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

Name: Bayer Crop Science K.K.
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Glufosinate herbicide tolerant and fertility restored oilseed rape (Modified *bar, barstar, Brassica napus* L.) (RF3, OECD UI :ACS-BNØØ3-6)
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 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 herbicide tolerant and fertility restored oilseed rape (modified *bar, barstar, Brassica napus* L., RF3, OECD UI :ACS-BNØØ3-6) (hereinafter referred to as "the recombinant oilseed rape RF3") 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 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 and *barstar* 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	Origin and function
barstar gene expression cassette			
PTA29	1.51	4763-3254	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 79).
barstar	0.27	3253-2981	It encodes ribo-nuclease inhibitor (hereinafter referred to as "BARSTAR protein"), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to ribo-nuclease, the product of *barnase* gene, (hereinafter referred to as "BARNASE protein") specifically, and inhibits its activity (Reference 31).
3'nos	0.26	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	1.73	2608-883	It is derived from *Arabidopsis thaliana*, and the promoter of rubisco small subunit gene. It induces expression selectively in the chlorenchyma (Reference 47).
Modified *bar*	0.55	882-331	It encodes phosphinothricin acetyl transferase (Modified PAT protein) derived from *Streptomyces hygroscopicus* and confers tolerance to glufosinate herbicide (Reference 92). The two codons (GTG, AGC) in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC, respectively.
3'g7	0.21	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 96).
Additional information			
LB	0.02	4809-4833	It is the left border of the T-DNA derived from pTiB6S3.
RB	0.02	1-25	It is the right border of the T-DNA derived from pTiB6S3.
Sm/Sp	1.01	5393-6403	It encodes *aminoglycoside adenyltransferase* (*aadA*) which confers streptomycin/spectinomycin tolerance, derived from *Escherichia coli* (Reference 24).
barstar	0.27	6754-7026	It encodes ribo-nuclease inhibitor (BARSTAR protein), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to ribo-nuclease, the product of *barnase* gene specifically, and inhibits its activity (Reference 31).
pVS1ori	3.77	7374-11145	It contains the replication origin of the plasmid pVS1 derived from *Pseudomonas sp*. (Reference 40).
pBRori	1.06	11146-12209	It contains the replication origin of the plasmid pBR322 derived from *Escherichia coli* (Reference 6).

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Figure 1 Nucleotide sequence of modified *bar* gene

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Figure 2 Nucleotide sequence of *barstar* 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 RF3 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

[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 die.

On the other hand, in the plant body to which the modified *bar* gene is transferred, phosphinothricin acetyl transferase (Modified PAT protein) 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 Modified 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 92). 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.

[BARSTAR protein]

BARSTAR protein is an intracellular inhibitor for BARNASE protein (ribo-nuclease), which is a product of *barnase* gene isolated from the bacterium *Bacillus amyloliquefaciens* (References 29 and 32). 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 30, 32, and 85). The BARNASE protein produced by the *barnase* gene under the control of the promoter PTA29 degrades RNA in the tapetum cell layer of the anther and destructs the cells, thereby expressing the trait of male sterility. The mechanism of restoration of fertility from the male-sterile trait by the recombinant oilseed rape RF3 is illustrated in Figure 4.

In general, the first cross cultivar (F1 cultivar) possesses stronger and higher productivity compared to the fixed cultivar and excellent uniformity (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 oilseed rape which possesses *barnase* gene to express specifically in the tapetum cell layer of the anther and inhibit the production of pollens (Reference 50) [glufosinate herbicide tolerant and male sterile oilseed rape (modified *bar, barnase, Brassica napus* L., MS8, OECD UI :ACS-BNØØ5-8) (hereinafter referred to as "the recombinant oilseed rape MS8")] with the male strain (the recombinant oilseed rape RF3 which possesses the trait to restore the fertility). 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 modified PAT protein and BARSTAR protein]

For the amino acid sequence of modified PAT protein and BARSTAR protein, homology search with known allergens in the database (Swiss Prot, PIR and HIV-AA) was conducted. In addition, shorter allergen epitope search (shorter sequences with 8 amino acids) was also conducted. Consequently, in the both searches, no homology with known toxins and allergens was observed.

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

[Modified PAT protein]

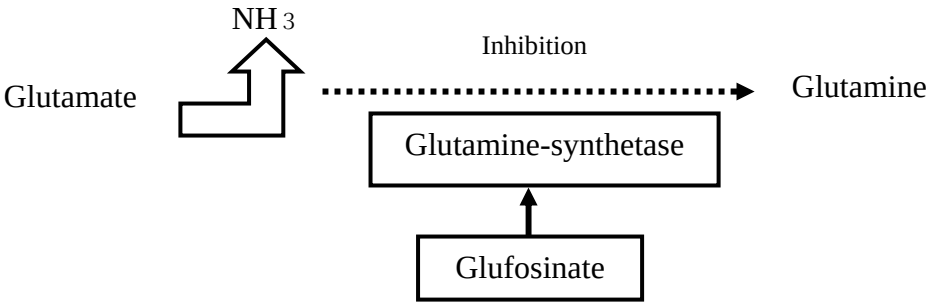
Since the modified PAT protein possesses high substrate specificity (Reference 92), it is considered that the PAT protein never causes any acetyl group transfer reaction to the compounds other than glufosinate. Therefore, it is considered that the modified PAT protein does not affect the metabolic system of the recipient organism.

