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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Glufosinate herbicide tolerant, male sterile and fertility restored oilseed rape (Modified *bar, barnase, barstar, Brassica napus* L.) （MS8RF3, OECD UI: ACS-BNØØ5-8×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

Glufosinate herbicide tolerant and male sterile and fertility restored oilseed rape (modified *bar, barnase, barstar, Brassica napus* L., MS8RF3, OECD UI: ACS-BNØØ5-8 x ACS-BNØØ 3-6) (hereinafter referred to as "MS8RF3") is a F1 cultivar obtained by a cross between glufosinate herbicide tolerant and male sterile oilseed rape (modified *bar*, *barnase*, *Brassica napus* L., MS8, OECD UI: ACS-BNØØ5-8) (hereinafter referred to as "MS8") and glufosinate herbicide tolerant and fertility restored oilseed rape (modified *bar*, *barstar*, *Brassica napus* L., RF3, OECD UI: ACS-BNØØ3-6) (hereinafter referred to as "RF3").

Composition of the donor nucleic acid that was used for the production of MS8 and RF3, and the origins of component elements are shown in Table 1-1 and Table 1-2 respectively.

Table 1-1 Composition of the donor nucleic acid used for the production of MS8 and the origins of component elements

Component elements	Size (kbp)	Origin and function
barnase gene expression cassette		
PTA29	1.5	A promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces specific expression only in the tapetum cell in the anther (Reference 77).
barnase	0.3	A gene derived from *Bacillus amyloliquefaciens* and encodes RNA degradation enzyme (BARNASE protein). It expresses in the tapetum cell in the anther under the control of PTA29, and confers the trait of male sterility (Refernce 27).
3'nos	0.3	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 15).
Modified *bar* gene expression cassette		
PSsuAra	1.7	It is derived from *Arabidopsis thaliana*, a rubisco small subunit gene promoter, and induces expression only in the chlorenchyma (Reference 45).
Modified *bar*	0.5	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *Streptomyces hygroscopicus* and conferring tolerance to glufosinate herbicide (Reference 90). The two codons in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC respectively.
3'g7	0.2	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 16 and 94).
Additional information		
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.
Sm/Sp	1.0	It encodes *aminoglycoside adenyltransferase* (*aadA*) which confers streptomycin/spectinomycin tolerance, derived from *Escherichia coli* (Reference 21).
barstar	0.3	It encodes ribo-nuclease inhibitor (BARSTAR protein), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to BARNASE protein specifically, and inhibits its activity (Reference 27).
pVS1ori	3.8	It contains the replication origin of the plasmid pVS1 derived from *Pseudomonas sp*. (Reference 37).
pBRori	1.1	It contains the replication origin of the plasmid pBR322 derived from *Escherichia coli* (Reference 6).

Table 1-2 Composition of the donor nucleic acid used for the production of RF3 and the origins of component elements

Component elements	Size (kbp)	Origin and function
barstar gene expression cassette		
PTA29	1.51	A promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces specific expression only in the tapetum cell in the anther (Reference 77).
barstar	0.27	It produces ribo-nuclease inhibitor (BARSTAR protein), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to ribo-nuclease (BARNARSE protein), the product of *barnase* gene specifically, and inhibits its activity (Reference 27).
3'nos	0.26	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 15).
Modified *bar* gene expression cassette		
PSsuAra	1.73	It is derived from *Arabidopsis thaliana*, a rubisco small subunit gene promoter, and induces expression only in the chlorenchyma (Reference 45).
Modified *bar*	0.55	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *Streptomyces hygroscopicus* and conferring tolerance to glufosinate herbicide (Reference 90). The two codons, GTG and AGC, in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC respectively.
3'g7	0.21	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 16 and 94).
Additional information		
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
Sm/Sp	1.01	It encodes *aminoglycoside adenyltransferase* (*aadA*) which confers streptomycin/spectinomycin tolerance, derived from *Escherichia coli* (Reference 21).
barstar	0.27	It encodes ribo-nuclease inhibitor, derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to ribo-nuclease, produced by *barnase* gene, specifically, and inhibits its activity (Reference 27).
pVS1ori	3.77	It contains the replication origin of the plasmid pVS1 derived from *Pseudomonas sp*. (Reference 37).
pBRori	1.06	It contains the replication origin of the plasmid pBR322 derived from *Escherichia coli* (Reference 6).

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

The modified *bar* gene is the modified type of the wild-type *bar* gene obtained from *Streptomyces hygroscopicus*, GTG was modified to ATG to conform to frequently-used codons in plant, and AGC was modified to GAC to improve efficiency of translation. Regarding the translated amino acid, methionine remains unchanged in the modification from GTG to ATG, though serine changes to asparatic acid in the modification from AGC to GAC. However, it is confirmed that the function of modified PAT protein produced by the modified *bar* gene remains unchanged in this modification (Reference 96).

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

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

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

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

2) Functions of component elements

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

Functions of component elements of donor nucleic acid which were used for the production of MS8 and RF3 are shown in Table 1-1 and Table 1-2 respectively.

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

[Modified PAT protein]

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

Phosphinothricin acetyl transferase (modified PAT protein) produced by the transferred modified *bar* gene acetylates the glufosinate to make N-acetylglufosinate, which inactivates the inhibition of glutamine synthetase by the glufosinate. Then ammonia is not accumulated in the plant body, and the crop does not die even if it is sprayed with glufosinate herbicide (Figure 2).

The modified PAT protein exhibits a high affinity to glufosinate. Glufosinate is classified into L-amino acid, though it does not cause any acetyl group transfer reaction to the other various amino acids and it has little affinity for the glutamic acid which has specifically high structural similarity to glufosinate and it causes virtually no transfer reaction *in vivo* (Reference 90). 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 96). As a result, it is considered that the modified PAT protein possesses high substrate specificity to glufosinate.

[BARNASE protein]

BARNASE protein is a single stranded protein consisting of 110 amino acids, and it degrades RNA by two-stage reaction mode. It breaks the 3',5'-phosphodiester bond of the polyribonucleotide strand, transfers the phosphate group to the 2'-OH group of the ribose and produces the 2',3'-cyclic nucleotide as an intermediate (the first-stage phosphotransfer reaction). Then, the BARNASE protein hydrolyzes the intermediate and produces the 3'-nucleotide specifically (the second-stage hydrolysis reaction) (Reference 30). 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 73).

Pollens are produced during the highly controlled process in an anther. Tapetum cell, one of the tissues of the anther, plays an important role such as providing nutrition at the time of pollen formation and during the growth of pollens. Therefore, it is considered that the absence of tapetum cells in the pollen production is the major factor of resulting male sterility (Reference 41).

The *barnase* gene expresses the ribo-nuclease (BARNASE protein) which hydrolyzes the single stranded RNA molecule in the tapetum cell layer of the anther under the control of the promoter PTA29. The BARNASE protein degrades the RNA in the tapetum cells and thus the plants are unable to produce pollens (References 19, 28 and 48). It is also shown that the *barnase* gene, under the control of the promoter PTA29, expresses stably even in the high-temperature condition (37℃ during the daytime) (Reference 2). It is not reported that the promoter PTA29 induces a temperature-dependent expression.

[BARSTAR protein]

BARSTAR protein is an intracellular inhibitor for BARNASE protein (References 25 and 28). 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 26, 28 and 82).

