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

Name: Bayer CropScience K.K.
Applicant: Gavin Marchant, President
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Glufosinate and Glyphosate herbicide-tolerant, male-sterile and fertility-restored oilseed rape (Modified *bar*, modified *cp4 epsps*, modified *gox v247*, *barnase*, *barstar*, *Brassica napus* L.) (MS8×RF3×RT73, OECD UI: ACS-BNØØ5-8×ACS-BNØØ3-6×MON-ØØØ73-7) (including the progeny lines isolated from the rapeseed lines that contain a combination of any of the transferred genes in the individual rapeseed lines MS8, RF3 and RT73 (except those already granted an approval regarding Type 1 Use Regulation))
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

The composition of donor nucleic acid and the origins of component elements used for the development of the parental lines (MS8, RF3 and RT73) of this stack oilseed rape line are shown individually in Table 1 to Table 3 (pp.14～16).

In MS8 and RF3, a *bar* gene which was the modified gene of the wild-type *bar* gene was introduced. In RT73, a *cp4 epsps* gene which was the modified gene of the wild-type *cp4 epsps* gene and a *gox* gene which was the modified gene of the wild-type *gox* gene were introduced. These genes are hereinafter referred to as "modified *bar* gene", "modified *cp4 epsps* gene" and "modified *gox v247* gene", respectively. In addition, the respective proteins being expressed are hereinafter referred to as "modified PAT protein", "modified CP4 EPSPS protein" and "modified GOX-v247 protein", respectively.

The modified PAT protein expressed in MS8 and RF3 had the following modification from the wild-type PAT protein encoded by the wild-type *bar* gene: GTG codon at the N terminal was modified to ATG to conform to codons frequently used in plants, and the second codon 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 aspartic acid in the modification from AGC to GAC.

The modified CP4 EPSPS protein expressed in RT73 had the following modification from the wild-type CP4 EPSPS protein: the second residue from the N terminal was modified from serine to leucine to produce a *Sph*I restriction enzyme cleavage site at the N terminal to facilitate cloning.

In the modified GOX v247 protein expressed in RT73, the 84th amino acid residue from the N terminal was modified from glycine to serine, the 153rd residue was modified from arginine to lysine, and the 334th residue was modified from arginine to histidine to facilitate glyphosate degradation.

Table 1 Component elements of the plasmid pTHW107 used for the development of MS8

Component elements	Size (kbp)	Origin and function
barnase gene expression cassette		
PTA29	1.51	A promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces expression in the tapetum cell in the anther (Seurinck *et al*., 1990).
barnase	0.34	A gene derived from *Bacillus amyloliquefaciens*. It encodes a ribonuclease (BARNASE protein) and is expressed in the tapetum cell in the anther under the control of PTA29 to confer male sterility (Hartley, 1988).
3'nos	0.26	The 3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Depicker *et al.*, 1982).
Modified *bar* gene expression cassette		
PSsuAra	1.73	The promoter of RuBisCo small subunit gene derived from *Arabidopsis thaliana*. It induces expression in the chlorenchyma (Krebbers *et al*., 1988).
Modified *bar*	0.55	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *Streptomyces hygroscopicus* and conferring tolerance to glufosinate herbicide (Thompson *et al*., 1987). The two codons in the N-terminal of wild-type PAT protein are replaced with 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 (Velten and Schell, 1985; Dhaese *et al*., 1983).
Others		
RB	0.03	The right border of the T-DNA derived from pTiB6S3 (Gielen *et al*., 1984).
LB	0.03	The left border of the T-DNA derived from pTiB6S3 (Gielen *et al*., 1984).
Sm/Sp	1.01	A region derived from *Escherichia coli* and encoding aminoglycoside adenyltransferase that confers streptomycin/spectinomycin tolerance (Fling *et al*., 1985).
barstar	0.27	A gene encoding ribonuclease inhibitor (BARSTAR protein), derived from *B. amyloliquefaciens*. BARSTAR protein specifically binds to BARNASE protein to inhibit its activity (Hartley, 1988).
pVS1ori	3.78	A region containing replication origin of plasmid pVS1 derived from *Pseudomonas sp*. (Itoh *et al*., 1984).
pBRori	1.16	A region containing replication origin of plasmid pBR322 derived

		from *E. coli* (Bolivar *et al*., 1977).

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

Table 2 Component elements of the plasmid pTHW118 used for the development of RF3

Component elements	Size (kbp)	Origin and Function
barstar gene expression cassette		
PTA29	1.51	A promoter of anther-specific gene TA29 derived from *N. tabacum*. It induces expression in the tapetum cell in the anther (Seurinck *et al*., 1990).
barstar	0.27	It produces ribonuclease inhibitor (BARSTAR protein), derived from *B. amyloliquefaciens*. BARSTAR protein specifically binds to BARNASE protein to inhibit its activity (Hartley, 1988).
3'nos	0.26	The 3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Depicker *et al.*, 1982).
Modified *bar* gene expression cassette		
PSsuAra	1.73	The promoter of RuBisCo small subunit gene derived from *Arabidopsis thaliana*. It induces expression in the chlorenchyma (Krebbers *et al*., 1988).
Modified *bar*	0.55	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *S. hygroscopicus* and conferring tolerance to glufosinate herbicide (Thompson *et al*., 1987). The two codons, GTG and AGC, in the N-terminal of wild-type PAT protein are replaced with ATG and GAC respectively.
3'g7	0.21	The 3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (Velten and Schell, 1985; Dhaese *et al*., 1983).
Others		
RB	0.03	The right border of the T-DNA derived from pTiB6S3 (Gielen *et al*., 1984).
LB	0.03	The left border of the T-DNA derived from pTiB6S3 (Gielen *et al*., 1984).
Sm/Sp	1.01	A region derived from *Escherichia coli* and encoding aminoglycoside adenyltransferase that confers streptomycin/spectinomycin tolerance (Fling *et al*., 1985).
barstar	0.27	A gene encoding ribonuclease inhibitor (BARSTAR protein), derived from *B. amyloliquefaciens*. BARSTAR protein specifically binds to BARNASE protein to inhibit its activity (Hartley, 1988).
pVS1ori	3.78	A region containing replication origin of plasmid pVS1 derived from *Pseudomonas sp.* (Itoh *et al*., 1984).
pBRori	1.16	A region containing replication origin of plasmid pBR322 derived from *E. coli* (Bolivar *et al*., 1977).

(Note: All the rights pertinent to the information in the table above and the responsibility for

the contents rest upon the applicant.)

Table 3 Component elements of the plasmid PV-BNGT04 used for the development of RT73

Component elements	Origin and function
Modified *gox v247* gene expression cassette	
P-FMV	35S promoter of *Figwort mosaic virus* (Gowda *et al*., 1989; Richins *et al*., 1987; Sheperd *et al*., 1987). It drives constitutive expression of the target gene in all tissues.
Arab-SSU1A /CTP1	N-terminal sequence of chlorophyll transit peptide sequence in small subunit 1A of ribulose-1,5-bisphosphate carboxylase derived from *Arabidopsis* (Timko *et al*., 1988) . It directs the target protein to chloroplasts.
Modified *gox v247*	A variant of glyphosate-degrading enzyme (glyphosate oxidoreductase; GOX) derived from *Ochrobactrum anthropi* LBAA strain (Barry *et al*., 1994; Woodward *et al*., 1994). It degrades glyphosate into aminomethylphosphonic acid (AMPA) and glyoxylate.
E9 3'	The 3' untranslated region of *rbcS E9* gene of *Pisum sativum*. It terminates polyadenylation of modified *gox v247* and modified *cp4 epsps* genes (Coruzzi *et al*., 1984; Morelli *et al*., 1985).
Modified *cp4 epsps* gene expression cassette	
P-FMV	35S promoter of *Figwort mosaic virus* (Gowda *et al*., 1989; Richins *et al*., 1987; Sheperd *et al*., 1987). It drives constitutive expression of the target gene in all tissues.
AEPSPS/CTP2	N-terminal sequence of chlorophyll transit peptide sequence in *EPSPS* gene derived from *Arabidopsis* (Klee *et al*., 1987). It directs the target protein to chloroplasts.
Modified *cp4 epsps*	5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* sp. strain CP4 (Padgette *et al*., 1993). Its expression product (modified CP4 EPSPS protein) confers high tolerance to glyphosate herbicide.
E9 3'	The 3' untranslated region of *rbcS E9* gene of *Pisum sativum*. It terminates polyadenylation of modified *gox v247* and modified *cp4 epsps* genes (Coruzzi *et al*., 1984; Morelli *et al*., 1985).
Other component elements	
Right Border (RB)	Restriction fragment derived from pTiT37 plasmid. It initiates transfer of T-DNA from *Agrobacterium tumefaciens* to plant genome (Depicker *et al*., 1982).
Left Border (LB)	Restriction fragment derived from Octopine Ti plasmid pTiA6, containing the left border sequence (25 bp) of T-DNA (Barker *et al*., 1983).
ori-V	Replication origin segment in *Agrobacterium* derived from the broad host range plasmid RK2 (Rogers *et al*., 1987).
ori-322	Replication origin of PV-BNGT04 in *E. coli* derived from pBR322 (Sutcliffe, 1979).
aadA	The bacterial promoter, coding region and terminator of 3'(9)-O-nucleotidyltransferase, an aminoglycoside-modifying enzyme, derived from transposon Tn 7. It confers spectinomycin/streptomycin resistance (Fling *et al*., 1985). (GenBank accession X03043)

rop	A sequence encoding the repressor of the primer protein. It regulates the plasmid copy number in *E. coli* (Giza and Huang, 1989).