[BARSTAR protein]

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). In addition, 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 BARSTAR 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). Its homology with the amino acid sequence of BARNASE protein is only 20 to 25%, though there is a report that the extra-cellular ribo-nuclease of Streptomyces, which has the similar protein structure, (Reference 35) is also inhibited by the BARSTAR protein (Reference 33). 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 31, 32, 35 and 85). Based on the above understanding, it is considered that BARSTAR protein would not affect the metabolic system of recipient organism.

A) Normal Plant

Since glufosinate herbicide inhibits glutamine synthetase, ammonia 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 modified 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.

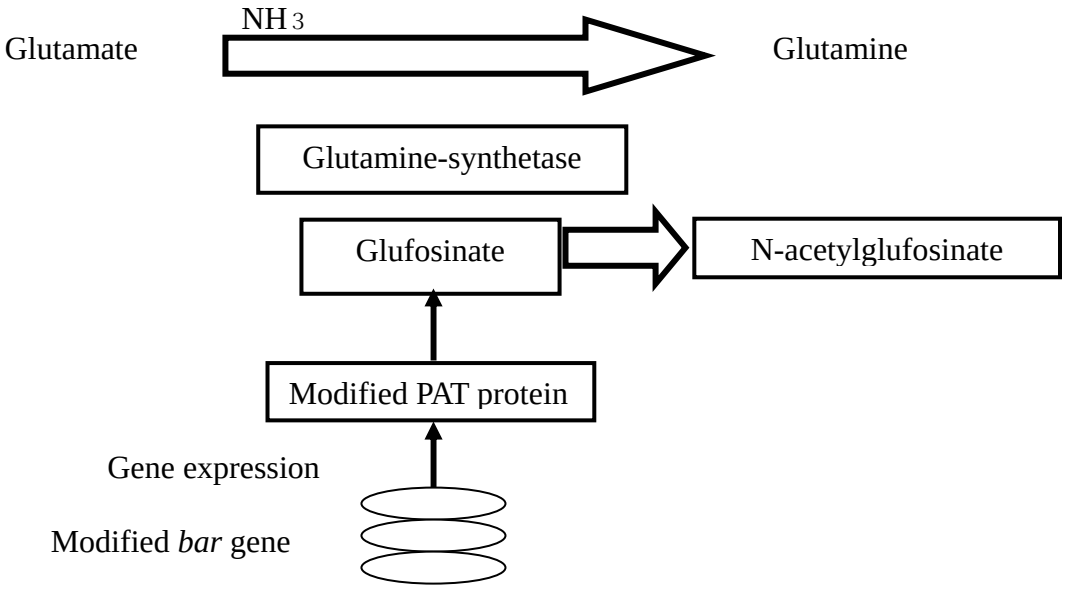


Figure 3 Mechanism of tolerance to glufosinate herbicide by the product of modified *bar* gene