In general, the first cross cultivar (F1 cultivar) possesses stronger and higher productivity and excellent uniformity compared to the fixed cultivar (Reference 44). However, it is hard to obtain the F1 cultivar without fail for self-fertile crops such as oilseed rape. It becomes possible to obtain the F1 seeds without fail by crossing the female strain (the recombinant oilseed rape MS8 to which *barnase* gene (Reference 48) was transferred to express specifically in the tapetum cell of the anther and inhibit the production of pollens) with the male strain (the recombinant oilseed rape RF3 which possesses the trait to restore pollen fertility). In the F1 generation, pollen fertility is restored by the function of BARSTAR protein which inhibits the BARNASE protein (Reference 49), therefore, the seed production in high-yield by self-pollination becomes available.

[Toxicity and allergenicity of modified PAT protein, BARNASE protein and BARSTAR protein]

For the amino acid sequence of modified PAT protein, BARNASE 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 (short amino acid sequence with 8 each) 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 90), it is considered that it does not cause any acetyl group transfer reaction to the compounds other than glufosinate. Therefore, it is considered that the modified PAT protein does not affect the metabolic pathway of the recipient organism.

[BARNASE protein]

The expression of the *barnase* gene is limited to the tapetum cells under the control of the promoter PTA29, and the *barnase* gene is unlikely to be expressed in any other tissue. The tapetum cell mostly develops at the tetrad stage of pollen production, and degrades/breaks along with the development of pollens (Reference 85). Therefore, it is considered that the possibility of the *barnase* gene to express in the tissues other than tapetum cells and to affect the metabolic pathway of the plant body is extremely low.

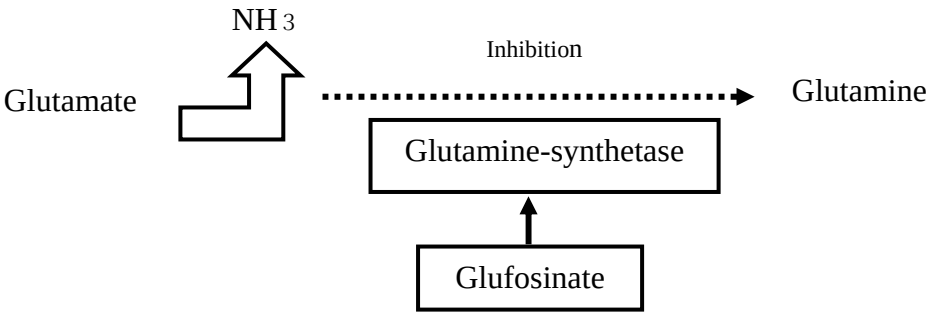
[BARSTAR protein]

The *barstar* gene is under the control of the promoter PTA29 and then it is unlikely to be expressed in any tissue other than tapetum cell. In addition, BARSTAR protein forms non-covalently bonded complex specifically with BARNASE protein in one-to-one correspondence, and the stability of the complex is high (References 47 and 50). Furthermore, the ribo-nuclease of bacteria and filamentous fungi is

found considerably homologous in the structure and sequence and thus, it is expected that these enzymes contain some inhibitors homologous with BARNASE protein. However, such inhibitors are known only in the ribo-nuclease BINASE protein produced by the *Bacillus intermedius*. The BINASE protein possesses high homology (85%) with the BARNASE protein, and it is inhibited by the BARSTAR protein (Reference 99). There is a report that the extra-cellular ribo-nuclease of *Streptomyces*, which has the similar protein structure although its homology with the amino acid sequence of BARNASE protein is only 20 to 25% (Reference 32), is also inhibited by the BARSTAR protein (Reference 29). 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 27, 28, 32 and 82). Based on the above understanding, it is considered that BARSTAR protein would not affect the metabolic system of the recipient organism.

A) Normal Plant

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



B) Modified 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.

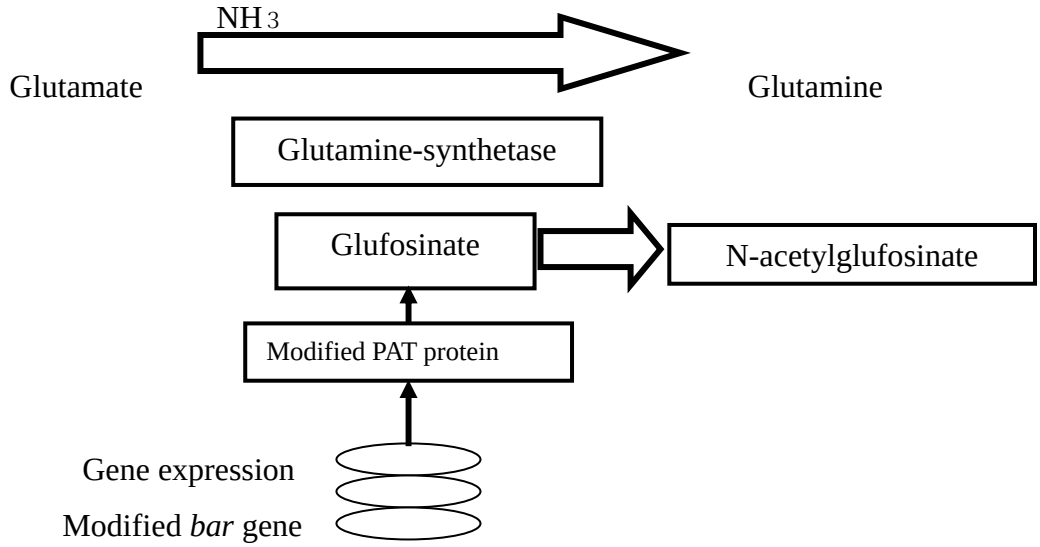


Figure 2 Mechanism of tolerance to glufosinate herbicide by the product of modified *bar* gene