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

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, RF3 and RT73 are shown in Table 1 to Table 3 (pp.14～16), 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. In detoxification of the produced ammonia, glutamine synthase plays a pivotal role. However, if sprayed to plants, glufosinate herbicide inhibits the glutamine synthase to allow the produced ammonia to accumulate, resulting in death of the plants.

The modified PAT protein, the product expressed from the modified *bar* gene, can acetylate glufosinate to produce N-acetyl-glufosinate, inactivating the inhibitory action of the glufosinate on glutamine synthase. This mechanism would prevent ammonia from accumulating, resulting in survival of plants, even if glufosinate herbicide were sprayed to the plants.

Modified CP4 EPSPS protein

Glyphosate herbicide serves as the active ingredient of Roundup, a non-selective herbicide, and inhibits the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis, by specifically binding to the enzyme (Steinrüken and Amrhein, 1980; Haslam, 1993). As a result, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. The modified *cp4 epsps* gene, a target gene of RT73, expresses the modified CP4 EPSPS protein which has high tolerance to glyphosate herbicide. The activity of the modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene is not inhibited in the presence of glyphosate; thus, recombinant plants expressing this protein have normal functions of shikimate pathway and can grow.

Modified GOX v247 protein

Glyphosate is degraded and inactivated by microorganisms in soil, which is attributed to the action of a glyphosate-degrading enzyme (Glyphosate Oxidoreductase; GOX) in microorganisms. This enzyme degrades glyphosate herbicide into aminomethylphosphonic acid (AMPA) and glyoxylate, both of which having no herbicidal activity, and has been found in a number of gram-negative and gram-positive bacteria degrading glyphosate (Pipke and Amrhein, 1988; Jacob *et al.*, 1988). Among the organisms assumed to have the potential of degrading glyphosate into AMPA and glyoxylate, *Ochrobactrum anthropi* (designation based on former classification: *Achromobacter* sp.) LBAA strain, which showed the highest capability of glyphosate degradation, was selected, and the *gox* gene was isolated (Hallas *et al.*, 1988; Barry *et al.*, 1994). The *Ochrobactrum anthropi* LBAA strain is one of the microorganisms most frequently found in the rhizosphere of plants (Joos *et al.*, 1988) and is capable of utilizing glyphosate as carbon and/or phosphorus sources (Barry *et al.*, 1994).

BARNASE protein

Pollens are produced through a highly controlled process in the anther. Tapetum cells, one of the tissues in the anther, play an important role of providing nutrition at the time of pollen formation and during the subsequent growth of pollens. Therefore, the lack of the tapetum cells is believed to be the major cause of male sterility (Kaul, 1988).

The *barnase* gene expresses a ribonuclease (BARNASE protein) that hydrolyzes single-stranded RNA molecules in the tapetum cells of the anther under the control of the promoter PTA29. The BARNASE protein degrades the RNA in the tapetum cells to destroy these cells, which leads to the inhibition of pollen formation (Drews and Goldberg, 1989; Hartley, 1989; Mariani *et al.*, 1990).

BARSTAR protein

Like the *barnase* gene in MS8, the *barstar* gene is under the control of the promoter PTA29 to express the BARSTAR protein in the tapetum cells of the anthers. The BARSTAR protein is an intracellular inhibitor for the BARNASE protein, forming a non-covalently bonded complex specifically with the BARNASE protein in one-to-one correspondence and inhibiting the ribonuclease activity of the BARNASE protein (Smeaton and Elliott, 1967; Hartley and Smeaton, 1973; Hartley, 1989).

In general, hybrid varieties (F1 hybrid) are stronger and exhibit higher productivity and excellent uniformity compared to pure-bred varieties (Koinuma, 2005). However, it is hard to obtain out-crossed F1 seeds without fail in self-fertile crops such as oilseed rape, as seeds are also formed through selfing. F1 seeds can be efficiently obtained by pollinating MS8, i.e.

the male-sterile line expressing the *barnase* gene, with pollens of RF3, i.e. the fertility-restored line expressing the *barstar* gene (Figure 1, p.17). In the F1 generation, pollen fertility is restored by the function of the BARSTAR protein inhibiting the BARNASE protein (Mariani *et al.*, 1992), realizing a high yield of seed production through self-pollination.

The stack line between MS8 and RF3, i.e. 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) (hereinafter referred to as "MS8RF3"), has been granted approval of the Type I Use Regulation in August 2007.

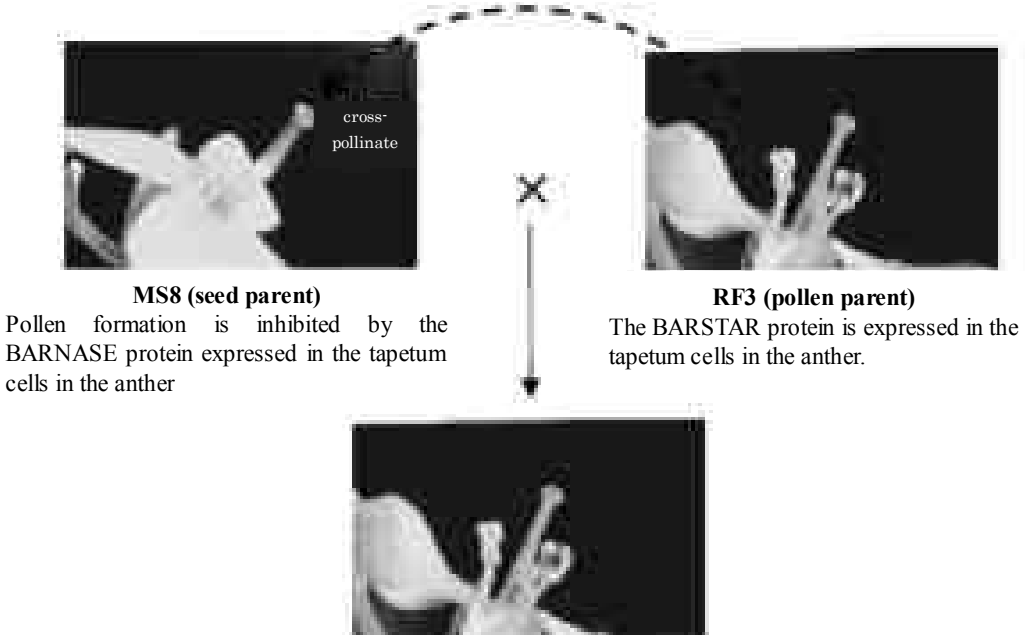

Figure 1 Schematic diagram of F1 cultivar production using male-sterile and fertility-restored oilseed rape

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

As a result of the amino acid sequence homology search conducted in 2009 using the database (AllergenOnline), it has been confirmed that the modified PAT protein, the BARNASE protein and the BARSTAR protein had no homology with known toxins or allergens.

In order to investigate whether the modified CP4 EPSPS protein and the modified GOX v247 protein share functionally important amino acid sequences with known allergens, the proteins were compared with allergens in the allergen database (AD_2009[1]), using FASTA algorithm and ALLERGENSEARCH algorithm. The results showed that these modified proteins did not share structurally related sequences with any known allergens examined.

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

Modified PAT protein

The modified PAT protein is an enzyme that inactivates glufosinate by transferring acetyl group to the herbicide. Glufosinate is classified under L-amino acids, but the modified PAT protein does not catalyze acetyl group transfer to other amino acids and hardly shows any affinity to glutamic acid, which structurally resembles glufosinate, inducing substantially no transfer reaction in vivo (Thompson *et al.*, 1987). Moreover, it has been reported that the acetyl group transfer to glufosinate induced by the modified PAT protein was not inhibited in the presence of excessive amounts of other amino acids (Wehrmann *et al.*, 1996). Therefore, it is considered that the modified PAT protein has high substrate specificity to glufosinate and would not affect the metabolic system of the recipient organism.

Modified CP4 EPSPS protein

EPSPS is one of the enzymes that catalyze the shikimate pathway for the biosynthesis of aromatic amino acids, which are specific to plants and microorganisms, and is located in chloroplasts or plastids in plants (Della-Cioppa *et al.*, 1986). The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants (Haslam, 1974; Haslam, 1993). This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It has been demonstrated that it is extremely unlikely that the stages involved in the synthesis of chorismic acid from DAHP through the production of 5-enol-pyruvylshikimate-3-phosphate (EPSP), which is catalyzed by EPSPS, are inhibited or suppressed by metabolic intermediates or end products of this pathway (Weiss and Edwards, 1980; Herrmann, 1983). This suggests that EPSPS is not the rate-determining enzyme, and thus it is considered that enhanced EPSPS activity will not increase the concentration of

[1] FARRP (Food Allergy Research and Resource Program): Database holding the sequences registered in the Allergen Online database (FARRP, 2009) as of January, 2009.

aromatic amino acids, the end products of this pathway. Actually, it has been reported that plant cells producing 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids (Smart *et al.*, 1985). Moreover, amino acid composition in seeds was determined as part of a food/feed safety evaluation conducted for Roundup herbicide-tolerant crops (soybean, cotton, maize) commercialized as products of Monsanto, but no difference was observed in the contents of aromatic amino acids, i.e. the end products of the shikimate pathway, between seeds of genetically modified crops and those of non-recombinant crops, which also supported that EPSPS is not a rate-determining enzyme of this pathway. Besides, EPSPS is an enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Levin and Sprinson, 1964) and is known to specifically react with these substrates (Gruys *et al.*, 1992). The only substance known to react with EPSPS other than these substrates is shikimate, an analogue of S3P, but the reactivity with shikimate is only one-two millionth of the reactivity with S3P, so it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

Modified GOX v247 protein

GOX protein is an enzyme catalyzing the degradation of glyphosate into AMPA and glyoxylate. The enzyme has high substrate specificity, and the only compounds known to react with the GOX protein other than the substrate are iminodiacetic acid (IDA) and 2-methylglyphosate, which are structurally similar to glyphosate but have not been found in plants (Padgette *et al.*, 1994). The modified GOX v247 protein expressed in RT73 has three amino acids modified for the purpose of increasing glyphosate degradation activity of the GOX protein. A component analysis of RT73 expressing the modified GOX v247 protein revealed no difference in composition compared to the non-recombinant oilseed rape used as a control, suggesting that the modified GOX v247 protein has substrate specificity similar to that of the GOX protein.