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

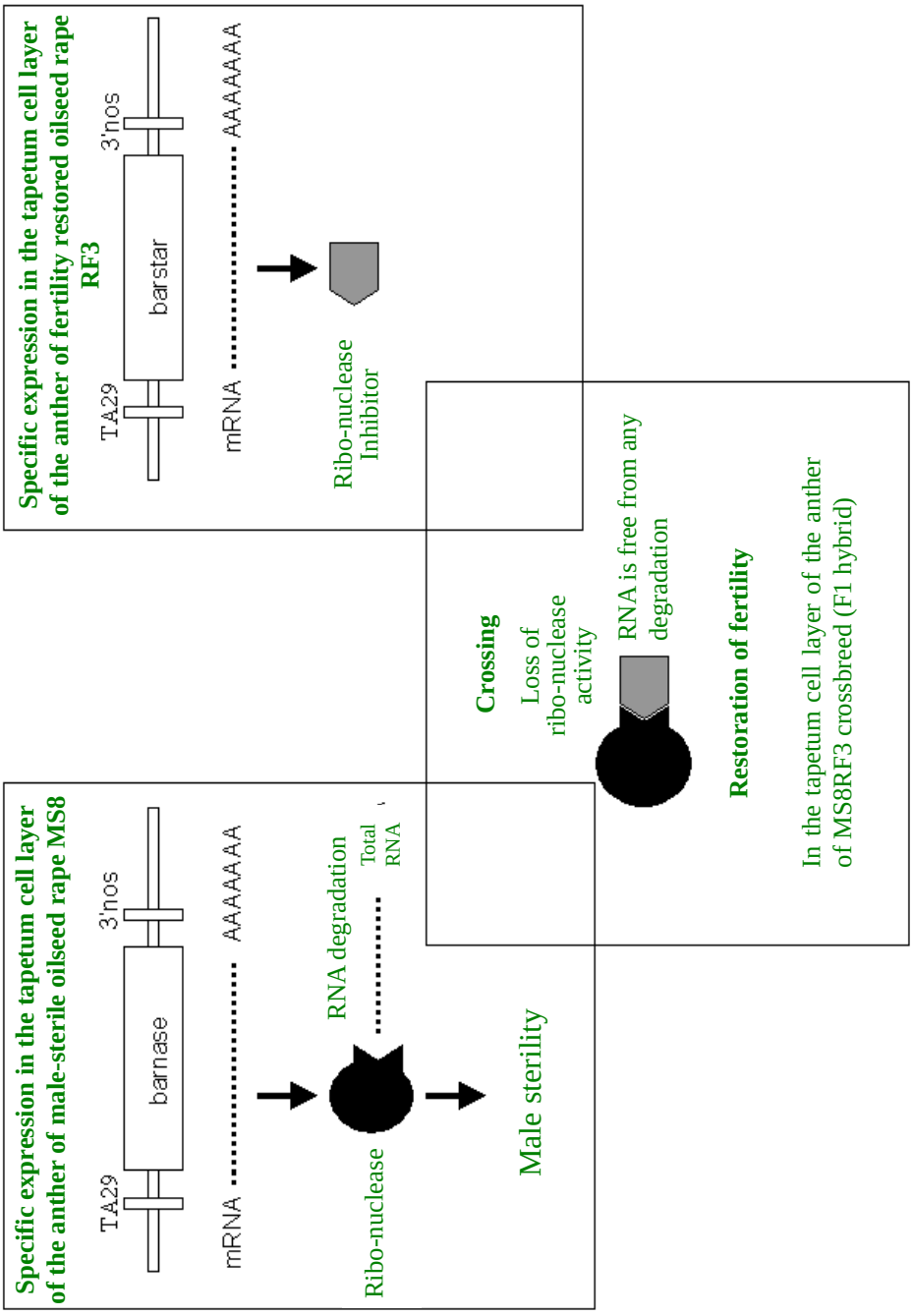


Figure 4 Mechanism of restoration of fertility by the product of *barstar* gene

In the case of crossing the female strain (male-sterile recombinant oilseed rape MS8) with the male strain (recombinant oilseed rape RF3)

(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 RF3 is the binary Ti plasmid vector pTHW118, which is produced from the vector pGSV1 derived from *Escherichia coli* (Reference 15).

2) Properties

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

The total number of base pairs of the plasmid pTHW118 is 12,508bp. Figure 5 shows the map of the plasmid. In addition, the entire nucleotide sequence is shown in Annex 6.

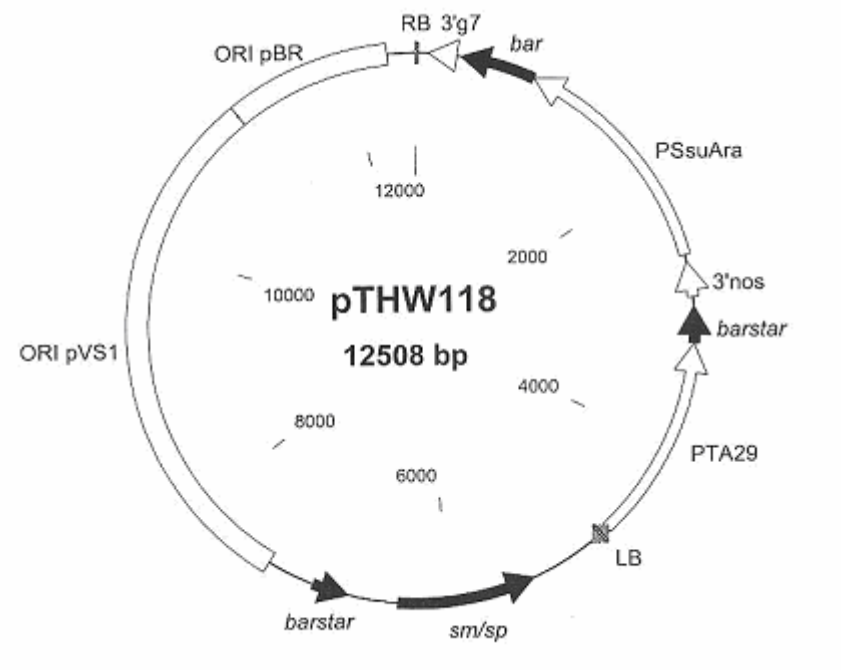


Figure 5 Physical Map of plasmid pTHW118

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 pTHW118 possesses the streptomycin/spectinomycin tolerance gene (Sm/Sp), the *barstar* gene, the pBRori and the pVS1ori outside the T-DNA region. The Sm/Sp gene was used as a selectable marker for the vector, though it expresses only in the bacteria and it does not express in any plant cells (References 16 and 95). In addition, the *barstar* gene has been present in the basic plasmid pGSV1 used for constructing this plasmid pTHW118, and the pBRori and the pVS1ori are replication origins, which function to cause autonomous 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 proofs which cover the region containing each sequence (Annex 7).