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

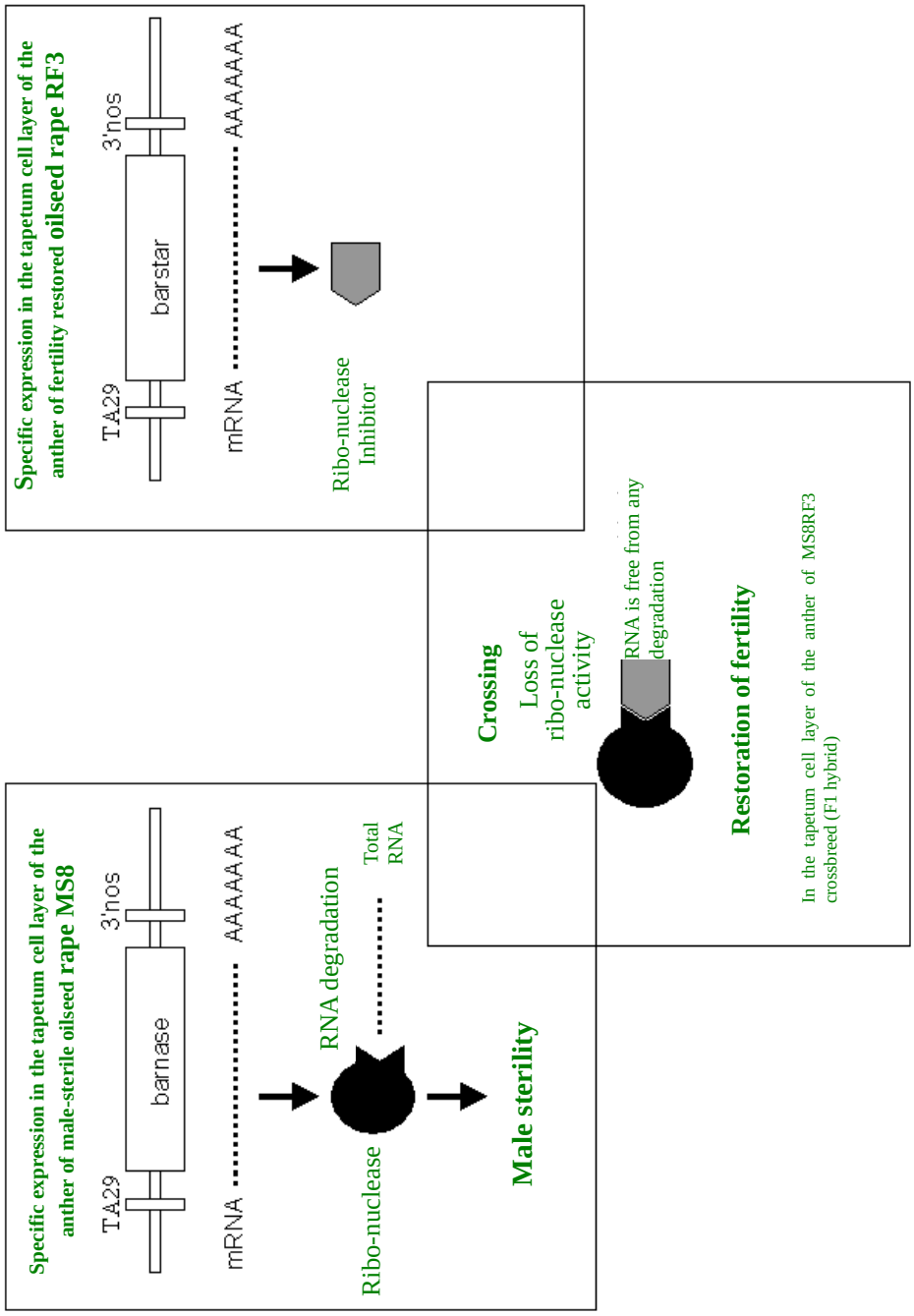


Figure 3 Mechanism of restoration of fertility

In the case of crossing the female strain (MS8) with the male strain (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 vector

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.* 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 12).

2) Properties

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

The total numbers of base pairs of the plasmid pTHW107 are 12,622bp and plasmid pTHW118 is 12,508bp. Figures 4-1 and 4-2 show the map of the plasmid of each. In addition, the entire nucleotide sequence of each vector is shown in Annex 1 [pTHW107、pTHW118].

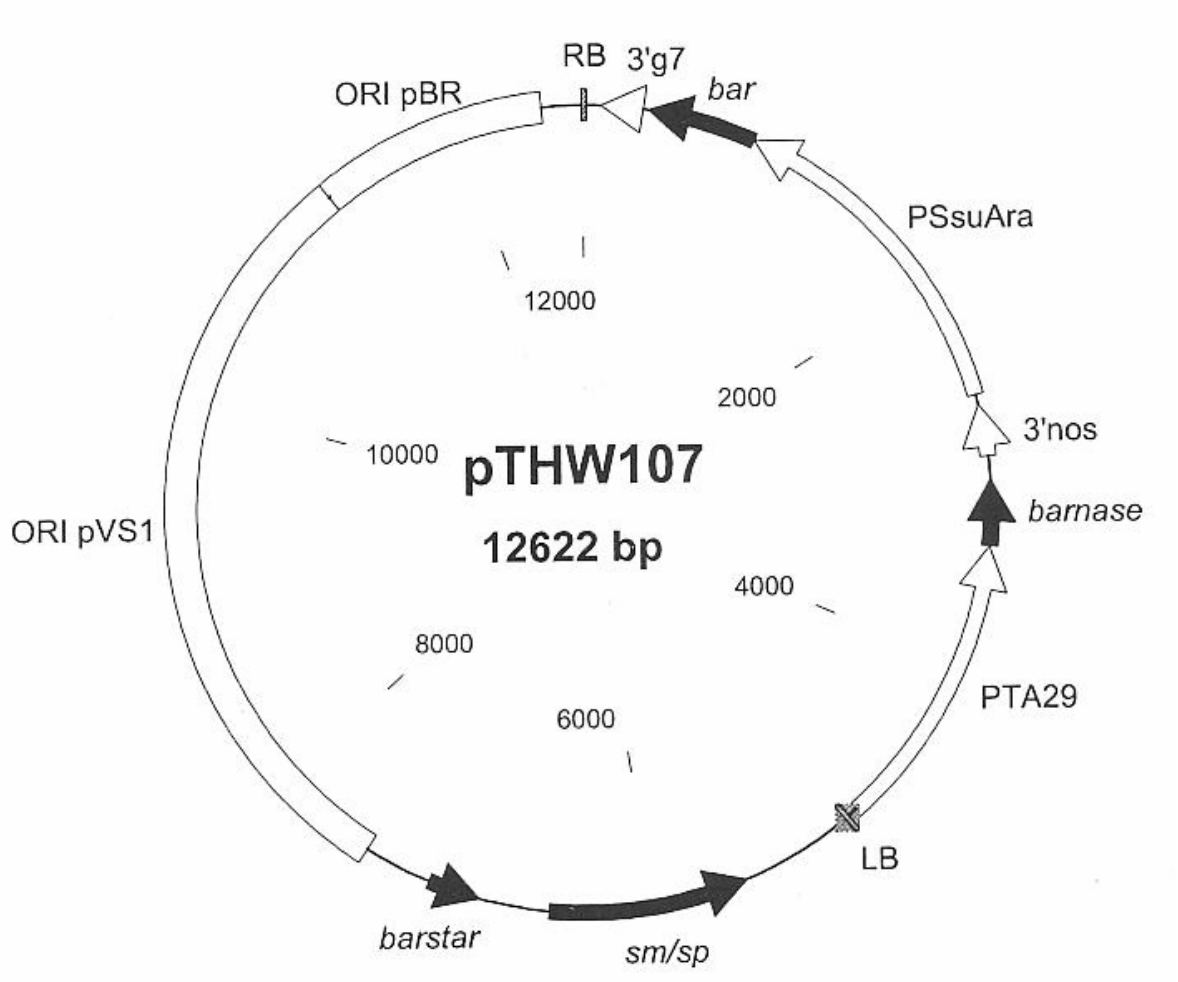


Figure 4-1 Physical Map of plasmid pTHW107
In the diagram, *bar* refers to the modified *bar* gene.