BARNASE protein

In the anther of oilseed rape transformed with the *barnase* gene under the control of the anther-specific promoter PTA29, only the tapetum cells were specifically destroyed (Mariani *et al.*, 1990); thus, it is unlikely that the *barnase* gene is expressed in any other tissue. The tapetum cells mostly develop at the tetrad stage during pollen development and degrade/break along with the development of microspores (Takahata, 2005). Therefore, it is extremely unlikely that the *barnase* gene is expressed in tissues other than the tapetum cells and that the BARNASE protein affects the metabolic pathway of the plant body.

BARSTAR protein

The *barstar* gene is under the control of the promoter PTA29 and is therefore unlikely to be expressed in tissues other than the tapetum cells. In addition, the BARSTAR protein forms a highly stable, non-covalently bonded complex specifically with the BARNASE protein in one-to-one correspondence (Martinez *et al.*, 1995). Furthermore, ribonucleases of bacteria and fungi are found to share considerable structural and sequence homology, so it is expected that inhibitors homologous to the BARSTAR protein exist for these enzymes. However, such inhibitor has only been found for BINASE protein, a ribonuclease produced by *Bacillus intermedius*. The BINASE protein shares a high homology (85%) with the BARNASE protein and is inhibited by the BARSTAR protein (Yakovlev *et al.*, 1995). In addition, there is a report that an extra-cellular ribonuclease of *Streptomyces*, which shares only 20 to 25% amino acid sequence homology with the BARNASE protein but is structurally similar to the protein (Hill *et al.*, 1983), is also inhibited by the BARSTAR protein (Hartley *et al.*, 1996). However, there is no report that the BARSTAR protein inhibits ribonuclease in plants. Based on the above findings, it is considered that BARSTAR protein would not affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

MS8： Binary Ti plasmid pTHW107 constructed based on pGSV1 derived from *E. coli* (Figure 2, p.23).

RF3： Binary Ti plasmid pTHW118 constructed based on pGSV1 derived from *E. coli* (Figure 3, p.24).

RT73： Plasmid vector PV-BNGT04 constructed from plasmids such as pBR322 derived from *E. coli* (Figure 4, p.25).

2) Properties

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

pTHW107：12,622 bp
pTHW118：12,508 bp
PV-BNGT04：11,491 bp

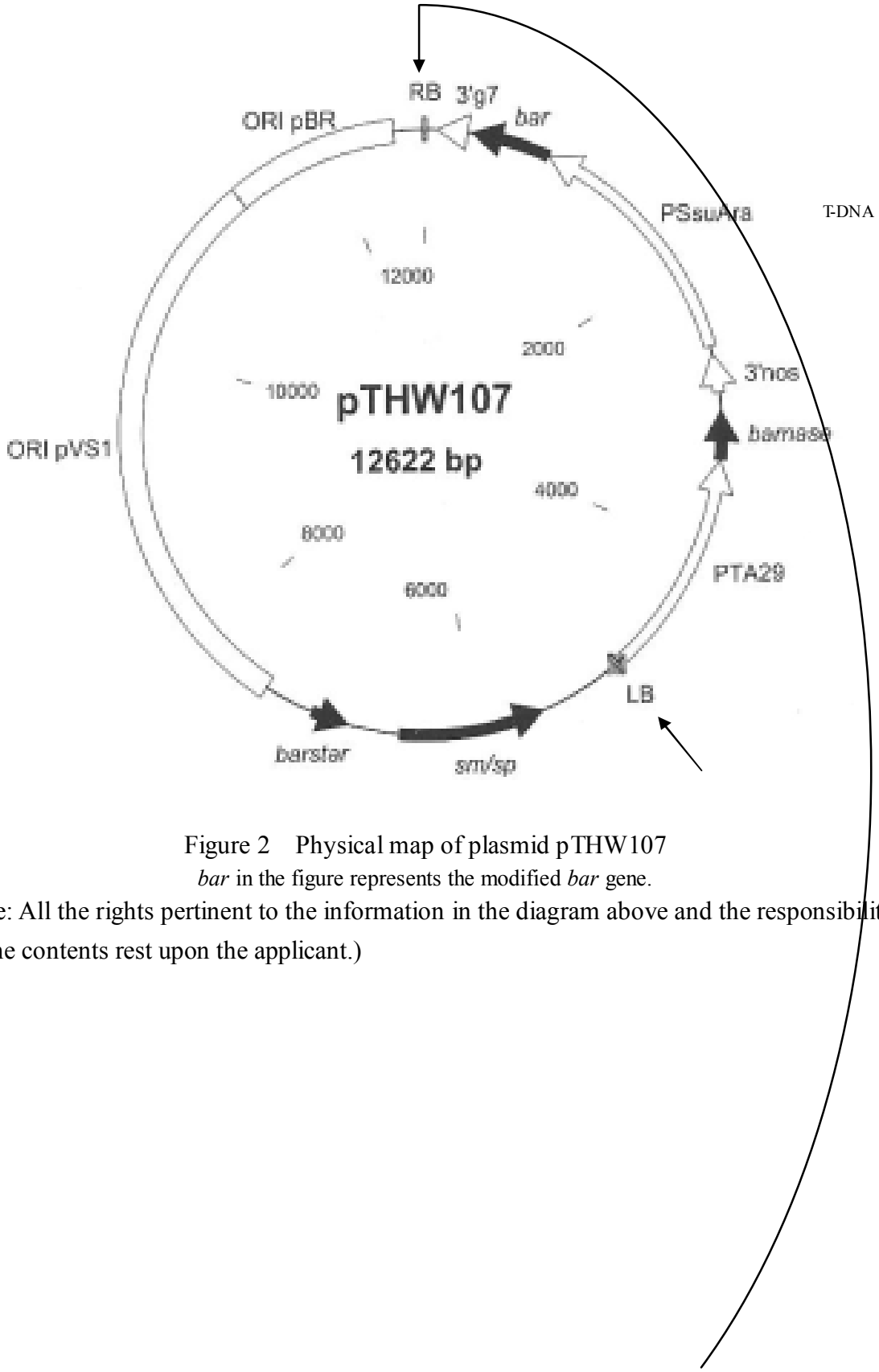

Figure 2 Physical map of plasmid pTHW107

bar in the figure represents 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.)