(c) Presence or absence of infectious characteristics of vector and, if present, the information concerning the range of recipient organisms

It is known that the range of recipient organisms for the autonomous replication of plasmid pTHW118 is limited to *Agrobacterium tumefaciens*, *Escherichia coli* and a few gram-negative bacteria, and the plasmid pTHW118 does not possess the infectious characteristics in plant bodies.

(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 RF3, the plasmid pTHW118 was used, in which the *barstar* gene expression cassette to confer the trait of fertility restoration and the modified *bar* gene expression cassette (PTA29-*barstar*-3'nos-PSsuAra-modified *bar*-3'g7) to confer the trait of glufosinate herbicide tolerance were located between the LB and RB on the vector.

The position and direction of component elements of the nucleic acid in the vector is shown in Figure 5. In addition, the restriction enzyme cleavage site is shown in Figure 6.

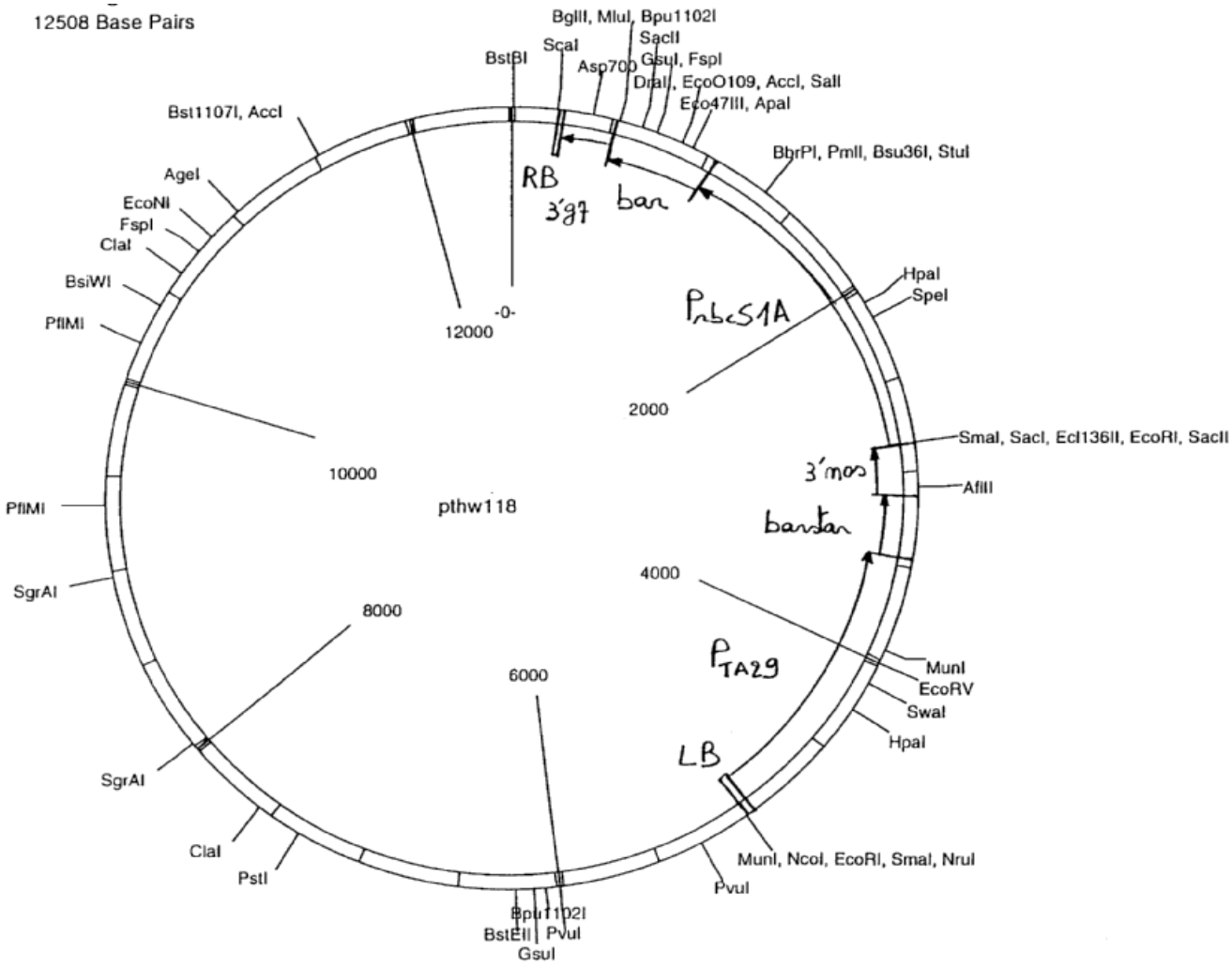


Figure 6 The restriction enzyme cleavage site

The "*bar*" refers to the modified *bar* gene, and the "PrbcS1A" refers to the PSsuAra.