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

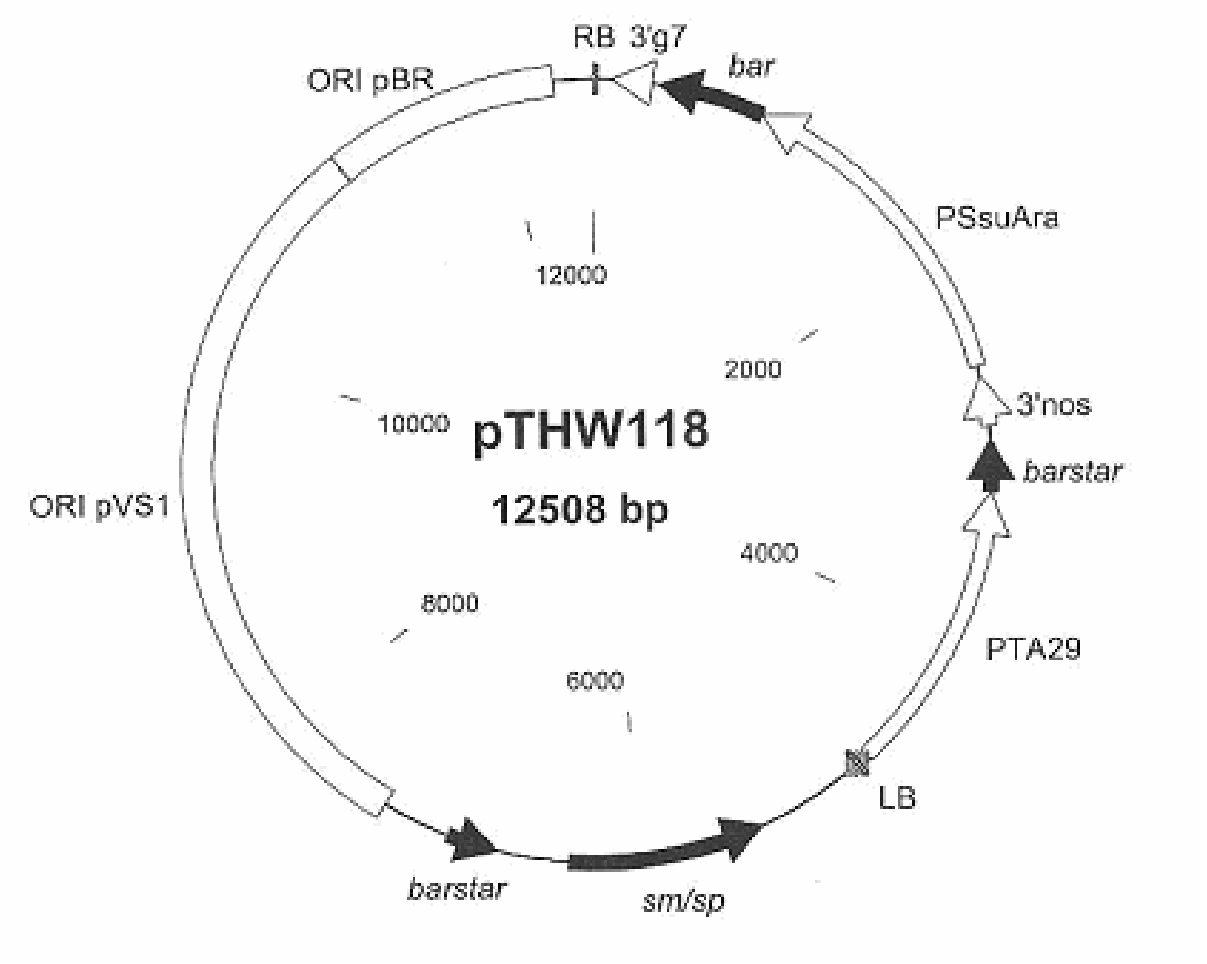


Figure 4-2 Physical Map of plasmid pTHW118
In the diagram, *bar* refers to the modified *bar* gene.

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

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

The plasmid pTHW107 and the plasmid pTHW118 both possess the streptomycin/spectinomycin tolerance gene (*Sm/Sp*), *barstar* gene, pBRori and pVS1ori outside the T-DNA region. The *Sm/Sp* gene was used as selectable markers for the vectors, though it expresses only in the bacteria and it does not express in any plant cells (References 13 and 93). In addition, the *barstar* gene has been present in the basic plasmid pGSV1. At the time of inserting the *barnase* gene to the plasmid by using *E.coli* for constructing the pTHW107, a small amount of BARNASE protein would express even though using the promoter for plants and the *E.coli* would die. Therefore, the *barstar* gene was used for inhibiting the enzyme activity of the BARNASE protein. The pBRori and the pVS1ori are replication origins, which function to cause autonomous replication in the *E. coli* and the *Pseudomonas aeruginosa*, respectively. These sequences locate outside the T-DNA region, and they are considered not to be transferred into the oilseed rape genomes of MS8 and RF3 (Annex 2).

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

It is known that the range of recipient organisms for the autonomous replication of plasmids pTHW107 and pTHW118 is limited to *Agrobacterium tumefaciens*, *E.coli* and gram-negative bacteria, and the plasmids do not possess the infectious characteristics in plants.

(3) Method of preparing living modified organisms

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

In the MS8, *barnase* gene expression cassette and modified *bar* gene expression cassette (PTA29-*barnase*-3'nos-PSsuAra-modified *bar*-3'g7) were transferred which are located between LB and RB on the pTHW107. In addition, in the RF3, *barstar* gene expression cassette and modified *bar* gene expression cassette (PTA29-*barstar*-3'nos-PSsuAra-modified *bar*-3'g7) were transferred which are located between LB and RB on the pTHW118. The position and direction of component elements of the nucleic acid in the vector is shown in Figures 4-1 and 4-2. In addition, the restriction enzyme cleavage site is shown in Figures 5-1 and 5-2. The nucleotide sequences of T-DNA regions transferred to MS8 and RF3 respectively are shown in Annex 3.