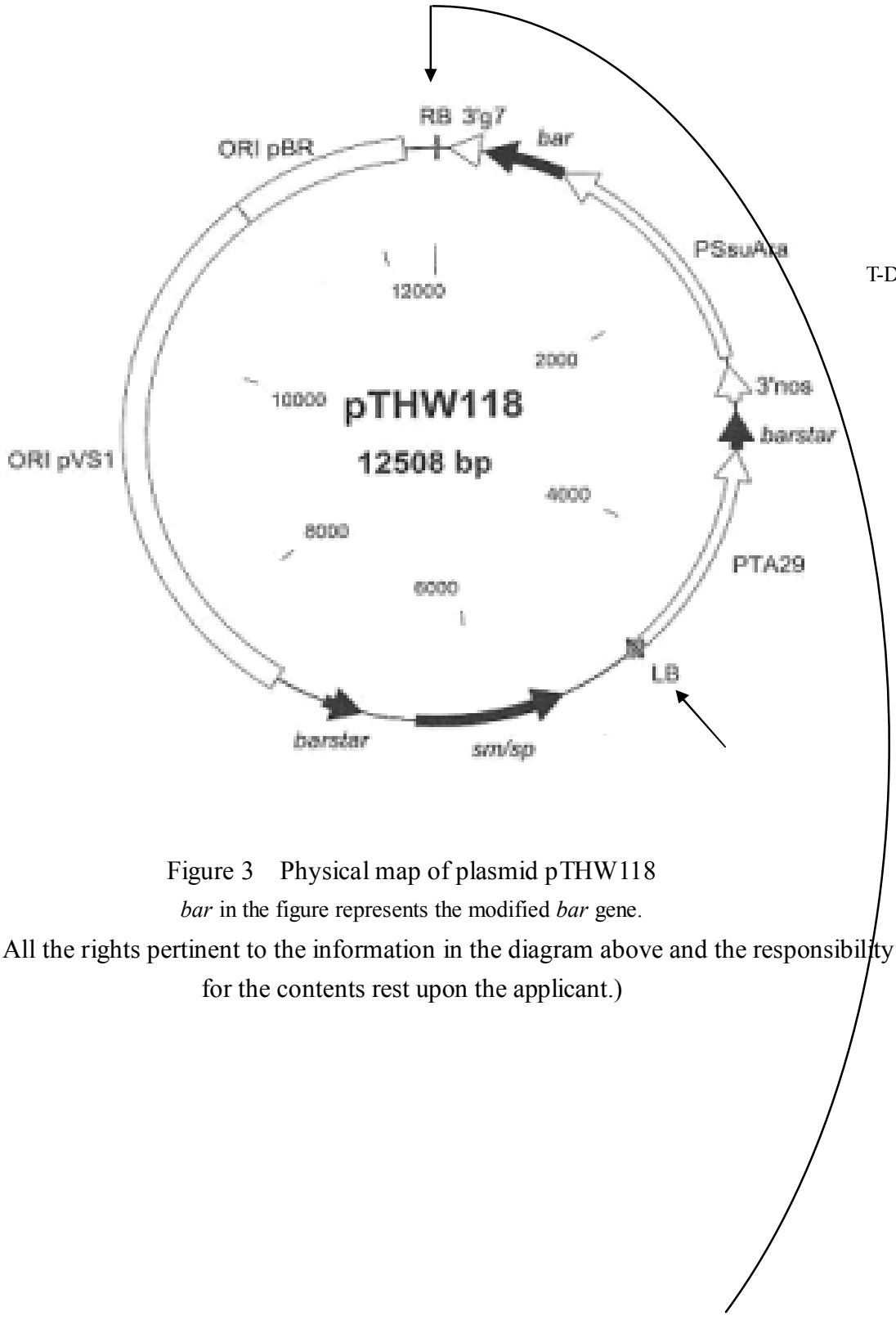

Figure 3 Physical map of plasmid pTHW118

bar in the figure represents 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.)

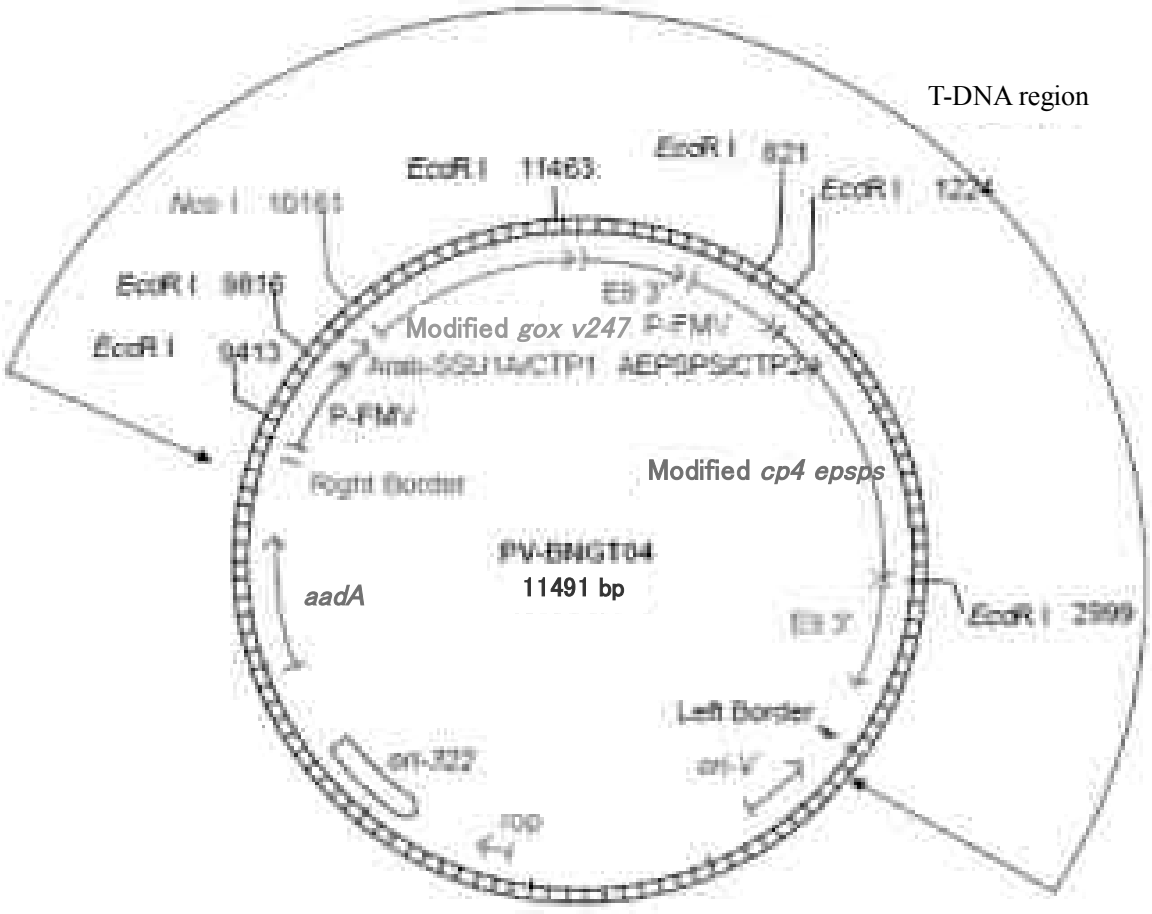

Figure 4　Physical map of plasmid PV-BNGT04

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

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

Component elements of the plasmids pTHW107, pTHW118 and PV-BNGT04 are shown in Table 1 to Table 3 (pp.14～16), respectively.

The *barstar* gene present outside the T-DNA region of pTHW107 and pTHW118 was established in the pGSV1 for the purpose of suppressing the activity of a small amount of BARNASE protein expressed despite the use of a plant promoter to protect the *E. coli* used for inserting the *barnase* gene into the plasmid when constructing pTHW107.

(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

pTHW107, pTHW118 and PV-BNGT4 do not possess any infectious characteristics.

(3) Method of preparing living modified organisms

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

MS8 : The T-DNA region of pTHW107 is composed of the *barnase* gene expression cassette and the modified *bar* gene expression cassette [PTA29]-[*barnase*]-[3'nos]-[PSsuAra]-[modified *bar*]-[3'g7] (Figure 2, p.23).

RF3 : The T-DNA region of pTHW118 is composed of the *barstar* gene expression cassette and the modified *bar* gene expression cassette [PTA29]-[*barstar*]-[3'nos]-[PSsuAra]-[modified *bar*]-[3'g7] (Figure 3, p.24).

RT73 : The T-DNA region of PV-BNGT04 is composed of the modified *gox v247* gene expression cassette [P-FMV]-[Arab-SSU1A/CTP1]-[modified *gox v247*]-[E9 3'] and the modified *cp4 epsps* gene expression cassette [P-FMV]-[AEPSPS/CTP2]-[modified *cp4 epspsp*]-[E9 3'] under the control of FMV promoter (Figure 4, p.25).

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

The *Agrobacterium* method was used for transferring the nucleic acid to the recipients MS8, RF3 and RT73.

c) Processes of rearing of living modified organisms

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

Cells containing the transferred nucleic acids were selected using media containing the following additives:

MS8 : Glufosinate
RF3 : Glufosinate
RT73 : Glyphosate

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

In the development of MS8 and RF3, *Agrobacterium* was removed by adding carbenicillin to the medium. Complete removal of *Agrobacterium* from MS8 and RF3 was confirmed by transferring MS8 and RF3 to the carbenicillin-free medium and checking the absence of *Agrobacterium* colony formation.

In the development of RT73, *Agrobacterium* was removed by adding carbenicillin and paromomycin to the medium. Complete removal of *Agrobacterium* from RT73 was confirmed by transferring RT73 to the carbenicillin-/paromomycin-free medium and checking the absence of *Agrobacterium* colony formation.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in 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

In the development of MS8 and RF3, plants regenerated after transformation were selected based on comprehensive evaluation of individual target traits, agronomic characters, etc.

In the development of RT73, further selection on the regenerated plants was carried out based on the analysis of transferred genes and the expression level of the modified CP4 EPSPS protein. Tests in climate chamber and greenhouse were then carried out, and the actual glyphosate tolerance and agronomic characters were examined in field tests. RT73 was selected upon a comprehensive evaluation of these results.

The process of rearing of this stack line is shown in Figure 5 (p.28). The subjects of this

application are this stack line produced through triparental cross of MS8, RF3 and RT73, and the progenies of this stack line.

The status of application for approval of the individual parent lines and this stack line in Japan is listed in Table 4 (p. 28).

Table 4 Status of approval of the individual parental lines and this stack line in Japan

	Food	Feed	Environment
MS8	March 2001 Safety verified	March 2003 Safety verified	September 2006 Approved for Type 1 Use Regulation
RF3	March 2001 Safety verified	March 2003 Safety verified	April 2007 Approved for Type 1 Use Regulation
RT73	March 2001 Safety verified	March 2003 Safety verified	March 2006 Approved for Type 1 Use Regulation
This stack line	Application submitted in November 2010	Notification submitted in November 2010	Application submitted in November 2010

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

Confidential: Not made available or disclosed to unauthorized persons

Figure 5 Process of rearing of this stack line

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

(a) Place where the replication product of transferred nucleic acid exists

It was confirmed that the nucleic acids transferred to MS8, RF3 and RT73 exist on the chromosome.

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

Results of Southern blotting analysis and sequence analysis demonstrated that one copy of T-DNA region was transferred into MS8.

