glufosinate herbicide (Ignite)-spraying tests using the F1, BC1F1 and BC2F1 generations to examine the segregation ratio for their tolerance, about 50% of individuals in all generations showed the glufosinate tolerance (Annex 3, Tables PC24 and PC33). Consequently, it is considered that the T-DNA region transferred into the recombinant oilseed rape MS8 is existing in a chromosome of the oilseed rape genome.

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 the recombinant oilseed rape MS8 (BC3 generation) was cleaved by the restriction enzymes Apa I/Nsi I, Hind III/EcoR I, Nsi I and BamH I/Hind III, and Southern blotting analysis using 3 types of probes (modified *bar*, PSsuAra, PTA29) which cover the entire region of T-DNA. As a result, the expected fragment was obtained in each analysis, and it was confirmed that the one copy of modified *bar* gene and one copy of *barnase* gene were transferred in the recombinant oilseed rape MS8 (Annex 3). In addition, as a result of determination of nucleotide sequence, it was proved that the *barnase* gene expression cassette and the modified *bar* gene expression cassette linked were transferred in linked-state (Annex 6).

Moreover, in order to confirm the stability of transferred gene in multiple generations, Southern blotting analysis was conducted for the DNA obtained from the BC1, BC3 and BC1F1 generations of the recombinant oilseed rape MS8. As a result, the identical fragments were detected in each generation, and the stability of the transferred gene in multiple generations was confirmed (Annex 3, Figure 2).

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

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

Northern blotting analysis was conducted for the RNA derived from leaves, flower buds and dried seeds of the recombinant oilseed rape MS8 (BC3 generation) to examine the expression of the modified *bar* gene and the *barnase* gene. As a result, the products of the modified *bar* gene were detected in leaves and flower buds, but it was under the detection limit in dried seeds (detection limit: 0.5pg). In addition, the products of the *barnase* gene were not detected in any tissues (detection limit: 0.5pg) (Annex 3).

The recombinant oilseed rape MS8 does not produce pollens due to the expression of the *barnase* gene. Therefore, seeds were produced by backcrossing with the non-recombinant control oilseed rape (Drakkar cultivar), and a segregation ratio between the recombinant body and the non-recombinant body in the seeds is 1:1. As mentioned in the “1) Place where the replication product of transferred nucleic

acid exists", as a result of glufosinate herbicide (Ignite)-spraying tests using the BC1, F1, BC1F1 and BC2F1 generations of the recombinant oilseed rape MS8 line to examine the segregation ratio for their tolerance, about 50% of individuals showed the glufosinate tolerance in all generations (Annex 3, Tables PC24 and PC33). In addition, in the BC2F1 and BC4F1 generations obtained by the backcrossing of the recombinant oilseed rape MS8 with the Cultivar A, the appearance rate of male-sterile strain was 100% of the strain which showed glufosinate tolerance (Annex 3).

Since the modified *bar* gene and the *barnase* gene is transferred in linked-state, it is considered that the characteristics of glufosinate tolerance and male sterility are inherited as one set. In practice, the individual which indicates the separation of these two gene expression cassettes was not found during the production and examination up to now.

Overall, based on the results discussed above, it was confirmed that the characteristic conferred by the transferred nucleic acid is stably expressed under a natural environment.

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

The recombinant oilseed rape MS8 contains 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

Specific detection method for the recombinant oilseed rape MS8 is available by PCR method using its flanking sequences of transferred DNA as primers. This PCR method is utilized effectively for cultivation management of the recombinant oilseed rape MS8 in general (Annex 7).

(6) Difference from the recipient organism or the 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

With the production of the PAT protein, tolerance to glufosinate herbicide is conferred to the recombinant oilseed rape MS8. With the production of the BARNASE protein, pollen production is inhibited and male sterility is conferred to the recombinant oilseed rape MS8.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between recombinant crop and the taxonomic species to which the recipient organism belongs, and the degree of difference, if any

In FY 1999, isolated field tests were conducted at the Vegetable Breeding Department, National Institute of Vegetable and Tea Science, National Agriculture Research Center, for comparison between the recombinant oilseed rape MS8 (BC5 generation) and the recipient organism, Drakkar cultivar (hereinafter referred to as "the non-recombinant control oilseed rape") (Annex 1). In the test plots of the recombinant oilseed rape MS8, there are the male-sterile strain (the recombinant body) and male-fertile strain (the non-recombinant body) showing a segregation ratio of 1:1. Therefore, the individuals which do not produce pollens were selected at flowering period, and the male-sterile strain and male-fertile strain of the recombinant oilseed rape MS8 were separately examined. Hereinafter, the recombinant oilseed rape MS8 refers to the male-sterile strain. In addition, the tests for productivity of harmful substances of the recombinant oilseed rape MS8 (BC5 generation) were conducted at the special screened greenhouse in Japan in FY 2005 (Annex 2).