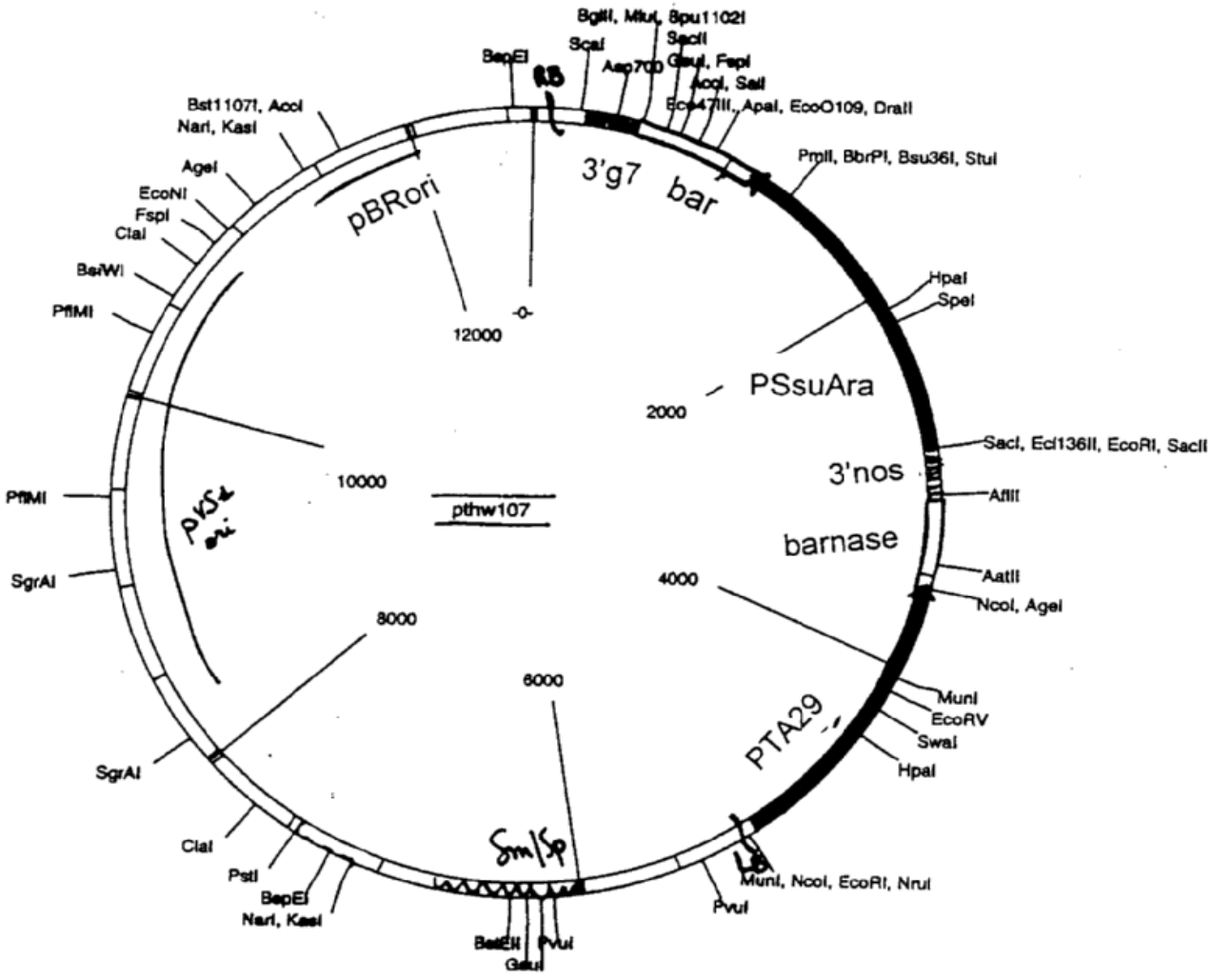


Figure 5-1 The restriction enzyme cleavage site of pTHW107
In the diagram, *bar* refers to the modified *bar* gene.

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

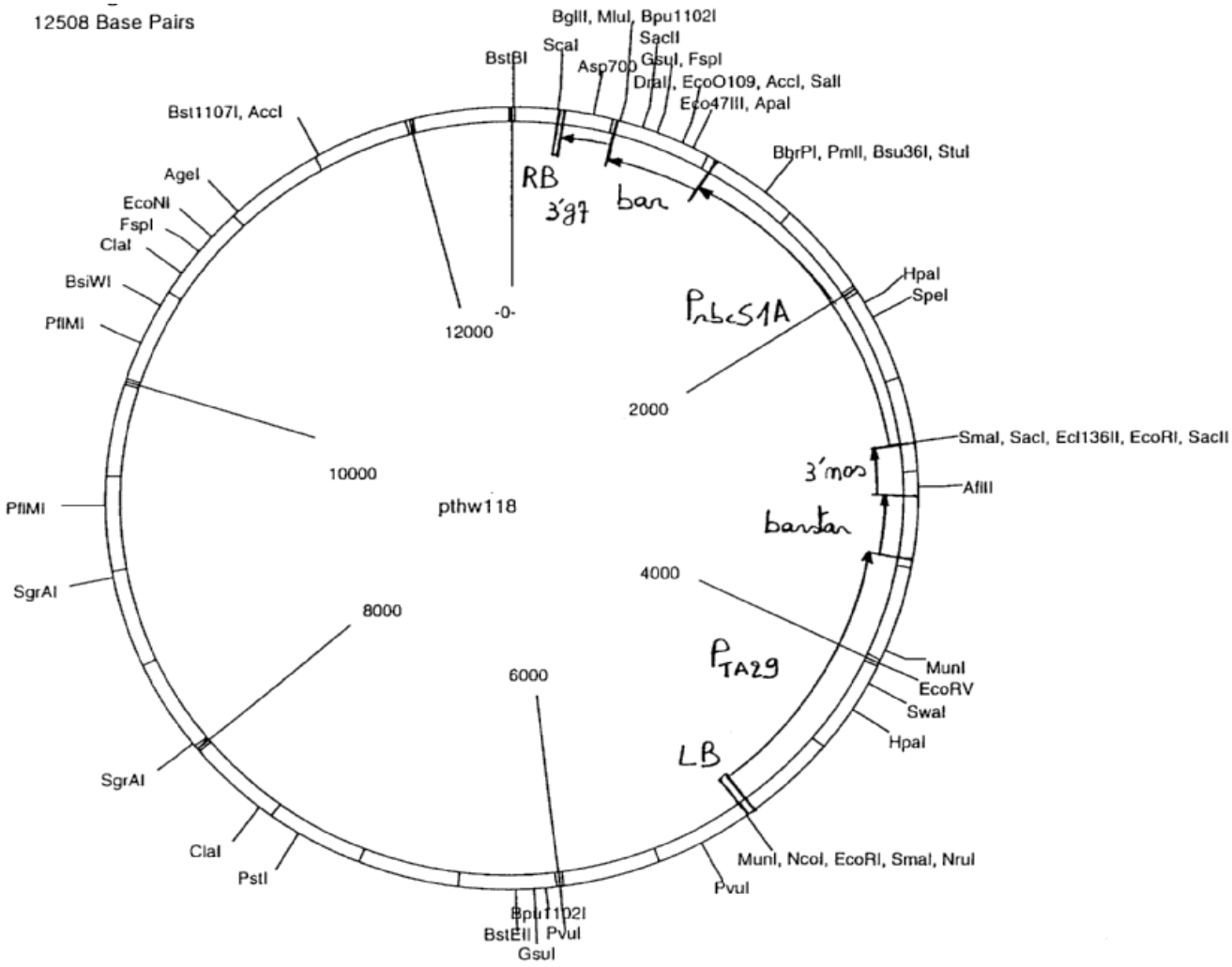


Figure 5-2 The restriction enzyme cleavage site of pTHW118

In the diagram, *bar* refers to the modified *bar* gene and PrbcS1A refers to the PSsuAra.

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

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

The *Agrobacterium*-mediated transformation method was used for transferring nucleic acid to both MS8 and RF3 (Reference 14).

The *E.coli* MC1061 strain which possesses either pTHW107 or pTHW118, the *E.coli* HB101 strain which possesses the transferable (helper) plasmid pRK2013, and the non-oncogenic *A.tumefaciens* C58C1Rif^R strain were coexistent. After the *A.tumefacines* C58C1Rif^R strain which possesses pTHW107 or pTHW118 was produced, a piece of hypocotylsof the recipient organism was infected with it, and the T-DNA region was transferred into the oilseed rape genomes (Reference 17).

3) Processes of rearing of living modified organisms

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

After transformation, a piece of hypocotyls was grown in a solid medium containing 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 14).

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

After transformation by the *Agrobacterium*, 500 mg/L of Carbenicillin was added to the medium and the remaining *Agrobacterium* cells were removed (Reference 14).

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

After transformation, the regenerated plant body of each of MS8 and RF3 was comprehensively examined for the target traits and agricultural characteristics. The process of rearing of MS8RF3 is shown in Figure 6. The MS8RF3 was produced by crossing the lines of BC2F1 or later generations of MS8 with the lines of T3, BC2F1 or later generations of RF3. The approvals of the MS8RF3 received from organizations in Japan are as follows.