Results of Southern blotting analysis and sequence analysis of the nucleic acid transferred into RF3 demonstrated that one copy of T-DNA region as well as a truncated PTA29, the *barstar* gene, 3'nos and a non-functional portion of PSsuAra were inserted adjacent to each other in inverted orientations.

Results of Southern blotting analysis and sequence analysis of the gene transferred into RT73 demonstrated that one copy of T-DNA region containing the modified *cp4 epsps* gene expression cassette and the modified *gox v247* gene expression cassette was transferred into the genomic DNA of RT73 at one site and that no other component element was transferred.

Stability of inheritance of the transferred genes in MS8, RF3 and RT73 was confirmed by Southern blotting analysis conducted on multiple generations.

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

This item is not applicable to MS8 and RT73 because only one copy each of T-DNA region was inserted.

The result of sequence analysis demonstrated that one copy of T-DNA region as well as a truncated PTA29, the *barstar* gene, 3'nos and a non-functional portion of PSsuAra were transferred adjacent to each other in RF3.

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

The stability of expression was confirmed as follows.

MS8: Confirming the expression stability by Northern blotting analysis, glufosinate herbicide spraying test and male sterility in individuals exerting tolerance to glufosinate herbicide

RF3: Confirming the expression stability by Northern blotting analysis, glufosinate herbicide spraying test and fertility restoration in MS8RF3

RT73: Confirming the expression stability by glyphosate herbicide spraying test

(e) 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 allowing transmission and thus, there is no possibility that the nucleic acid transferred to the oilseed rape lines could be transmitted to any other wild animals and plants under natural conditions. Moreover, PV-BNGT04 used for developing RT73 can undergo autonomous replication only in gram-negative bacteria such as *E. coli* and *A. tumefaciens*, so it is unlikely that the plasmid would be transmitted to any wild animals and plants under natural conditions.

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

Specific detection of MS8 and RF3 is available by PCR method using primers based on the flanking sequences of DNA transferred into MS8 and RF3 respectively.

As a method for detection and identification of RT73, a qualitative PCR methodhas been developed, in which the DNA sequences of the transferred genes and the flanking regions in the plant genome are used as primers. RT73 can be specifically detected using this method.

For detection and identification of this stack line, the above-mentioned methods must be applied to individual seeds or plants.

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

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

This stack line is given the following traits carried by the parent lines.

MS8: Tolerance to glufosinate herbicide and male sterility

RF3: Tolerance to glufosinate herbicide and fertility restoration

RT73: Tolerance to glyphosate herbicide

The modified PAT protein is an enzyme that inactivates glufosinate by transferring acetyl group to the herbicide. Glufosinate is classified under L-amino acids, but the modified PAT protein does not catalyze acetyl group transfer to other amino acids and hardly shows any affinity to glutamic acid, which structurally resembles glufosinate, inducing substantially no transfer reaction in vivo (Thompson *et al*., 1987). Moreover, it has been reported that the acetyl group transfer to glufosinate induced by the modified PAT protein was not inhibited in the presence of excessive amounts of other amino acids (Wehrmann *et al*., 1996). Therefore, it is considered that the modified PAT protein has high substrate specificity to glufosinate and would not affect the metabolic system of the recipient organism.

As the wild-type EPSPS protein, the modified CP4 EPSPS protein acts as an enzyme catalyzing the shikimate pathway for aromatic amino acid biosynthesis. EPSPS protein is not the rate-determining enzyme in this pathway, and thus it is considered that enhanced EPSPS activity will not increase the concentration of aromatic amino acids, the end products of this pathway. Moreover, EPSPS protein is known to specifically react with its substrates, PEP and S3P (Gruys *et al*., 1992). The only substance known to react with EPSPS other than these substrates is shikimate, but, calculated based on the report by Gruys *et al*, the reactivity with shikimate is only one-two millionth of the reactivity with S3P. Thus, it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

GOX protein is an enzyme catalyzing the degradation of glyphosate into AMPA and glyoxylate. The enzyme has high substrate specificity, and the only compounds known to react with the GOX protein other than glyphosate are iminodiacetic acid (IDA) and 2-methylglyphosate, which are structurally similar to glyphosate but have not been found in plants (Padgette *et al*., 1994). The modified GOX v247 protein expressed in RT73 has three amino acids modified for the purpose of increasing glyphosate degradation activity of GOX protein. A component analysis of RT73 expressing the modified GOX v247 protein revealed no difference in composition compared to the non-recombinant oilseed rape used as a control, suggesting that the modified GOX v247 protein has substrate specificity similar to that of the GOX protein.

BARNASE protein is a ribonuclease that hydrolyzes single-stranded RNA molecules and is expressed only in the tapetum cells under the control of the promoter PTA29. Thus, it is considered that BARNASE protein would not affect other metabolic pathways in the plant.

BARSTAR protein is an intracellular inhibitor for the BARNASE protein, forming a highly stable, non-covalently bonded complex specifically with the BARNASE protein in one-to-one correspondence (Makarov *et al.*, 1993; Martinez *et al.*, 1995). Furthermore, ribonucleases of bacteria and fungi are found to share considerable structural and sequence homology, so it is expected that inhibitors homologous to BARSTAR protein exist for these enzymes. However, such inhibitor has only been found for BINASE protein, a ribonuclease produced by *Bacillus intermedius*. The BINASE protein shares a high homology (85%) with the BARNASE protein and is inhibited by the BARSTAR protein (Yakovlev *et al.*, 1995). In addition, there is a report that an extra-cellular ribonuclease of *Streptomyces*, which shares only 20% to 25% amino acid sequence homology with the BARNASE protein but is structurally similar to the protein (Hill *et al.*, 1983), is also inhibited by the BARSTAR protein (Hartley *et al.*, 1996). However, there is no report that the BARSTAR protein inhibits ribonuclease in plants. Based on the above findings, it is considered that BARSTAR protein would not affect the metabolic system of the recipient organism.

As can be seen, the modified PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein act independently from each other through different mechanisms. Meanwhile, the BARNASE protein and the BARSTAR protein interact with each other but are unlikely to interact with the modified PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein. Moreover, both the BARNASE protein and the BARSTAR protein are expressed in the tapetum cells in the anthers under the control of the promoter PTA29 and specifically form a complex with each other. Thus, it is unlikely that these expressed proteins would exhibit any unintended functional interaction in this stack line.

The absence of functional interaction between these expressed proteins was actually confirmed through the following bioassays.

Glufosinate herbicide spraying test

In 2010, a test was conducted in Belgium by spraying glufosinate herbicide at a normal dosage (0.2 g active ingredient per 1.5 m^2) or at a 5-times higher dosage onto seedlings (1st or 2nd leaf-stage) of this stack line, MS8RF3, MS8×RT73 and MS8 cultivated in pots under greenhouse conditions. The degree of tolerance of the seedlings to the herbicide was scored and subjected to statistical analysis. At either of the concentrations tested, no statistically significant difference was observed between lines sharing the same genetic background, i.e. this stack line and MSRF3, and MS8×RT73 and MS8 (Table 5, p.33).

The results showed that the tolerance to glufosinate herbicide had not been modified through crossing, suggesting that there is no functional interaction between the modified

PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein.

Glyphosate herbicide spraying test

In 2010, a test was conducted in Belgium by spraying glyphosate herbicide at a normal dosage (0.108 g active ingredient per 1.5 m^2) or at a 5-times higher dosage onto seedlings (1st or 2nd leaf-stage) of this stack line and RT73 cultivated in pots under greenhouse conditions. The degree of tolerance of the seedlings to the herbicide was scored and subjected to statistical analysis. At either of the concentrations tested, no statistically significant difference was observed between this stack line and RT73 (Table 5, p.33).

The results showed that the tolerance to glyphosate herbicide had not been modified through crossing, suggesting that there is no functional interaction between the modified CP4 EPSPS protein or the modified GOX v247 protein and the modified PAT protein, the BARNASE protein or the BARSTAR protein.

Table 5 Degree of tolerance to herbicides [1)]

Lines tested	Glufosinate herbicide				
	Normal dosage [2)]			5-times dosage	
	Average±Standard deviation		Significant difference [4)]	Average±Standard deviation	Significant difference
This stack line	5.0 ± 0		ns	5.0 ± 0	ns
MS8RF3	5.0 ± 0			5.0 ± 0	
MS8×RT73	4.8 ± 0.45		ns	3.0 ± 0	ns
MS8	4.6 ± 0.55			3.0 ± 0	
Non-recombinant	1.0 ± 0		-	1.0 ± 0	-

Lines tested	Glyphosate herbicide				
	Normal dosage [3)]			5-times dosage	
	Average±Standard deviation		Significant difference	Average±Standard deviation	Significant difference
This stack line	4.6 ± 0.55		ns	3.2 ± 0.45	ns
RT73	4.0 ± 0			3.0 ± 0	
Non-recombinant	1.0 ± 0		-	1.0 ± 0	-

[1)]The test was conducted in 5 plots. Approximately 51 seeds (since the male-sterile MS8 was maintained by a cross with a non-recombinant oilseed rape, in theory, the transferred gene would be transmitted to only 50% of the seeds produced by MS8. Thus, the numbers of seeds sown were doubled for MS8 and MS8×RT73.) were sown per plot. The herbicide was sprayed onto the germinated seedlings (1st or 2nd leaf stage), and the tolerance to the herbicide was individually evaluated for each plot. The average and standard deviation of the scores among the 5 plots (n=5) are shown in the table. The indices for tolerance were as follows: 1: very low tolerance (herbicide injury 100-80%); 2: low tolerance (herbicide injury 79-60%); 3: moderate tolerance (herbicide injury 59-40%); 4: high tolerance (herbicide injury 39-20%); 5: very high tolerance or no herbicide injury observed (herbicide injury 19-0%).

