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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Oilseed rape tolerant to glyphosate herbicide (Modified *cp4 epsps*, *Brassica napus* L.) (MON88302, OECD UI: MON-883Ø2-9)
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	—

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Outline of the Biological Diversity Risk Assessment Report

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

5

1. Information concerning preparation of living modified organisms

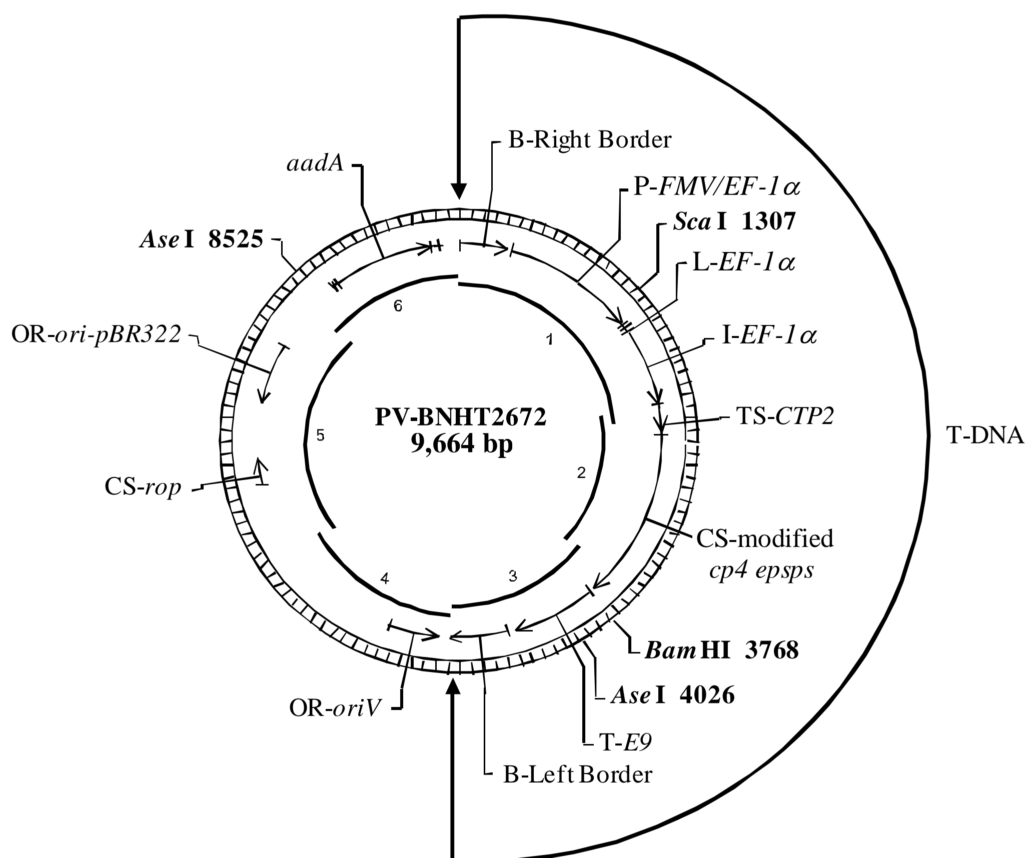
Monsanto Company has developed the oilseed rape tolerant to glyphosate herbicide MON88302 (modified *cp4 epsps*, *Brassica napus* L.)(MON88302, OECD UI: MON-883Ø2-9) (hereinafter
10 referred to as "this recombinant oilseed rape"), in order to provide a weed control system more effective than conventional ones and for a low cost and labor saving cultivation of oilseed rape,

(1) Information concerning donor nucleic acid

15 1) Composition and origins of component elements

The composition of donor nucleic acids and the origins of component elements used for the development of this recombinant oilseed rape are shown in Figure 1 (p. 3) and Table 1 (p. 4-13).