(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 pTHW118, 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 pTHW118 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 pTHW118 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 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 RF3 was produced. The pedigree tree of the recombinant oilseed rape RF3 is shown in Figure 7.

The approvals of the recombinant oilseed rape RF3 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, 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, 2003.

[Environmental safety]

In 1998, 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 7 Pedigree tree of the recombinant oilseed rape RF3

(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 RF3 (the original gene transformant) is heterozygote for the transferred gene locus. Therefore, it is expected that in the S1 generation raised by self-fertilization, a segregation ratio of 3:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals. In addition, it is expected that the glufosinate-tolerant individuals would contain homozygote and heterozygote in a proportion of 1:2.

As a result of examination on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals in the S1 of recombinant oilseed rape RF3, the segregation ratio showed a good agreement with the theoretical segregation ratio of 3:1 (Table 2). In addition, as a result of investigation on the number of glufosinate-tolerant plants in the S2 generation individuals raised by self-fertilization of S1 generation individuals exhibiting the tolerance to glufosinate herbicide, the ratio of approx. 1:2 was observed between the S1 individuals confirmed to exhibit the fixation of glufosinate tolerance in the S2 generation and

the S1 individuals in the S2 generation showing the segregation ratio in agreement with 3:1 in the progeny. It is expected that the gene locus would be homozygote for the former S1 strain and heterozygote for the latter (Table 3). Consequently, it is considered that the T-DNA region transferred into the recombinant oilseed rape RF3 exists in a chromosome of the oilseed rape genome.

Table 2 Segregation of glufosinate-tolerant strains in the S1 generation

Plant examined	Total number of strains examined	Number of glufosinate-tolerant strains	Chi^2
S1 generation plants	38	31	Chi^2 (1/4 glufosinate-sensitive) NS

NS：No significant difference

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

Table 3 Segregation of glufosinate-tolerant strains in the S2 generation obtained from self-fertilization of S1 generation glufosinate-tolerant plants

S1 generation ID No.	Total number of strains examined	Number of glufosinate-tolerant strains	Expected type of zygote*
1	51	38	Hetero
2	54	54	Homo
3	55	39	Hetero
4	57	57	Homo
5	52	44	Hetero
6	50	50	Homo
7	45	45	Homo
8	53	45	Hetero
9	50	38	Hetero
10	53	39	Hetero
11	50	50	Homo
12	48	48	Homo
13	53	38	Hetero
14	50	37	Hetero
15	52	40	Hetero
16	52	41	Hetero

* Regarding the transferred gene locus, type of zygote was estimated.

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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, Southern blotting analysis (Annex 3, Table 1; Figures 2a to 2d), PCR method and sequence analysis (Annex 4) were conducted. As a result, it was confirmed that one complete copy of T-DNA region and one incomplete copy of T-DNA region in which modified *bar* gene is not contained were transferred.

In addition, in order to confirm the stability of inheritance of transferred nucleic acid, Southern blotting analysis was conducted for the DNA obtained from the S1, S3 and BC1F1 generations of the recombinant oilseed rape RF3, cleaved by the restriction enzyme EcoRV which contains a cleavage site in the T-DNA region (Figure 6), using PTA29 as a probe. As a result, the identical band pattern was observed in all the generations, and it was confirmed that the transferred gene is inherited stably in multiple generations (Annex 3, Figure 2).

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

In order to identify the position relationship between 2 copies of T-DNA transferred to the recombinant oilseed rape RF3, PCR and sequence analysis were conducted. As a result, it was found that one copy of complete T-DNA region and one copy of incomplete T-DNA region are arranged opposite to each other in the repeated structure (Annex 4). In addition, in the incomplete T-DNA region, the halfway-broken PTA29, *barstar* gene, 3'nos and the PSsuAra containing no functional parts are arranged. Their position relationship is shown in Figure 8.

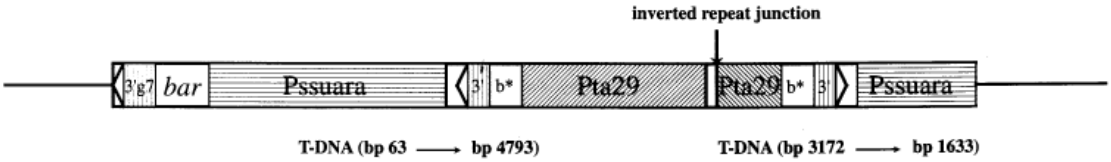

Figure 8 Map of the entire T-DNA region transferred to the recombinant oilseed rape RF3

The "bar" refers to the modified *bar* gene, "b*" refers to the *barstar* gene, and "3'" refers to the 3'nos.

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

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, pollens and dried seeds of the recombinant oilseed rape RF3 (S3 generation), using the modified *bar* and the *barstar* as probes. As a result, the modified *bar* mRNA was detected in leaves and flower buds, but it was under the detection limit in pollens and dried seeds (detection limit: 0.25pg). In addition, the *barstar* mRNA was detected only in flower buds (detection limit: 0.5pg) (Annex 3).