[2)]Glufosinate herbicide; active ingredient 0.2 g/1.5 m^2.

[3)]Glyphosate herbicide; active ingredient 0.108 g/1.5 m^2.

[4)]Statistical analysis was conducted by Mann-Whitney U test (significance level: 5%). ns: Not statistically significant. -: Not statistically evaluated.

Examination of male sterility and fertility restoration

In 2008, tests were conducted at 5 sites in Canada (2 sites in Saskatchewan and 3 sites in Alberta). For each line tested, 400 plants per site (2000 plants in total) were examined. This stack line was tested in two plots, one treated with glufosinate and glyphosate and the other untreated. MS8RF3, MS8×RT73, MS8 and RF3 were treated with glufosinate, while non-recombinant plants were untreated.

The results of the examination showed that substantially all plants of this stack line and

MS8RF3 produced fertile pollens, with no statistically significant difference observed between the two lines. In contrast, substantially all plants of MS8×RT73 and MS8 were male sterile, with no statistically significant difference observed between the two lines. Substantially all plants of RF3 and non-recombinant plants were fertile, with no statistically significant difference observed between the two lines (Table 6, p.34).

The results showed that male sterility and fertility restoration had not been modified through crossing, suggesting that there is no functional interaction between the BARNASE protein and the modified CP4 EPSPS protein or the modified GOX v247 protein, or between the BARSTAR protein and the modified CP4 EPSPS protein or the modified GOX v247 protein.

Table 6 Examination of male sterility and fertility restoration

Lines tested	Herbicide treatment[1)]	Expected number of plants		Actual number of plants		χ^2 value[2)]	p value
		Sterile	Fertile	Sterile	Fertile		
This stack line	Gly + Glu	0	2000	1	1999	0.33 (ns)	0.564
This stack line	—	0	2000	0	2000	2.00 (ns)	0.157
MS8RF3	Glu	0	2000	2	1998		
MS8×RT73	Glu	2000	0	2000	0	1.00 (ns)	0.317
MS8	Glu	2000	0	1999	1		
RF3	Glu	0	2000	8	1992	0.69 (ns)	0.405
Non-recombinant	—	0	2000	5	1995		

At 5 sites, 1600 seeds (since the male-sterile MS8 was maintained by a cross with a non-recombinant oilseed rape, in theory, the transferred gene would be transmitted to only 50% of the seeds produced by MS8. Thus, the numbers of seeds sown were doubled for MS8 and MS8×RT73.) were sown for each line tested. Among the plants germinated from the seeds, 400 plants per site (2000 plants in total) were examined for fertility. Meanwhile, MS8×RT73 and MS8 plants were treated with glufosinate herbicide during the vegetative period to select lines possessing the modified *bar* gene, and 400 plants per site (2000 plants in total) were examined for fertility in the flowering period.

[1)] Details of individual treatments are as follows:

Gly + Glu: Seedlings were treated with glyphosate (active ingredient 450 g/ha) once during the cotyledon stage to 2nd leaf stage and with glufosinate (active ingredient 500 g/ha) once during the 4th to 6th leaf stage.

Glu: Seedlings were treated with glufosinate (active ingredient 500 g/ha) once during the 2nd to 4th leaf stage.

[2)] Assuming that the transferred gene would not affect pollen fertility, the null hypothesis is rejected when χ^2 value is 3.84 (p=0.05) or larger.

Based on the above results, it is unlikely that expressed proteins would exhibit any

unintended functional interaction in this stack line and that the traits derived from the parent lines would be changed.

With respect to physiological or ecological characteristics, differences between this stack line and the taxonomic species to which the recipient organism belongs were evaluated based on the results obtained from the examination of individual parent lines, i.e. MS8, RF3 and RT73.

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

Information regarding the following 7 items (a to g) was collected by the following means:

MS8: An isolated field test (FY 1999, Vegetable Breeding Research Team, National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization) and a test conducted in special screened greenhouses in Japan (2005)

RF3: An isolated field test (FY 1999, Vegetable Breeding Research Team, National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization) and tests conducted in special screened greenhouses in Japan (2005 and 2006)

RT73: An isolated field test (from May 1995 to March 1996, National Institute for Agro-Environmental Sciences) and a test conducted in non-containment greenhouses (from July 1994 to February 1995, Monsanto Japan Limited)

a. Morphological and growth characteristics

Comparison was made for the items listed in Table 7 (p.36) between MS8, RF3 and RT73 and their respective non-recombinant control cultivars.

Comparison between MS8 and the relevant control cultivar revealed a statistically significant difference in the pod setting rate (Appendix 1: not made available or disclosed to unauthorized persons). Rapeseed mainly produces seeds through self pollination, but since MS8 produces no pollen, it requires pollens from other lines to form seeds. Thus, the fewer chances of pollination compared to the control cultivar seemed to have affected the pod setting in MS8.

RF3 showed a slightly but statistically significantly lower number of seed setting than the relevant control cultivar (Appendix 3: not made available or disclosed to unauthorized persons). This could be attributed to the slightly lower number of ovules per pod in RF3 than in the control, although the difference was not statistically significant.

Comparison between RT73 and the relevant control cultivar revealed no difference in all items examined (Appendix 7: not made available or disclosed to unauthorized persons).

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

In order to assess the cold tolerance at the early stage of growth in MS8 and RF3, seeds of MS8, RF3 and their respective control cultivars were sown in an isolated field in October 1998. The survival rates obtained in March 1999 were 100% for all lines tested (Appendix 1: not made available or disclosed to unauthorized persons; Appendix 3: not made available or disclosed to unauthorized persons). Regarding the heat tolerance at the early stage of growth, seeds of MS8, RF3 and their respective control cultivars were sown in a naturally ventilated, special screened greenhouse in summer (July). The dry weights of the plants harvested about 2 months after sowing were compared, and no statistically significant difference was observed between the recombinants and their relevant controls (Appendix 2: not made available or disclosed to unauthorized persons; Appendix 4: not made available or disclosed to unauthorized persons).

The cold tolerance of RT73 was assessed by examining the plant height and the growth stage of RT73 and the relevant control cultivar at around the 1st or 2nd leaf stage, subsequently growing them in a climate chamber in which the temperature was set at 5℃ (humidity 35%, 3,500lx, 12-hour day length) and examining the plant height and growth stage 30 days later. The results revealed no statistically significant difference in growth between RT73 and the control cultivar (Appendix 8: not made available or disclosed to unauthorized persons). Furthermore, an isolated field test was conducted to compare summer and winter growth between RT73 and the control cultivar. For assessing the summer growth, seedlings at the 5th or 6th leaf stage were planted on July 25, 1995 to examine their subsequent growth, and seeds were sown on August 1, 1995 to examine the germination rate. For assessing the winter growth, seeds were sown in autumn (sowing date: October 6) to examine the germination rate and the initial growth (Appendix 7: not made available or disclosed to unauthorized persons). The results of the planting and sowing tests conducted in summer revealed no statistically significant difference between RT73 and the control cultivar in terms of survival rate, plant height, germination rate, etc. (Appendix 7: not made available or disclosed to unauthorized persons). Moreover, no statistically significant difference could be found between RT73 and the control cultivar in terms of germination

rate and initial growth after autumn sowing (Appendix 7: not made available or disclosed to unauthorized persons).

Table 7 Test items and summary of results regarding morphological and growth characteristics

Test items	MS8	RF3	RT73
Time of bolting	○	○	—
Flowering period	○	○	—
Maturation period	○	○	—
Initiation of flowering	—	—	○
Completion of flowering	—	—	○
Harvesting time	—	—	○
Uniformity of germination	—	—	○
Germination rate	—	—	○
Number of primary branches	○	○	○
Plant height	○	○	○
Plant shape	○	○	○
Color of leaves	○	○	—
Number of open flowers	—	—	○
Number of pods	○	○	○
Rate of pod dehiscence	—	—	○
Number of sterile pods per ear	○	○	—
Number of ovules per pod	○	○	—
Pod setting rate	○ *	○	—
Pod length	○	○	○
Pod width	—	—	○
Number of seed setting (number of seeds per pod)	○	○ *	○
Seed setting percentage (%)	○	○	—
Seedcolor (color of seed)	○	○	○
Uniformity of seed size	○	○	○
Shape of hilum	—	—	○
Fresh weight of aerial parts at harvesting period (aerial fresh weight)	○	○	○
Fresh weight of underground parts at harvesting period	—	—	○
Dry weight of aerial parts	○	○	○
Dry weight of underground parts	—	—	○
Dry matter ratio (%)	○	○	—
Seed yield	○	○	—
1000-seed weight	○	○	—

○: Examined. －: Not examined *: Statistically significant difference (significance level: 5%) observed.
(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

c. Wintering ability or summer survival of the matured plant

The summer survival of matured MS8 and RF3 plants were examined by sowing seeds of MS8 and RF3 and their respective control cultivars in October 1998 and leaving the germinated plants in the field after they reached maturation in June. In all lines tested, all plants were found withered and dead by July 30 (Appendix 1: not made available or disclosed to unauthorized persons; Appendix 3: not made available or disclosed to unauthorized persons).