20 Due to the insertion of a restriction enzyme cleavage site in the cloning process, in the amino acid sequence of the CP4 EPSPS protein, for which the *cp4 epsps* gene introduced into this recombinant oilseed rape codes, serine at the 2nd position from the N-terminal is replaced by leucine, compared to the amino acid sequence of the CP4 EPSPS protein derived from *Agrobacterium* sp. CP4 strain. Therefore, the *cp4 epsps* gene transferred into this recombinant oilseed rape and the expressed
25 protein are defined as the "modified *cp4 epsps* gene" and the "modified CP4 EPSPS protein," respectively. The modified CP4 EPSPS protein expressed in this recombinant oilseed rape has been developed by Monsanto Company and it is the same protein as the one expressed in the modified oilseed rape RT73, which has already been approved for the Type 1 Use Regulation, and other crops tolerant to glyphosate herbicide. The deduced amino acid sequences of the modified CP4 EPSPS
30 protein expressed in this recombinant oilseed rape are shown in Annex 1.



5 Figure 1 Plasmid map of PV-BNHT2672 used for developing this recombinant oilseed rape.[1]

EF-1α is identical to *Tsf1* shown in Figure 1 (p. 33) in Annex 4.

[1] All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 1 Origins and functions of each component of PV-BNHT2672 used for developing this recombinant oilseed rape[2]

Component elements	Position in the plasmid	Origins and functions
	T-DNA	
B[Note 1]-Right Border	1~357	DNA region derived from *Agrobacterium tumefaciens*. It is a sequence containing the right border sequence used during transfer of the T-DNA. (Depicker et al., 1982; Zambryski et al., 1982)
Intervening Sequence	358~427	Sequence used in DNA cloning.
P[Note 2]-*FMV/ EF-1α*[Note 3]	428~1,467	Chimeric promoter in which an enhancer element of the 35S promoter of Figwort Mosaic Virus(FMV) (Richins et al., 1987) was bound to the *Tsf1* promoter derived from *Arabidopsis thaliana* (thale cress) (Axelos et al., 1989). It is involved in the constitutive expression of the target genes.
L[Note 4]- *EF-1α*	1,468~1,513	5' untranslated leader region (exon 1) of the *Tsf1* gene derived from *A. thaliana* (thale cress) (coding for the translated elongation factor EF-1 alpha) (Axelos et al., 1989). It promotes the expression of the target genes.
I[Note 5]- *EF-1α*	1,514~2,135	Intron sequence of the *Tsf1* gene derived from *A. thaliana* (thale cress) (coding for the translated elongation factor EF-1 alpha) (Axelos et al., 1989). It promotes the expression of the target genes.
Intervening Sequence	2,136~2,144	Sequence used in DNA cloning.
TS[Note 6]-*CTP2*	2,145~2,372	Sequence coding for the chloroplast transit peptide derived from the S*hkG* gene coding for the EPSPS of *A. thaliana* (thale cress) (Klee et al., 1987; Herrmann, 1995). It transports the modified CP4EPSPS protein to the chloroplast.
CS[Note 7]-modified *p4 epsps*	2,373~3,740	Coding sequence of the *aroA* gene coding for 5-enolpyruvylshikimate-3-phosphate synthase of *Agrobacterium* CP4 strain (CP4 EPSPS), a Gram-negative bacterium (Padgette et al., 1996b; Barry et al., 2001).
Intervening Sequence	3,741~3,782	Sequence used in DNA cloning.
T[Note 8]-*E9*	3,783~4,425	3'-terminal untranslated region derived from the *rbcS2* gene coding for a small subunit of the ribulose-1,5-bisphosphate carboxylase of *Pisum sativum* (pea). It directs mRNA polyadenylation (Coruzzi et al., 1984).
Intervening Sequence	4,426~4,468	Sequence used in DNA cloning.
B-Left Border	4,469~4,910	DNA region derived from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983).

[2] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 1 (continued) Origins and functions of each component of PV-BNHT2672 used for developing this recombinant oilseed rape

Component elements	Position in the plasmid	Origins and functions
Vector backbone region (Not present in this recombinant oilseed rape)		
Intervening Sequence	4,911~4,996	Sequence used in DNA cloning.
OR [Note 9]- *ori V*	4,997~5,393	Origin of replication from the broad host range plasmid RK2, which confers autonomous replication ability to the vector in *Agrobacterium* (Stalker et al., 1981).
Intervening Sequence	5,394~6,901	Sequence used in DNA cloning.
CS-*rop*	6,902~7,093	Coding sequence for repressor of primer protein derived from the ColE1 plasmid, for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).
Intervening Sequence	7,094~7,520	Sequence used in DNA cloning.
OR-*ori-pBR322*	7,521~8,109	Origin of replication isolated from pBR322. It confers autonomous replication ability to the vector in *E. Coli (*Sutcliffe, 1979).
Intervening Sequence	8,110~8,639	Sequence used in DNA cloning.
aadA	8,640~9,528	Bacterial promoter, coding sequence and 3' untranslated region of, 3''(9)-O-nucleotidyltransferase (aminoglycoside-modifying enzyme), derived from transposon Tn7 (Fling et al., 1985)。 It confers spectinomycin and streptomycin resistances.
Intervening Sequence	9,529~9,664	Sequence used in DNA cloning.