[Food safety]

Based on the "Guideline for food safety assessment of food and food additives derived from recombinant-DNA technology", the conformity to the Guideline for safety of use as food regarding MS8RF3 was approved in December, 1997 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]

Based on the "Guideline for feed safety assessment of recombinant feed", the compatibility to the Guideline regarding glufosinate herbicide tolerant canola (MS8RF3) in December, 1997 was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, along with legislating, passing through the "Procedures for feed safety assessment of feed and feed additives derived from recombinant-DNA technology", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in March, 2003.

[Environmental safety]

In April 1997, based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries," the compatibility to the guideline regarding the isolated field test on the recombinant oilseed rape was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, in January 1998, the compatibility to the guideline regarding the glufosinate herbicide tolerant, male sterile and fertility restored oilseed rape (MS8RF3) 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 MS8RF3

(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 MS8 (the original gene transformation) is heterozygote for the transferred gene locus. In addition, MS8, which is conferred the male sterile trait, is maintained by a cross with the non-recombinant oilseed rape and then, it is expected that a segregation ratio of 1:1 for the posterity would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals under the control of single gene locus. As a result of examination on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals in various generations of MS8, the F1, BC1F1 (obtained by the backcrossing with Cultivar A) and BC2F1 generations of MS8 exhibited the segregation ratio as expected, about 50% individuals showed glufosinate herbicide tolerance (Annex 5, Table PC24 and Table PC33). Consequently, it is considered that the T-DNA region transferred into the recombinant oilseed rape MS8 exists at one site on a chromosome of the oilseed rape genome.

It is expected that the genetic locus of RF3 (the original gene transformant) is heterozygote for the transferred gene locus. Therefore, it is expected that in the T1 generation raised by self-pollination, 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 T1 of recombinant oilseed rape RF3, the segregation ratio showed a good agreement with the theoretical segregation ratio of 3:1 (Annex 5, Table $93GNB033_1$). In addition, as a result of investigation on the number of glufosinate-tolerant plants in the T2 generation individuals raised by self-pollination of T1 generation individuals exhibiting the tolerance to glufosinate herbicide, the ratio of approx. 1:2 was observed between the T1 individuals confirmed to exhibit the fixation of glufosinate tolerance in the T2 generation and the T1 individuals in the T2 generation showing the segregation ratio in agreement with 3:1 in the progeny (Annex 5, Table $93GNB033_2$). Consequently, it is considered that the T-DNA region transferred into the recombinant oilseed rape RF3 exists at one site on 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 to MS8, Southern blotting analysis (Annex 5) and sequence analysis (Annex 4) were conducted using BC2F1 generation. As a result, it was confirmed that one copy of modified *bar* gene expression cassette and one copy of *barnase* gene expression cassette were transferred in connecting condition (Annex 4).

In order to identify the number of copies of transferred nucleic acid to RF3, Southern blotting analysis (Annex 5) and sequence analysis (Annex 4) were conducted using T3 generation. 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 (Annex 4).

In addition, in order to confirm the stability of inheritance of transferred nucleic acid to MS8 and RF3, Southern blotting analysis was conducted for the each generation, F1, BC2F1 and BC1F1 (obtained by the backcrossing with Cultivar B) generations of the recombinant oilseed rape MS8, and T1, T3 and BC1F1 generations of the recombinant oilseed rape RF3. 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 5, Figure 3).

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

In MS8, modified *bar* gene expression cassette and *barnase* gene expression cassette were transferred in connecting condition , so this item is not applicable for the recombinant oilseed rape MS8.

On the other hand, in order to identify the position relationship between 2 copies of T-DNA transferred to the recombinant oilseed rape RF3, Southern blotting analysis

and sequence analysis were conducted as mentioned before. 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. 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 (Annex 4).

MS8RF3 contains one copy of modified *bar* gene on the chromosome derived from the parent plants and then, possesses two copies of modified *bar* gene.

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

[Glufosinate herbicide tolerance]

In the special screened greenhouse tests conducted in 2006, all seedlings germinated from the test seeds [MS8 (BC5F1)×RF3(T9)] exhibited glufosinate herbicide tolerance (Annex 7, Table 21), therefore, it is considered that this trait is expressed stably under natural condition.

[Male sterility and fertility restoration]

Regarding MS8, RF3 and MS8RF3 which had been selected by glufosiante herbicide, the segregation ratio for male-sterile individuals and male-fertile individuals was examined. As a result, about 100% of individuals of MS8 showed male sterility, about 100% of individuals of RF3 showed male fertility, and all individuals of MS8RF3 showed male fertility (Annex 5, Table $FBN9501_4$). Therefore, it is considered that this trait is expressed stably under natural condition.

[Expression of transferred gene]

In addition, Northern blotting analysis was conducted to examine the expression of modified *bar* gene, *barnase* gene and *barstar* gene of MS8RF3 with use of young leaves, matured leaves, roots, flower buds, pollens, dry seeds, and RNA derived from unmatured seeds. As a result, modified *bar* mRNA was detected in young leaves, matured leaves, roots, flower buds and unmatured seeds, and not detected in pollens and dry seeds (detection limit: 0.5 pg). In addition, *barnase* mRNA and *barstar* mRNA were both detected only in flower buds (detection limit: 1.0 pg and 0.5pg respectively) (Annex5, Table 3).

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

MS8 and RF3 contain no DNA sequence which possesses transferring factor and therefore, there is no possibility of transmission of nucleic acid transferred to wild animals and wild plants under a natural environment.

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

Detection of MS8RF3 is available by PCR method using the flanking sequences of DNA transferred in MS8 and RF3 respectively for each plant body (seed). This PCR method is utilized effectively for cultivation management of individual events (Annex 8).

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

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

MS8RF3 exhibits the trait of tolerance to glufosinate harbiside. The parent lines, MS8 is given the trait of male sterility, and RF3 is given the trait of fertility restoration. In MS8RF3, the F1 cultivar of both lines, the ribo-nuclease activity of BARNASE protein derived from MS8 is inhibited by the BARSTAR protein derived from RF3, and pollens would be produced.

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

In FY 1997, the isolated filed tests were conducted at the Hokkaido Agricultural Research Center (Natural Agricultural Research Center for Hokkaido Region of National Agricultural and Food Research Organization, currently) to compare morphological and growth characteristics, summer survival of matured plant, production of the seed, and other trait of MS8RF3 to the recipient organism cultivar Drakkar (hereinafter referred to as "Drakkar") (Annex 6).

Moreover, in 2006, in the special screened greenhouse in Japan, comparison was made between MS8RF3 [MS8 (BC5F1) ×RF3 (T9)] and Drakkar regarding the heat-tolerance at the early stage of growth, fertility and size of the pollen, productivity of harmful substances, and other traits (Annex 7).

For references of evaluation, results of cultivation tests conducted in foreign countries at several areas in 1995 were used (Annex 5).