RT73 and the control cultivar were planted on May 31, 1995 and left cultivated after the harvesting time to examine the wintering ability of the matured plants. All plants were found brown and dead by November 22, 1995, and no difference was observed between the two lines (Appendix 7: not made available or disclosed to unauthorized persons).

d. Fertility and size of the pollen

Pollen formation was not observed in MS8 (Appendix 1: not made available or disclosed to unauthorized persons). There was no difference in fertility, shape and size of the pollens between RF3 and the control cultivar (Appendix 3: not made available or disclosed to unauthorized persons).

Comparison of pollens of RT73 with those of the control cultivar revealed no significant difference in pollen fertility (Appendix 8: not made available or disclosed to unauthorized persons) and no difference in pollen size (Appendix 8: not made available or disclosed to unauthorized persons).

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

Seed production

As mentioned in section a, a comparison between MS8 and the relevant control cultivar revealed a statistically significant difference in the pod setting rate (Appendix 1: not made available or disclosed to unauthorized persons). This could be attributed to the fewer chances of pollination of MS8 compared to the control cultivar, as MS8 produces no pollen and requires pollens from other lines to form seeds.

As mentioned in section a, RF3 showed a slightly but statistically significantly lower number of seed setting than the relevant control cultivar (Appendix 3: not made available or disclosed to unauthorized persons). This could be attributed to the slightly lower number of ovules per pod in RF3 than in the control, although the difference was not statistically significant.

Comparison was made between RT73 and the relevant control cultivar regarding the number of pods and the number of seeds per pod (number of seed setting), but no statistically significant difference was observed between the two lines (Appendix 7: not made available or disclosed to unauthorized persons). Moreover, no statistically significant difference was observed between RT73 and the relevant control cultivar regarding the number of seeds, the number of pods and the pod setting rate obtained through artificial pollination and natural crossing (Appendix 7: not made available or disclosed to unauthorized persons).

Shedding habit

MS8 and RF3 were compared with their respective control cultivars for pod dehiscence, but no difference or statistically significant difference was found between the recombinants and their relevant controls (Appendix 1: not made available or disclosed to unauthorized persons; Appendix 3: not made available or disclosed to unauthorized persons).

RT73 and the relevant control cultivar showed no statistically significant difference in the rate of pod dehiscence (Appendix 7: not made available or disclosed to unauthorized persons).

Dormancy and germination rate

Seeds of MS8 and the relevant control cultivar cultivated and harvested in an isolated field showed 100% germination (Appendix 1: not made available or disclosed to unauthorized persons).

Germination rate has not been examined for RF3 seeds in Japan, but a test was conducted in Belgium in 1995 on seeds of RF3 and the relevant control cultivar which had been stored at room temperature after harvest. One hundred seeds each for RF3 and the control were sown in 6 replicates, and 91 and 89 seeds germinated on average, respectively, demonstrating the equivalence of the germination rate between the two lines (Appendix 6: not made available or disclosed to unauthorized persons).

Germination rates of the seeds of RT73 and the relevant control cultivar obtained through artificial pollination were 100% and 99%, respectively, which were not statistically significantly different from each other (Appendix 8: not made available or disclosed to unauthorized persons).

f. Crossability

Since MS8 produces no pollen, crosses mediated by MS8 pollens do not occur. Meanwhile, the female gametes of MS8 are normal and thus can be cross-fertilized by

pollens from surrounding plants. Therefore, crossability and seed production potential are likely to depend on the surrounding conditions.

The crossability of RF3 with non-recombinant rapeseed (Mie Nagashima Natane) and *B. rapa* (native rapeseed: *Seitei*) cultivated adjacent to RF3 in the presence of honey bees was examined. Seedlings derived from the seeds harvested from each line were sprayed with glufosinate herbicide. As a result, 1.8% to 2.0% and 0% of the seedlings showed tolerance to the herbicide in non-recombinant oilseed rape and *B. rapa*, respectively (Appendix 3: not made available or disclosed to unauthorized persons), which did not exceed the existing data on the outcrossing rate of oilseed rape (5-30%; Hühn and Rakow, 1979; Rakow and Woods, 1987) and the crossability between oilseed rape and *B. rapa* (0.4-1.5%: Scott and Wilkinson, 1998).

The crossability of RT73 was examined in an isolated field using a non-recombinant control cultivar, *B. juncea* (Leaf mustard) and *B. rapa* (rapeseed) cultivated in zones 0, 2, 5 and 10 m north, south, east and west of the cultivation zone of RT73. The crossability of RT73 with the control cultivar was highest in the adjacent 0 m zone and was 1% or lower in the 10 m zone. In the adjacent 0 m zone, the maximum crossability throughout all crossing tests was 21% (Appendix 7: not made available or disclosed to unauthorized persons), which was substantially the same as the natural crossability between non-recombinant oilseed rape cultivated adjacent to each other (approximately 20%, *Hatasaku Zensho*, Rural Culture Association Japan). Moreover, the crossability of RT73 with *B. juncea* (Leaf mustard) or *B. rapa* (native rapeseed) was also highest in the adjacent 0 m zone and was 0% in the 10 m zone (Appendix 7: not made available or disclosed to unauthorized persons).

In a pollen dispersal experiment conducted in a field in Canada in 1989 (Appendix 8: not made available or disclosed to unauthorized persons), the incidences of hybrid plants among control cultivar plants cultivated in zones 50 m, 100m and 150-225 m away from RT73 cultivation zone were 0.19% (average of 3 zones), 0.12% (average of 3 zones) and 0.08% (average of 4 zones), respectively (Appendix 8: not made available or disclosed to unauthorized persons).

In a non-containment greenhouse, the distance of pollen dispersal by artificial wind and the flower-visiting behavior of honey bees were examined, and no statistically significant difference was found between RT73 and the relevant control cultivar (Appendix 8: not made available or disclosed to unauthorized persons).

Furthermore, in a non-containment greenhouse, *B. juncea* (Leaf mustard) and *B. rapa* (native rapeseed), which were used in the crossability test, were artificially pollinated with RT73 and the control cultivar, and the number of fertile seeds per siliqua obtained were counted to examine their cross compatibility with RT73 and the control. In both *B. juncea* (Leaf mustard) and *B. rapa* (rapeseed), the cross compatibility with RT73 was substantially

the same as that with the control cultivar (Appendix 7: not made available or disclosed to unauthorized persons).

Moreover, the germination rate was examined for seeds obtained from all combinations of crosses involving *B. juncea* (Leaf mustard), *B. rapa* (rapeseed), RT73 and the relevant control cultivar. The results showed that crossing with RT73 did not affect the dormancy of progeny seeds (Appendix 7: not made available or disclosed to unauthorized persons).

g. Productivity of harmful substances

A succeeding crop test, a plow-in test and a soil microflora test were carried out for MS8 and the relevant control cultivar in a special screened greenhouse. In all tests, no statistically significant difference could be found between MS8 and the control cultivar in all items tested (Appendix 2: not made available or disclosed to unauthorized persons).

A succeeding crop test, a plow-in test and a soil microflora test were carried out for RF3 and the relevant control cultivar in a special screened greenhouse. In all tests, no statistically significant difference could be found between RF3 and the control cultivar in all items tested (Appendix 4: not made available or disclosed to unauthorized persons). However, in the plow-in test, the germination rate of the test plant (radish) showed a statistically insignificant difference between the RF3 plot (59.0%) and the control cultivar plot (86.0%) (Appendix 4: not made available or disclosed to unauthorized persons), so an additional plow-in test was carried out. The additional plow-in test examined the effects of the plowed plants on test plants over time (immediately, one week, two weeks and four weeks after mixing), but no statistically significant difference was observed in the germination rate, root length, fresh weight or dry weight of radish. Meanwhile, a statistically significantly difference was observed between the RF3 plot and the control cultivar plot in the plant height of radishes grown on soils immediately and 4 weeks after mixing (Appendix 5: not made available or disclosed to unauthorized persons). The plant height of radishes grown in the soil immediately after mixing was slightly lower in the RF3 plot than in the control cultivar plot, while the plant height in the soil four weeks after mixing was higher in the RF3 plot than in the control cultivar plot (Appendix 5: not made available or disclosed to unauthorized persons).

For RT73, a soil microflora test was carried out in an isolated field, and succeeding crop test, a plow-in test, an assay using leaf leachate and a soil microflora test were carried out in non-containment greenhouses. For all items tested, no statistically significant difference was observed between RT73 and the control cultivar (Appendix 7: not made available or disclosed to unauthorized persons; Appendix 8: not made available or disclosed to unauthorized persons).

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

Name of the Type of Living Modified Organism: Glufosinate and Glyphosate herbicide-tolerant, male-sterile and fertility-restored oilseed rape (Modified *bar*, modified *cp4 epsps*, modified *gox v247*, *barnase*, *barstar*, *Brassica napus* L.) (MS8×RF3×RT73, OECD UI: ACS-BNØØ5-8×ACS-BNØØ3-6×MON-ØØØ73-7) (including the progeny lines isolated from the oilseed rape lines that contain a combination of any of the transferred genes in the individual oilseed rape lines MS8, RF3 and RT73 (except those already granted an approval regarding Type 1 Use Regulation.))
Content of the Type 1 Use: Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.
Applicant: Bayer CropScience K.K.