In addition, as a result of examination of S1 and F1 generations for the tolerance to glufosinate herbicide, all the individuals exhibited the glufosinate tolerance in all the generations examined. Consequently, it was confirmed that the modified *bar* gene is stably expressed in multiple generations under a natural environment (Annex 3, Table 1； Table 95GBN016).

Moreover, regarding the recombinant oilseed rape RF3, the recombinant oilseed rape MS8, and the glufosinate herbicide tolerant and male sterile and fertility restored oilseed rape obtained by crossing the recombinant oilseed rape MS8 with the recombinant oilseed rape RF3 (modified *bar, barnase, barstar, Brassica napus* L., MS8RF3, OECD UI :ACS-BNØØ5-8×ACS-BNØØ3-6) (hereinafter referred to as "the recombinant oilseed rape MS8RF3"), the segregation ratio for fertility and sterility was examined. As a result, for the recombinant oilseed rape MS8, virtually 100% of individuals showed sterility, while for the recombinant oilseed rape RF3, almost 100% of individuals showed fertility. In addition, for the recombinant oilseed rape MS8RF3, fertility was completely restored, and the stability of expression of *barstar* gene under a natural environment was confirmed (Annex 3, Table FBN9501$_4$).

Overall, based on the results discussed above, it was confirmed that the modified *bar* gene and the *barstar* gene are stably expressed across individuals or generations 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 RF3 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 RF3 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 RF3 in general (Annex 5).

(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

The recombinant oilseed rape RF3 exhibits tolerance to glufosinate herbicide. In addition, it restores fertility in the F1 hybrid by a cross with the male-sterile genetically modified oilseed rape containing the *barnase* gene.

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 RF3 and the recipient organism, Drakkar cultivar (hereinafter referred to as “the non-recombinant control oilseed rape”) (Annex 1). In addition, in order to identify the crossability, the reference cultivar "Mie Nagashima Rapeseed", which belongs to *B.napus*, (oilseed rape currently prevailing in Mie Prefecture as “Nabana”) and the bok choy "Seitei", which belongs to *B.rapa*, were cultivated adjacent to the recombinant oilseed rape RF3 in a greenhouse in which honeybees were released (see the layout plan in Annex 1). Moreover, in 2005 and 2006, in the special screened greenhouse in Japan, the tests for productivity of harmful substances of the recombinant oilseed rape RF3 were conducted (Annex 2 and Annex 8).

(a) Morphological and growth characteristics

Comparison was made between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape for time of bolting, flowering period, maturation period, plant type, color of leaves, plant height, the number of primary branches, the number of pods formation, the number of pods unformed per an ear, the rate of pods formation [the number of pods formation/(the number of pods formation + the number of pods unformed per an ear)], the number of ovules per a pod, the number of seed setting, the rate of seed setting (the number of seed setting/the number of ovules per a pod), length of pod, 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 seeds (Annex 1, Tables 2-1 to 2-5).

As a result, the number of seed setting per a pod was found 17.8 for the non-recombinant control oilseed rape and 15.3 for the recombinant oilseed rape RF3, showing a significant difference (Annex 1, Table 2-3). On the other hand, regarding the time of bolting, flowering period, maturation period, plant type, color of leaves, the rate of dry matter, property of open pods, color of seed and the uniform excellence of seeds, no difference was observed between the non-recombinant control oilseed rape and the recombinant oilseed rape RF3 (Annex 1, Table 2-1; Tables 2-4 and 2-5). In addition, regarding the plant height, the number of primary branches, the number of pods formation, the number of pods unformed per an ear, the rate of pods formation, the number of ovules per a pod, the rate of seed setting, length of pod, fresh weight of aerial part, dry weight of aerial part, seed yield and 1000-seeds weight, no significant difference was observed between the non-recombinant control oilseed rape and the recombinant oilseed rape RF3 (Annex 1, Table 2-2; Table 2-3, and Tables 2-4 and 2-5).

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

In the tests conducted in FY 2005, in the summer-season special screened greenhouse controlled only by the natural ventilation, the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape were sown in July 27, 2005 and harvested in September 21, 2005 to compare the dry weight of plant body. As a result, no significant difference was observed (Annex 2, Table 8). Based on the above, heat-tolerance of the recombinant oilseed rape RF3 and

the non-recombinant control oilseed rape at the early stage of growth is considered to be equal.

In addition, wintering ability (survival rate) of the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape sowed in the isolated field in October 6, 1998 was both 100% at the observation in March 5, 1999 (Annex 1, Table 4). 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 RF3 and the non-recombinant control oilseed rape both show cold-tolerance at the early stage of growth.

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

The recombinant oilseed rape RF3 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 RF3 and the non-recombinant control oilseed rape was not found (Annex 1, Table 4).