(a) Morphological and growth characteristics

The comparison was made between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape for time of bolting, flowering period, maturation period, the number of primary branches, plant height, plant shape or plant type, color of leaves, the number of pods formation, the number of pods unformed per an ear, the number of ovules per a pod, the rate of pods formation , length of pod, the number of seed setting, the rate of seed setting, fresh weight of aerial part, dry weight of aerial part, the rate of dry matter, property of open pods, seed yield, 1000-seeds weight, color of seed and the uniform excellence of seedss. As a result, the significant difference was found between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape only for the rate of pods formation (Annex 1, Table 2-3). It is considered that the difference in the rate of pods formation was caused by the fact that the oilseed rape produces seeds mainly by self-pollination, but the recombinant oilseed rape MS8 cannot produce pollens resulting in no self-pollination.

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

In the isolated field tests, wintering ability (survival rate) of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape sowed in October 6, 1998 was both 100% at the observation in March 5, 1999 (Annex 1, Table 3). The average temperature of January and February was about 5℃, the highest temperature was about 10℃, and the lowest temperature was sometimes about 0℃ (Annex 1, Figure 1 of Weather map). Based on the above, it is considered that the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape both show cold-tolerance at the early stage of growth.

In the special screened greenhouse tests conducted in FY 2005, the comparison for the dry weight was made between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape sowed in July 27, 2005 in the greenhouse which is controlled only with natural ventilation and harvested in September 21, 2005, no significant difference was observed between them (Annex 2, Tables 7-10). Based on the above, heat-tolerance of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape at the early stage of growth is considered to be equal.

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

The recombinant oilseed rape MS8 and the non-recombinant control oilseed rape sowed in October, 1998 and cultivated in the isolated field were left after the maturation period in June, 1999, and were observed in July 30, 1999. As a result, all lines dried and all individuals died. Consequently, summer survival of the matured plants of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape was not found (Annex 1, Table 3).

(d) Fertility and size of the pollen

The flowers of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape were collected at flowering period, and the presence of pollens was confirmed. As a result, it was confirmed that there is no pollens in the recombinant oilseed rape MS8 (Annex 1).

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

Among the items examined in the isolated field [described in "(a) Morphological and growth characteristics"], the following items were examined for the production of seeds; the number of pods formation, the number of pods unformed per an ear, the number of ovules per a pod, the rate of pods formation, the number of seed setting, the rate of seed setting (the number of seed setting/the number of ovules per a pod), seed yield and 1000-seeds weight. The comparison was made between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape for each items. As a result, the rate of pods formation was low in the male-sterile strain, and the significant difference was found between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape (Annex 1, Table 2-3). It is considered that the difference in the rate of pods formation was caused by the fact that the oilseed rape produces seeds mainly by self-pollination, but the recombinant oilseed rape MS8 which does not produce pollens cannot conduct self-reproduction.

Regarding the property of open pods of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape, the difficulty level was evaluated in 5-stage (1: most difficult- 5: most easy). As a result, both showed level 4 (relatively easy), and it is considered the shedding habit is equivalent for the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape (Annex 1).

To evaluate the germination rate, seeds obtained from the recombinant oilseed rape MS8 cultivated in the isolated field were examined. As a result, the germination rate was 100% (Annex 1, Table 4). Consequently, it is considered that the seeds of the recombinant oilseed rape MS8 do not possess dormancy.

(f) Crossability

The recombinant oilseed rape MS8 is conferred male sterility and does not produce pollen (Annex 1). Therefore, crossing with pollens of the recombinant oilseed rape MS8 never occurs. On the other hand, since female gamete of the recombinant oilseed rape MS8 is normal, cross-fertilization would occur with surrounding pollens. In the isolated field test, the recombinant oilseed rape MS8 showed the lower rate of pods formation than that of the non-recombinant control oilseed rape (Annex 1, Table 2-3). In addition, for the grain yield per a strain, there was no significant difference between the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape, but the recombinant oilseed rape MS8 showed slightly lower value compared to the non-recombinant control oilseed rape (Annex 1, Table 2-4). Based on the above understanding, it is considered that the productivity of the seed for the recombinant oilseed rape MS8 would be extremely low under certain surrounding condition.

(g) Productivity of harmful substances

In order to check the substances excreted from the roots of the recombinant oilseed rape MS8 which can affect other plants, the substances exist in the plant body which can affect other plants after dying, the substances excreted from the roots which can affect microorganisms in soil, succeeding crop test, plow-in test, and soil microflora test were carried out, respectively, at the special screened greenhouse in 2005 (Annex 2).

Succeeding crop test : After cultivating the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape, radishes were cultivated as test samples in the soil respectively, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight. As a result, there was no significant difference confirmed in any items (Annex 2, Tables 1-6).

Plow-in test : The dried powder of plant body of the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape 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 significant difference confirmed in any items (Annex 2, Tables 7-14).