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

This stack line was produced by crossing the glufosinate herbicide-resistant, male-sterile oilseed rape (MS8) carrying the modified *bar* gene (encoding the modified PAT protein) and the *barnase* gene, the glyphosate herbicide-resistant, fertility-restored oilseed rape (RF3) carrying the modified *bar* gene and the *barstar* gene and the glyphosate herbicide-resistant oilseed rape (RT73) carrying the modified *cp4 epsps* gene and the modified *gox v247* gene. The Committee on Adverse Effect on Biological Diversity judged that each of these parent lines would not result in an Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stack line.

Since the modified PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein have high specificity to their respective substrates, it is considered that the expression of these proteins in this stack line would not affect the metabolic system of the recipient organism.

Moreover, in this stack line, the *barnase* gene and the *barstar* gene are both under the control of the anther-specific promoter PTA29, and the BARNASE protein and the BARSTAR protein expressed in the tapetum cells in the anther specifically form a complex with each other, which inhibits the ribonuclease activity of the BARNASE protein and results in fertility restoration. Meanwhile, the BARNASE protein and the BARSTAR protein are unlikely to interact with the modified PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein. Based on these understandings, it is considered that the expression of these proteins in this stack line would not affect the metabolic system of the recipient organism to produce unintended metabolites.

Since the degrees of glufosinate and glyphosate herbicide-resistances, male sterility and fertility restoration of this stack line were equivalent to those in the relevant parent lines, it is unlikely that these proteins derived from the relevant parent lines would exhibit any unintended functional interaction with each other and that notable changes in traits would occur, except for the traits received from the parent lines.

(1) Competitiveness

Oilseed rape (*Brassica napus* L.) to which the recipient organism belongs is reportedly growing on river banks, along railroads, 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 shrubs in any conditions of location other than roadsides, bluffs or riverside areas where disturbances could occur on a regular basis. Japan has a long history of importing oilseed rape seeds, but there has been no report that the species has affected preservation of wild animals or plant species or their populations in Japan.

Various traits related to competitiveness were examined in the parent lines of this stack line. For some of the traits examined, statistically significant differences were observed between the parent lines and their respective control cultivars, but these differences were not considered to increase the competitiveness of the parent lines.

This stack line is tolerant to glufosinate and glyphosate herbicides, but it is unlikely that these herbicides would function as selection pressures in the natural environment. Therefore, it is unlikely that these traits would increase the competitiveness of this stack line. MS8 and RF3, which are the parent lines of this stack line, exhibit male sterility and fertility restoration, respectively; in this stack line, however, the activity of the BARNASE protein derived from MS8 would be inhibited by the BARSTAR protein derived from RF3. Thus, this stack line has the same potential as a non-recombinant oilseed rape to produce pollens. The male sterility trait does not provide any competitive advantage to the host. Meanwhile, the fertility restoration trait can play its intended role when crossed with a male-sterile individual expressing the BARNASE protein but is absolutely functionless in the absence of such individual. Thus, the fertility restoration trait does not provide any competitive advantage to the host.

Based on the above understanding, it was judged that the conclusion made by the applicant that the use of this stack line and the progeny lines isolated from this rapeseed containing a combination of any of the transferred genes in MS8, RF3 and RT73 poses no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

Conventional oilseed rape is known to produce erucic acid and glucosinolate in the seeds

recognized as harmful substances to animals. The recipient organism of this stack line is one of the cultivars known as Canola featuring reduced contents of erucic acid and glucosinolate achieved by selective breeding and thus is unlikely to affect the habitat or growth of wild animals.

This stack line produces the modified PAT protein, the modified CP4 EPSPS protein, the modified GOX v247 protein, the BARNASE protein and the BARSTAR protein, but it has been confirmed that these proteins do not contain any sequence which is structurally analogous to known allergens.

Moreover, the modified PAT protein, the modified CP4 EPSPS protein and the modified GOX v247 protein have high substrate specificity, so it is considered that these proteins expressed in this stack line would not affect the metabolic system of the recipient organism. The BARNASE protein and the BARSTAR protein are both under the control of PTA29, a promoter specifically expressed in the tapetum cells in the anther, to be expressed in the tapetum cells. The two proteins specifically form a complex with each other to inhibit the ribonuclease activity of the BARNASE protein, but there is no report that the BARSTAR protein inhibits ribonucleases in plants. Therefore, it is considered that these proteins expressed in this stack line would not affect the metabolic system of the recipient.

Based on these understandings, it is considered unlikely that the modified PAT protein, the modified CP4 EPSPS protein, the modified GOX v247 protein, the BARNASE protein and the BARSTAR protein produce any harmful substances in this stack line.

In addition, productivity of harmful substances (including the substances secreted from the roots which can affect other plants and microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying) of MS8, RF3 and RT73 was examined by conducting a succeeding crop test, a plow-in test, a soil microflora test and other tests. As a result, in a plow-in test examining the effects of RF3 on test plants over time, statistically significantly differences were observed in some plots. However, since no statistically significant differences were observed in other plots and since no consistent trend could be found among the observed differences, it is considered that RF3 has not newly acquired any productivity of harmful substances. Therefore, it is unlikely that this stack line would newly produce any harmful substance.

Based on these understandings, it was judged that the conclusion made by the applicant that the use of this stack line and the progeny lines isolated from this rapeseed containing a combination of any of the transferred genes in MS8, RF3 and RT73 poses no risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is valid.

(3) Crossability

Among the related wild species that can be crossed with oilseed rape, none is native to

Japan. Therefore, no wild animal or plant species likely to be affected by crossability of this stack line could be specified.

Among the related wild species that can be naturally crossed with oilseed rape other than oilseed rape itself, Leaf mustard (*B. juncea*), black mustard (*B. nigra*), Rapeseed (*B. rapa*), Hoary mustard (*Hirschfeldia incana*), Wild radish (*Raphanus raphanistrum*) and charlok (*Sinapis arvensis*) are known to grow in Japan, all of which are introduced species and are not specified as wild species likely to be affected.

Based on the above understanding, it was judged that the conclusion made by the applicant that the use of this stack line and the progeny lines isolated from this rapeseed containing a combination of any of the transferred genes in MS8, RF3 and RT73 poses no risk of Adverse Effect on Biological Diversity attributable to crossability is valid.

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(4) Additional information

The possible indirect Adverse Effect on Biological Diversity attributable to crossing with oilseed rape and the related species described above was evaluated. The possible indirect Adverse Effect on Biological Diversity refers to that; i) crossbreds produced by crossing would become competitive and destroy populations of other wild animals and plants, and ii) related species populations would decrease due to the effect of transferred gene spread by crossing, thereby affecting the preservation of populations of insects and other wild animals and plants which are dependent on the related species.

(a) As a result of the crossability tests of the parent lines (RF3 and RT73) of this stack line with non-recombinant rapeseed etc., it has been confirmed that the crossability did not exceed or differ from the existing data

(b) The crossability of oilseed rape with these introduced related species is low, and even in the event of crossing, the chances are low that the resulting hybrid becomes dominant in a natural environment, due to low fertility etc.

(c) It is unlikely that plants possessing glufosinate and glyphosate herbicide-tolerance and fertility restoration would become competitive in a natural environment

(d) It has been reported that plants possessing male sterility as a dominant trait would rapidly decrease its population over generations

Consequently, it is judged that the possibility that this stack line would cross with introduced related species and the resulting cross-progeny would become competitive in a natural environment is as low as that of oilseed rape to which the recipient organism belongs. In addition,

(e) 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* while screening with glufosinate herbicide, it was reported that there was no difference in the pollen fertility, the survival and the amount of seeds produced.

(f) Even if the *barnase* gene become out of control of the promoter PTA29 and acquire a promoter which would express constitutively or site-specifically in a plant, the possibility that the 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 unlikely that the genes would spread in introduced related species populations and affect preservation of the populations.

Based on these understandings, this stack line and the progeny lines isolated from this oilseed rape containing a combination of any of the transferred genes in MS8, RF3 and RT73 poses no risk of indirect Adverse Effect on Biological Diversity attributable to crossability is valid.

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 and the progeny lines isolated from this oilseed rape containing a combination of any of the transferred genes in MS8, RF3 and RT73 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 valid.

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Appendix List

Appendix 1：【MS8】FY 1999 Report on isolated field test of recombinant rapeseed
Confidential: Not made available or disclosed to unauthorized person

Appendix 2：【MS8】Test report　Productivity of harmful substances of glufosinate herbicide-tolerant, male-sterile rapeseed line MS8
Confidential: Not made available or disclosed to unauthorized person

Appendix 3：【RF3】FY 1999 Report on isolated field test of recombinant rapeseed
Confidential: Not made available or disclosed to unauthorized person

Appendix 4：【RF3】Test report　Productivity of harmful substances of glufosinate herbicide-tolerant, fertility-restored rapeseed line RF3
Confidential: Not made available or disclosed to unauthorized person

Appendix 5：【RF3】Test report　Temporal observation of productivity of harmful substances of glufosinate herbicide-tolerant, male-sterile rapeseed line RF3 (plow-in test)
Confidential: Not made available or disclosed to unauthorized person

Appendix 6：【RF3】Examination on agronomic traits conducted overseas
Confidential: Not made available or disclosed to unauthorized person

Appendix 7：【RT73】Confidential: Not made available or disclosed to unauthorized person

Appendix 8：【RT73】Confidential: Not made available or disclosed to unauthorized person