[1] B-Border (border sequence)

5 [2] P-Promoter (promoter)

[3] *EF-1α* is identical to *Tsf1* shown in Table 1 (p. 29-30) in Annex 4.

[4] L-Leader (leader sequence)

[5] I-Intron (intron)

[6] TS-Targeting Sequence (targeting sequence)

10 [7] CS-Coding Sequence (coding sequence)

[8] T-Transcription Termination Sequence (transcript termination sequence)

[9] OR-Origin of Replication (replication initiation region)

2) Functions of the component element

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

The functions of individual component elements of the donor nucleic acids used for developing this recombinant oilseed rape are shown in Table 1 (p. 4-13).

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

The glyphosate herbicide inhibits 5-enolpyruvylshikimate 3-phosphate synthase (enzyme number: E.C.2.5.1.19, hereinafter referred to as "EPSPS protein",) one of the enzymes in the shikimic acid pathway, the aromatic amino acid synthetic pathway in plants, resulting in plant death (Franz et al., 1997). This recombinant oilseed rape has tolerance to glyphosate herbicide by the modified CP4 EPSPS protein expressed by the modified *cp4 epsps* gene transferred to this plant.

In order to examine whether or not the modified CP4 EPSPS protein shared similar amino acid sequences with the known allergens, homology was searched among eight amino acids following the FASTA type algorithm using the allergen database (AD_2012)[3]. The results showed that there were no similar sequences to the known allergens.

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

The EPSPS protein is one of the enzymes, which catalyze the shikimic acid pathway, the aromatic amino acid synthetic pathway to aromatic amino acids distinctive of plants and microorganisms, and present in the chloroplasts or plastids (Della-Cioppa et al., 1986). The shikimic acid pathway is the important metabolic pathway, which is thought to be involved in one-fifth of the carbon fixed by plants (Haslam, 1974; Haslam, 1993). It has been revealed that the shikimic acid is regulated by the 3-deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase, which is involved in the first stage of this pathway, while in the stage of generating chorismic acid from DAHP, this pathway is unlikely to be inhibited or suppressed by intermediary metabolites and end products (Weiss and Edwards, 1980; Herrmann, 1983). It suggests that the EPSPS protein is not a rate-limiting enzyme in this pathway. Therefore, it is concluded that even with increased activity of the EPSPS protein, the concentration of the aromatic amino acids, the end products of this pathway, is not raised or increased (Padgette et al., 1996a; Ridley et al., 2002). Actually, it was reported that the aromatic amino acids were not oversynthesized in the plants producing the EPSPS protein 40 times more than usual (Smart et al.,

[3] Database consisting of sequences registered on the FARRP (Food Allergy Research and Resource Program) AllergenOnline database (as of December, 2011). It contains 1,603 amino acid sequences.

1985). Moreover, in the evaluation processes of safety of the foods and feeds from the crops tolerant to glyphosate herbicide (soybeans, oilseed rape, cotton, maize, alfalfa and beets), which Monsanto Company has ever commercialized, analyses of the amino acid composition of the seeds of those modified crops confirmed that there were no differences in the contents of the aromatic amino acids between those modified crops and non-modified crops. These results support that the EPSPS protein is not a rate-limiting enzyme in this pathway.

The EPSPS protein is an enzyme catalyzing the reversible reaction, in which 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Pi) are generated from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (hereinafter referred to as "S3P") (Levin and Sprinson, 1964) and is known to react specifically to those substrates (Gruys et al., 1992). The only one, which is known to react the EPSPS protein, other than those substrates, is shikimic acid, an analog of S3P. However, as for the reaction of the EPSPS protein with shikimic acid and S3P, the comparison by the specificity constant (k_{cat}/K_m), which represents the degree of occurrence of reaction, showed that the reaction specificity between the EPSPS protein and shikimic acid is one to two millionth of that between the EPSPS protein and S3P (Gruys et al., 1992), and shikimic acid is highly unlikely to react as a substrate of the EPSPS protein. Therefore, it is concluded that the modified CP4 EPSEPS protein changes the metabolic system of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The plasmid vector PV-BNHT2672 used for the development of this recombinant oilseed rape was constructed from several vectors including vector pBR322 (Sutcliffe, 1979) derived from *E. coli*.