(a) Morphological and growth characteristics

Comparison was made between MS8RF3 and Drakkar for the plant height, the number of primary branches, plant shape, time of bolting, flowering period, maturation period, rate of pods formation, length of pod, the number of seed setting, and color of seed. As a result, the significant difference was found in the plant height (the plant height of MS8RF3 was 7cm shorter compared to that of Drakkar). Also, the significant difference was found in the length of pod and the number of seed setting (MS8RF3 showed lower values for both items compared to Drakkar). For the dry weight of aerial parts (stems and leaves), MS8RF3 was 31g lighter. For the number of primary branches, MS8RF3 and

Drakkar showed 8 and 9 respectively. For the time of bolting, the flowering period, and the maturation period, 4 days, 5days and 2 days shorter in MS8RF3 respectively compared to those of Drakkar. For the rate of pods formation, MS8RF3 showed 3.0% lower than that of Drakkar. For the plant shape, the color of leaf and the color of seed, there was no difference between them (Annex 6, Tables 1 and 2).

In cultivation tests conducted in several areas (Bergium, 2 areas in the U.K., France, Canada, and Sweden) in foreign countries in 1995, germination and seedling, plant power, time of flower initiation, degree of maturation and plant height were examined. As a result, there was almost no difference found between MS8RF3 and the control cultivar in the germination and seedling, the plant power and the degree of maturation. For the time of flower initiation there was the minor difference (one to two days) between MS8RF3 and the control cultivar depending on the area, but it was not the significant difference. For the plant height, MS8RF3 was compared to the control cultivar in four areas, and as a result, MS8RF3 showed 5 to 14cm higher value in three areas, and MS8RF3 showed 1cm shorter value in one area (Annex 5, Table $FBN9501_{1,2,3,5,6}$).

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

Seedlings of one week after germination of MS8RF3 and Drakkar were raised under the conditions (35℃ and 12-hours day length and 12-hours night length) and as a result, it was confirmed at six weeks later observation that all the individuals had died (Annex 7, Table 20). Therefore, it is considered that both oilseed rapes show no heat tolerance at the early stage of growth.

The oilseed rape varieties sown in autumn in Japan are generally known to grow even in winter in both warm and cold districts, though the rate of growth varies (Reference 79).

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

As a result of observation for the summer survival in the isolated fields, no difference was observed between the recombinant oilseed rape MS8RF3 and the other varieties examined.

It is generally known that oilseed rape shows high cold tolerance and high snow endurance (Reference 79).

(d) Fertility and size of the pollen

Pollens were collected from the MS8RF3 and Drakkar cultivated in the special screened greenhouse, and stained with acetocarmine solution and observed under a microscope. As a result, 99% of the pollens from MS8RF3 and Drakkar were both found stained, showing a high fertility of the pollens (Annex 7, Figure 3). In addition, as a result of comparison of size of pollen, no statistically significant difference was observed between them (Annex 7, Table 19).

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

Comparison was made for MS8RF3 and Drakkar in seed yield per plant (g/plant) and 1000-seed weight, and as a result, MS8RF3 showed 1.6g lower value in the seed yield per plant, and MS8RF3 showed heavier value in the 1000-seed weight compared to Drakkar, and there was a significant difference between them (Annex 6, Table 2). In addition, calculation was made for the number of seed per plant from the values of the seed yield per plant and 100-seeds weight, and as a result, MS8RF3 showed 1.41×10^4 and Drakkar showed 1.88×10^4.

In addition, seed production (kg/ha) was examined in five areas (Bergium, 2 areas in the U.K., France and Canada) in foreign countries in 1995, and as a result, MS8RF3 showed higher value compared to the control cultivar in three areas, and less in other two areas. It is considered that there is no stable pattern for the seed yield (Annex 5, Table FBN9501$_7$). For 1000-seed weight, the comparison was made in two areas (the U.K. and Bergium), and there was no difference found between MS8RF3 and the control cultivar in both areas (Annex 4, Table FBN9501$_8$).

Regarding the property of open pods of MS8RF3 and Drakkar, the difficulty level was evaluated in 5-stage (1: most difficult- 5: most easy) in isolated fields. As a result, both showed level 4 (relatively easy), and it is considered that the shedding habit is equivalent for MS8RF3 and Drakkar (Annex 6, Table 2).

To evaluate the germination rate, seeds obtained from MS8RF3 and Drakkar in the special screened greenhouse were sown. As a result, one week after sowing, the germination rate was found 100% (20/20 grains) for MS8RF3 and 85.0% (17/20 grains) for Drakkar (Annex 7, Table 21). In addition, the seeds of Drakkar that were found not germinated were evaluated for life or dead based on the tetrazolium method after removal from the soil and they were confirmed to be dead. Based on the understanding, it is considered that the dormancy of the seeds of MS8RF3 and Drakkar are extremely low.

(f) Crossability

In the isolated field, Drakkar, Karafuto, and *B.juncea* (leaf mustard) were cultivated adjacent to the MS8RF3 under the environment where honeybees were released, to evaluate by transferring the trait of glufosinate herbicide tolerance to them. The seeds harvested from the individual varieties were sown and then, the seedlings germinated were sprayed with glufosinate herbicide. As a result, the glufosinate tolerance was observed in 18.4 to 21.2% of Drakkar, 3.1 to 7.3% of Karafuto, and 0.1% of leaf mustard (Annex 6, Table 1).

It is reported that the out-crossing rate of oilseed rape is 5 to 30% (References 34 and 66) and then, the crossability with Drakkar and Karafuto was found not exceeding the existing findings. Also the ratio of tolerant individual in leaf

mustard was found not exceeding the known crossability of 0.3 to 1.1% between *B.juncea* and oilseed rape (Reference 5).

(g) Productivity of harmful substances

In order to check whether the substances are excreted from the roots of MS8RF3 which can affect other plants, exists in the plant body which can affect other plants after dying, and are excreted from the roots which can affect microorganisms in soil, the succeeding crop test, plow-in test and soil microflora test were carried out respectively in the special screened greenhouse.

Succeeding crop test： After cultivating the recombinant oilseed rape MS8RF3 and Drakkar for about two months, radishes were cultivated as test plants in the remaining soil respectively, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed (Annex 7, Tables 9-14). Therefore, it is considered that the recombinant oilseed rape MS8RF3 has not acquired any productivity of the substances excreted from the roots which can affect other plants.

Plow-in test：The dried powder of plant body of the MS8RF3 and Drakkar cultivated about three months after sowing was mixed with soil (1%), respectively, and seeds of radish were sowed in the soil and cultivated. Then the comparison was made for germination rate, plant height, root length, fresh weight and dry weight. As a result, there was no statistically significant difference observed in any items (Annex 7, Tables 9-14). Therefore, it is considered that MS8RF3 has not newly acquired any productivity of the substances which can affect other plants after dying.

Soil microflora test：The soil was obtained after cultivating the recombinant oilseed rape MS8RF3 and Drakkar for about two months, and was diluted by adding sterilized phosphate buffer solution. Bacteria and Actinomyces were incubated in PTYG medium, and filamentous fungi were incubated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, there was no statistically significant difference observed in any items between MS8RF3 and Drakkar (Annex 7, Tables 15-17). Based on the above results, it is considered that MS8RF3 has not newly acquired any productivity of the substances excreted from the roots which can 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.

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

MS8RF3 is produced by a cross between the glufosinate herbicide tolerant and male sterile oilseed rape (MS8) and the glufosinate herbicide tolerant and fertility restored oilseed rape (RF3). MS8RF3 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS8, and the BARSTAR protein derived from RF3.