(d) Fertility and size of the pollen

Regarding the fertility of the pollen, the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape were compared for the number of surviving pollens and the germination rate over time. The examination was conducted using the improved Hdgkin media (Reference 84) to count the number of pollens germinated, which have the pollen tube growing up to two times the diameter of pollen after two-hour culture at room temperature. As a result, regarding the germination rate over time, no specific difference was observed between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape, in both of which no pollen tube grew to two times the diameter of pollen even after 28 hours had elapsed from the collection of pollens, and no difference was observed in the germination rate of pollens (Annex 1, Table 3-1). Therefore, the fertility of pollen is considered equivalent between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape. In addition, also regarding the shape of flower and pollen, no difference was observed (Annex 1, Figure 4; Figure 5).

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

Regarding seed production, the differences between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape were examined for the number of pods formation, the number of pods unformed per an ear, the rate of pods formation, the number of ovules per a pod, 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. As a result, as mentioned in "(a) Morphological and growth characteristics", the number of seed setting was found 15.3 for the recombinant oilseed rape RF3 and 17.8 for the non-recombinant control oilseed rape, showing a slightly lower rate of seed

setting in the recombinant oilseed rape RF3, and then the significant difference was observed (Annex 1, Table 2-3). However, in all the other items, no significant difference was observed between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape (Annex 1, p.7, Table 2-2; Table 2-3; Tables 2-4 and 2-5).

Regarding the property of open pods of the recombinant oilseed rape RF3 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 that the shedding habit is equivalent for the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape (Annex 1, Table 2-4).

Regarding germination of the seed, no examination has been conducted in Japan, so the test results obtained in Belgium are provided. One hundred (100) seeds of each of the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape, which had been stored at room temperature after harvesting, were sown in their own plots of 1 m^2 each and the number of individuals germinated was compared. As a result, in terms of the mean value per 6 replications, the number of individuals germinated was 91 for the recombinant oilseed rape RF3 and 89 for the non-recombinant control oilseed rape, showing the equivalent results (Annex 3, Table $FBN9516_2$). In addition, based on the above results, the recombinant oilseed rape RF3 is considered not to possess higher dormancy than the non-recombinant control oilseed rape.

(e) Crossability

Examination was made to identify whether the trait of glufosinate tolerance is transferred to the "Mie Nagashima Rapeseed" (oilseed rape) and "Seitei" (*B.rapa*) cultivated adjacent to the recombinant oilseed rape RF3. These varieties were cultivated adjacent to each other in the isolated field greenhouse in which approximately 2,000 honeybees were released (see the layout plan in Annex 1); the seeds harvested from individual strains were sown in the open ground in the greenhouse; and then at the time when the first foliage leaf becomes half opened, glufosinate herbicide was sprayed and 4 days later, the number of surviving individuals was identified. The honeybees released in the greenhouse were confirmed to visit all the flowers, flying to every corner of the greenhouse to make hybridization to be easily attained.

As a result, the survival rate was found 1.8 to 2.0% for the "Mie Nagashima Rapeseed" (oilseed rape) and 0% for the "Seitei" (*B.rapa*) (Annex 1, Tables 3-3 and 3-4). It is reported that the out-crossing rate of oilseed rape is 5 to 30% (References 37, 60, and 68) and the crossability of *B.rapa* with oilseed rape under natural conditions is 0.4 to 1.5% (Reference 78), therefore the results obtained from above-mentioned tests do not exceed the existing findings.

(g) Productivity of harmful substances

In order to check whether the substances are excreted from the roots of the

recombinant oilseed rape RF3 which can affect other plants, exist in the plant body which can affect other plants after dying, and are 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). In addition, since the plow-in test showed the difference in the germination rate of the test plant of radish, additional plow-in test was conducted in 2006 in the same special screened greenhouse (Annex 8).

[Succeeding crop tests]

After cultivating the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape, 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. As a result, there was no significant difference confirmed in any items. The mean value of root length of radish showed a slight difference between the recombinant oilseed rape RF3 (7.3 cm) and the non-recombinant control oilseed rape (5.9 cm), though all other items were found equivalent between them. Consequently, regarding the productivity of harmful substances (secretion from roots to affect other plants), the recombinant oilseed rape RF3 is considered not to be different from the non-recombinant control oilseed rape (Annex 2, Tables 1 to 6).

[Plow-in tests]

The dried powder of plant body of the recombinant oilseed rape RF3 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 10, 12 and 14). However, the mean value of germination rate of radish showed a slight difference between the plots for the recombinant oilseed rape RF3 (59%) and the non-recombinant control oilseed rape (86%) (Annex 2, Table 9), so additional plow-in test was carried out.

The additional plow-in test examined the effects of plowed plant body on the other plants over time (Annex 8). The dried powder of plant body of the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape was mixed with soil, respectively, and immediately (the same day of mixing), one week, two weeks and four weeks after mixing, seeds of radish were sown in the soil and cultivated. Then comparison was made for the germination rate, plant height, root length, fresh weight and dry weight of radish between individual radish cultivating plots.