2) Properties

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

The number of base pairs in the plasmid vector PV-BNHT2672 used for the development of this recombinant oilseed rape is 9,664 bp.

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

As a marker gene for selecting the constructed vector in *E. coli*, the *aadA* gene derived from the transposon Tn7 of *E. coli* conferring resistance to spectinomycin and streptomycin is present outside the T-DNA region.

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

concerning the host range

The infectivity of this vector is not known.

(3) Method of preparing living modified organisms

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

The component elements of the plasmid vector transferred to the recipient organism are listed in Table 1 (p. 4-13). The positions of the component elements of the donor nucleic acid and sites cleaved by restriction enzymes in the vector are shown in Figure 1 (p. 3).

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

The Agrobacterium method was used to transfer the T-DNA region in PV-BNHT2672 into the hypocotyl of the conventional oilseed rape cultivar, Ebony.

3) Process of rearing of living modified organisms

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

The hypocotyl of the conventional oilseed rape cultivar, Ebony, was co-cultivated with *A.tumefaciens* ABI strain containing the plasmid vector PV-BNHT2672, and subsequently incubated on the medium containing glyphosate herbicide to select the cells.

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

Carbenicillin and ticarcillin-clavulanic acid were added to the medium to remove any residual *Agrobacterium* used for transformation. Then, at the R3 generation of this recombinant oilseed rape, PCR analysis was conducted for the backbone region of the plasmid vector PV-BNHT2672 used for transformation. As a result, the plasmid vector backbone region was not detected from this recombinant oilseed rape (Annex 2), and thus, it was confirmed that there was no residual *Agrobacterium* used for transformation in this recombinant oilseed rape.

(c) Process 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 Effects on Biological Diversity

The selected regenerated individuals (R0) were transplanted to soil and then self-pollinated to develop R1 seeds. It was confirmed that R0 and R1 individuals had tolerance to glyphosate herbicide and the modified *cp4 epsps* gene expression cassette and that the sequence of backbone region (*ori V*) of PV-BNHT2672 was absent in both plants. Then, R2 individuals containing a single copy of the T-DNA region in a homozygous state were selected based on application of glyphosate herbicide, PCR and Southern blot analysis. Based on the superior phenotype and the state of existence of transgenes this recombinant oilseed rape was selected as the final line.

The analysis of genes transferred in this recombinant oilseed rape, stability of expression of the transgenes, and the generations used in isolated field tests in Japan are shown in Figure 2, Process of rearing of this recombinant oilseed rape (p. 10). The scope of approval for Type 1 Use Regulation covers the R3 generation and all cross progeny lines derived from the R3 generation as shown in Figure 2 (p. 10).