In the MS8RF3, the BARNASE protein and the BARSTAR protein interact with each other in the anther tapetum cell (Note 2) (non-covalently bonding specifically to each other in one-to-one correspondence) and as a result, the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein and the male fertility is restored. As mentioned above, MS8RF3 exhibits the interaction of the traits derived from the both parent lines and then, evaluation has been focused on the stack line oilseed rape rather than the both parent lines.

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

(Note 2) By a cross between the female strain (male-sterile MS8) and the male strain (fertility restored RF3), seeds of the first cross cultivar (F1) is obtained which possesses the male fertility.

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

(1) Competitiveness

Oilseed rape (*Brassica napus* L.) to which the recipient organism belongs was introduced to Japan in early Meiji period, and it is reportedly growing on river banks, along roadsides, in the surroundings of seed off-loading harbors, and in other such areas. It is generally known that oilseed rape would be eventually replaced with perennial plants and shrub in the environmental conditions without any regular disturbance such as roadsides, cliffs and riverside areas.

In the isolated fields and special screened greenhouses in Japan, a total of 22 traits relating to the competitiveness were examined.

There was a statistically significant difference between MS8RF3 and Drakker in the plant height, the length of pod and the number of seed setting (MS8RF3 showed lower value compared to that of Drakkar) and for 1000-seed weight (MS8RF3 showed heavier value compared to that of Drakkar). For the dry weight of aerial parts (stems and leaves), MS8RF3 was lighter than Drakkar, and for the number of primary branches, MS8RF3 showed the number of one less branch. For the time of bolting, the flowering period, and the maturation period, 4 days, 5days and 2 days shorter in MS8RF3 respectively compared to those of Drakkar. For the rate of pods formation, MS8RF3 showed 3.0% lower than that of Drakkar. Also, the significant difference was found in the length of pod and the number of seed setting (MS8RF3 showed lower values for both items compared to Drakkar). For the seed yield per plant MS8RF3 showed 1.6g

lower value, and for the 1000-seed weight, MS8RF3 showed heavier value compared to Drakkar, and there was a significant difference between them. However, as a result of calculation made for the number of seed per plant from the values of the seed yield per plant and 100-seeds weight, MS8RF3 showed less value (1.41×10^4) compared to that of Drakkar (1.88×10^4). However, these differences are considered not to cause MS8RF3 to become competitive.

MS8RF3 is given;

(i) a trait to be tolerant to glufosinate herbicide, though it is generally considered that the glufosinate does not exert selective pressure under a natural environment.

(ii) MS8RF3 produces pollens due to the interaction between the BARNASE protein derived from MS8 and the BARSTAR protein derived from RF3, though the pollens of MS8RF3 are found to have equivalent fertility as those of Drakkar, and for the size of the pollens, it is confirmed that there is no significant difference from the control plants.

(iii) The BARNASE protein degrades RNA in the tapetum cells of the recipient organism, though there is no report that it possesses activity against any other substrates.

(iv) There is no report that the BARSTAR protein possesses any function other than that which inhibits the ribo-nuclease of the BARNASE protein.

Therefore, it is considered unlikely that these traits could cause MS8RF3 to become competitive under a natural environment.

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

(2) Productivity of harmful substances

It has been confirmed that the contents of erucic acid and glucosinolate in the MS8RF3, which are recognized as harmful substances to human and other mammals, fall within the ranges for the cultivars known as canola, in which the erucic acid and glucosinolate content was reduced by selective breeding.

MS8RF3 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS8, and the BARSTAR protein derived from RF3. The modified PAT protein possesses high substrate specificity and then, it is considered unlikely to transfer the acetyl group to any substances other than the substrate glufosinate. In addition, in the MS8RF3, the BARNASE protein and the BARSTAR protein non-covalently bind specifically to each other in the anther tapetum cells in one-to-one correspondence, and the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein. Therefore, it is considered unlikely that these proteins affect any other metabolic systems in the plant body and newly produce any harmful substances.

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

In the special screened greenhouses in Japan, the succeeding crop test, soil microflora test and plow-in test have been conducted to check the harmful substances productivity of this recombinant oilseed rape (the substances excreted from the roots which can affect other plants, the substances excreted from the roots which can affect microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying). However, there was no significant difference between MS8RF3 and Drakkar.

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-1

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

In concrete,

(i) It was confirmed as a result of test on the crossability between MS8RF3 and the non-recombinant oilseed rape that the crossability of MS8RF3 with the non-recombinant oilseed rape does not exceed any existing findings on the crossability between oilseed rape varieties.

(ii) Regarding the crossability with related species, it is reported that it would be hard to produce hybrid, and even if hybrid is produced, the progeny would possess low fertility.

(iii) It is considered unlikely that MS8RF3 which possesses glufosinate tolerance and fertility restoration would become competitive under a natural environment.

(iv) It is reported that the cross-progeny of plants which possess male sterility as dominant trait would decrease its population rapidly over generations.

Consequently, it is judged that the possibility that MS8RF3 would cross with related species to produce hybrid and the progeny would become competitive under a natural environment is as low as oilseed rape to which the recipient organism belongs.

In addition,

(v) In the comparison between the individuals tolerant and not tolerant to glufosinate herbicide in the BC3 generation obtained by three-time repeated backcrossing of *B. rapa* with the hybrid between the recombinant oilseed rape, which contains both the modified *bar* gene and the *barstar* gene, and the *B. rapa*, through screening with glufosinate herbicide, it was reported that there was no difference in the fertility of pollen, survivability and the amount of seeds produced.

(vi) Even if the *barnase* gene become out of control of the promoter PTA29 and acquire the promoter which would express constitutively or site-specifically in plant, the possibility that plant would grow properly is low, and it is considered unlikely that the gene would spread extensively in the related species population.

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

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

(5) Additional information-2

The harvested seed (F2 generation) of the F1 generation of MS8RF3 is considered to include seeds that possess the same characteristics as the parent lines, MS8 and RF3, which are produced by the separation of MS8RF3. Therefore, in case to use MS8RF3 in accordance with the Type 1 Use Regulation for Living Modified Organism, the seeds would be released in environment in Japan. However, for MS8 and RF3, the approvals

have been already made for the Type 1 Use Regulation(Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them). Based on the above understanding, it was judged that it is unlikely that the use of this stack line recombinant oilseed rape (MS8RF3) in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stack line recombinant oilseed rape (MS8RF3) in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex

Annex 1：Nucleotide sequence of vectors pTHW107 and pTHW118

Confidential: Not made available or disclosed to unauthorized person

Annex 2：Southern blotting analysis for genes existing outside of T-DNA region
(MS8/RF3 – Proof of absence of sequences derived from the 'vector'-part of the construct.)

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Annex 3：Nucleotide sequences of T-DNA regions on vectors
(TDNA insert of pTHW107/pTHW118)

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Annex 4：Result of sequence analysis of transferred genes in MS8 and RF3
（Sequence of the *Brassica napus* elite event Ms8/Rf3 insert）

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Annex 5：Material for molecular analysis and trait expression in MS8 and RF3

Confidential: Not made available or disclosed to unauthorized person

Annex 6：Isolated field test report in 1997

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Annex 7：Report on the tests in the special screened greenhouse in 2006

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Annex 8：Event Identifying Method (MS8 and RF3)

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