As a result, regarding the germination rate, no significant difference was observed in any of the plots including those immediately after mixing in the first test (Annex 8, Tables 4 to 7). In addition, also regarding the root length (Annex 8, Tables 10 to 13), fresh weight and dry weight (Annex 8, Tables 16 to 19), no significant difference was observed in any of the plots. On the other hand, the plant height was found higher in the plots for the non-recombinant

oilseed rape immediately after mixing, though no significant difference was observed one week and two weeks after mixing, and 4 weeks after mixing, the plots for the recombinant oilseed rape RF3 showed larger values (Annex 8, Tables 8 and 9). The plant height depression effect of recombinant oilseed rape RF3 observed immediately after mixing in the additional test was 5% or less. In the first test using the soil immediately after mixing, no significant difference was observed in the depression effect. Moreover, in all the subsequent plots, no depression effect was observed. Consequently, it is considered that there is no difference between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape regarding the productivity of harmful substances which can affect other plants after dying.

[Soil microflora tests]

The soil was obtained after cultivating the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape for about 2 months, and was diluted by adding sterilized phosphate buffer solution. Bacteria and Actinomyces were incubated in PTYG medium, and mold fungi were incubated 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 16 and 17). Therefore, it is considered that there is no difference between the recombinant oilseed rape RF3 and the non-recombinant control oilseed rape regarding the productivity of harmful substances (secretion from the roots to affect 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.

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 replaced eventually by perennial plants or shrub in any conditions of location other than roadsides, bluffs or riverside areas where disturbances could occur on a regular basis.

This recombinant oilseed rape is given;

1) 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.

2) a trait to restore the fertility, though it functions as intended only when it crosses with the recombinant oilseed rape which is conferred the male-sterile trait by the ribo-nuclease.

Therefore, it is considered unlikely that these traits could cause this recombinant oilseed rape to 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 difference in the number of seed setting by a slightly smaller mean value 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.

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 the recipient organism.

This recombinant oilseed rape produces the modified PAT protein which confers the trait to be tolerant to glufosinate, and the BARSTAR protein which inhibits the ribo-nuclease activity.

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.

In addition, it is considered that the BARSTAR protein does not affect the metabolic system of recipient organism other than exhibiting the trait to restore the fertility.

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

The tests (succeeding crop tests, soil microflora tests and plow-in tests) 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 exist in the plant body which can affect other plants after dying), at the special screened greenhouse in Japan. In the succeeding crop test and soil microflora test, no difference was observed from the plots for the non-recombinant control oilseed rape. In the plow-in test, regarding the plant height of the test plant of radish, the plant height of radish in the second test (Note) was found slightly larger in the plots for the non-recombinant control oilseed rape immediately after start of the test, and in the plots of this recombinant oilseed rape 4 weeks after start of the test, showing a significant difference. However, in the first test, there was no significant difference observed. In addition, regarding the root length, fresh weight and dry weight of radish, in both of the two plow-in tests, no significant difference was observed.

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.

(Note) In the plow-in test, the recombinant oilseed rape showed a slightly smaller mean value of germination rate of the test plant of radish compared to the non-recombinant control oilseed rape. Then additional test was carried out. In the second test, no difference was observed in the germination rate of test plant.

(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-reconmbinant 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.

a) It was confirmed that the crossability of this recombinant oilseed rape with the non-recombinant control oilseed rape or *B. rapa* does not exceed any existing findings.

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.

c) as discussed in (1), it is judged that this recombinant oilseed rape which possesses glufosinate tolerance and fertility restoration would not become competitive under a natural environment.

Consequently, it is judged that the possibility that hybrid obtained by crossing would become competitive and exterminate the population of the other wild animals and wild plants is extremely low.

In addition, 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. Therefore, it is considered unlikely that the modified *bar* gene and the *barstar* gene become a burden and they can affect the retention of population of inter-specific hybrid individuals within a short period of time.

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

Not made available or disclosed to unauthorized person

Annex List

Annex 1：FY 1999 report on the isolated field tests on genetically modified oilseed rape

Not made available or disclosed to unauthorized person

Annex 2：Test report Productivity of harmful substances of glufosinate herbicide tolerant and fertility restored oilseed rape RF3

Not made available or disclosed to unauthorized person

Annex 3：Reference data based on the results of environmental safety test carried out in foreign countries on the recombinant oilseed rape RF3

Not made available or disclosed to unauthorized person

Annex 4：Analysis on DNA transferred to the recombinant oilseed rape RF3

Not made available or disclosed to unauthorized person

Annex 5：Event identifying method

Not made available or disclosed to unauthorized person

Annex 6：Nucleotide sequences of plasmid pTHW118　(pTHW118 vector sequence)

Not made available or disclosed to unauthorized person

Annex 7：Southern blot analysis of genes existing outside of T-DNA region

(RF3 – Proof of absence of sequences derived from the ‘vector’-part of the construct.)

Not made available or disclosed to unauthorized person

Annex 8：Test report　Productivity of harmful substances of glufosinate herbicide tolerant and fertility restored oilseed rape RF3 (plow-in test)

Not made available or disclosed to unauthorized person

Annex 9：Erucic acid and glucosinolate content of seed

Not made available or disclosed to unauthorized person