Soil microflora test : The soil was obtained after cultivating the recombinant oilseed rape MS8 and the non-recombinant control oilseed rape for about 2 months, and was diluted by adding sterilized phosphate buffer solution. Bacteria and Actinomyces were cultivated in PTYG medium, and mold fungi were cultivated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, there was no significant

difference confirmed in any items (Annex 2, Tables 15-17).

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.

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 could grow voluntarily in the environments such as roadsides and abandoned factory sites maintained with regular care though it could not survive the competition with perennial wild plants and thus it is difficult to grow voluntarily under a natural environment left almost uncontrolled, therefore it is not regarded as an aggressive introduced species which could exterminate Japanese native species of plants and cause Adverse Effect on Biological Diversity.

This recombinant oilseed rape 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, and (ii) a trait to be male sterile, and it is generally considered that this recombinant oilseed rape cannot produce posterity by self-pollination. Therefore, it is judged that this recombinant oilseed rape would not become competitive under a natural environment.

In addition, the traits for competitiveness of this recombinant oilseed rape were examined in the isolated field in Japan, and this recombinant oilseed rape was found to show the significant low value for the rate of pods formation compared to the non-recombinant control oilseed rape. However, there was no significant difference for the other items examined. Therefore, it is judged that this recombinant oilseed rape would not 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

Conventional oilseed rape is known to produce erucic acid and glucosinolate in the seeds recognized as harmful substances to human and other mammals. The cultivar of the recipient organism of this recombinant oilseed rape is one of the canola cultivar, in which the erucic acid and glucosinolate content was reduced by breeding. In the

componential analysis of this recombinant oilseed rape, it was confirmed that the erucic acid and glucosinolate content is within the range of canola cultivar.

This recombinant oilseed rape produces the modified PAT protein which confers the trait to be tolerant to glufosinate, and the BARNASE protein which is the RNA-degrading enzyme.

There is no report that the PAT protein possesses Adverse Effect to wild animals and wild plants. In addition, it is suggested that the PAT protein possesses high substrate specificity. Therefore, it is considered that the PAT protein does not affect the metabolic system of the recipient organism.

Moreover, regarding the morphological and growth characteristics, this recombinant oilseed rape which is given male sterility showed the difference only for the rate of pods formation compared to the non-recombinant control oilseed rape, therefore it is considered that the BARNASE protein (RNA-degrading enzyme) does not affect the metabolic system of cells other than anther.

In addition, for the amino acid sequences of PAT protein and BARNASE protein, comprehensive homology search and allergen epitope homology search were conducted. Consequently, no homology with known allergen was observed.

The tests have been conducted to check the harmful substances productivity (the substances excreted from the roots of the recombinant oilseed rape MS8 which can affect other plants, the substances excreted from the roots which can affect microorganisms in soil, and the substances exist in the plant body which can affect other plants after dying), at the special screened greenhouse in Japan. As a result, there is no significant difference between this recombinant oilseed rape and the non-recombinant control oilseed rape.

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 the related species that can be crossed with oilseed rape 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) this recombinant oilseed rape does not produce pollens due to the male sterility, and can not produce posterity as a pollen parent. In addition, this recombinant oilseed rape can produce progeny only if it is as a seed parent. In this case, a) regarding the crossability with oilseed rape, it is reported that the cross-progeny of the oilseed rape which possesses male sterility as dominant trait would decrease its population rapidly over generations, and b) 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; and

(ii) as discussed in (i), it is judged that this recombinant oilseed rape which possesses glufosinate tolerance and male sterility would not become competitive under a natural environment.

Consequently, it is judged that the possibility of hybrid obtained by crossing would become competitive and exterminate the population of the other wild animals and wild plants is extremely low. In addition, based on the same reason, it is judged that the possibility of transferred gene of this recombinant oilseed rape spread by crossing would decrease the related species population, and wild animals and wild plants such as insects which are dependent on the related species would be affected for maintenance of their population, is extremely 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 recombinant oilseed rape in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex List

Annex 1：Isolated field test report

Confidential: Not made available or disclosed to unauthorized person

Annex 2：Test report for productivity of harmful substances

Confidential: Not made available or disclosed to unauthorized person

Annex 3：Material based on the result of environmental safety test for the male-sterile line oilseed rape (recombinant oilseed rape MS8) conducted outside Japan

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Annex 4：Nucleotide sequence of plasmid pTHW107
(pTHW107 vector sequence)

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Annex 5：Southern blotting analysis of genes existing outside of T-DNA region

(MS8 – Proof of absence of sequences derived from the ‘vector’-part of the construct.)

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Annex 6：Determination of transferred nucleic acid in the recombinant oilseed rape MS8
(Determination of inserted transgenic sequences in *Brassica napus* elite event MS8)

Confidential: Not made available or disclosed to unauthorized person

Annex 7：Event identifying method

Confidential: Not made available or disclosed to unauthorized person

Annex 8：Erucic acid and glucosinolate content of seed

Confidential: Not made available or disclosed to unauthorized person