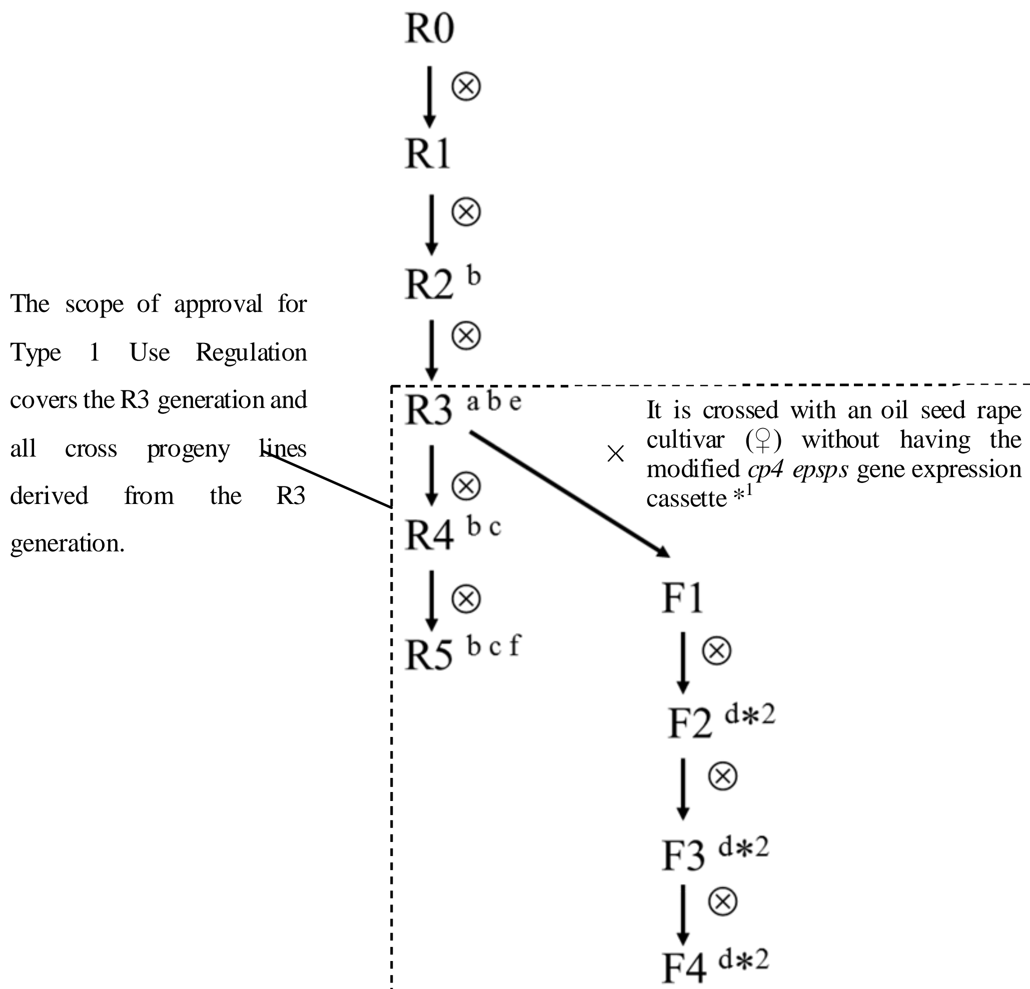


Figure 2 Process of rearing of this recombinant oilseed rape[4]

⊗ Self-pollination

[a] Generation subjected to the analysis of transgenes (Southern blot analysis and base sequence analysis)

[b] Generation subjected to the stability test among generations of transgenes (Southern blot analysis) and expressed proteins (Western blot analysis)

[c] Generation subjected to the confirmation of protein expression by ELISA

[d] Generation used for the segregation ratio test of transgenes

[e] Generation used for rearing commercialized cultivars

[f] Generation subjected to isolated field tests in Japan

*1 For the analysis of progeny segregation ratio, this recombinant oilseed rape was crossed with the conventional oilseed rape cultivar without having the modified *cp4 epsps* gene expression cassette (65037) to develop the F1 generation (heterozygote).

*2 The F2, F3 and F4 generations were developed by self-pollination of only individuals which are heterozygous for the modified *cp4 epsps* gene expression cassette of a previous generation.

[4] All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited.

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

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

In order to confirm whether the transferred gene exists on the chromosome of this recombinant oilseed rape, this recombinant oilseed rape having the homozygous transgenes (R3 generation) was crossed with the oilseed rape cultivar not containing the modified *cp4 epsps* gene expression cassette to develop
10 the F1 individuals. The F1 individuals were subsequently self-pollinated to produce the F2 generation. The F2 individuals were tested for the presence of the modified *cp4 epsps* gene expression cassette by real-time TaqMan PCR, and heterozygous F2 individuals were selected and self- pollinated to produce the F3 generation. Similarly, heterozygous F3 individuals were selected and self-pollinated to produce the F4 generation. The resulting F2, F3 and F4 generations were subjected to real-time TaqMan PCR,
15 and the segregation ratio of the modified *cp4 epsp* gene expression cassette in this recombinant oilseed rape was determined based on zygosity (Table 2, p 12; Annex 3). In this case, the segregation ratio of the modified *cp4 epsp* gene expression cassette was expected to be 1:2:1 (homozygote + +: heterozygote + -: homozygote - -) according to Mendel's law.

20 As a result, regarding the F2, F3 and F4 generations, no statistically significant difference was found between the observed and expected segregation ratios based on the Chi-square test (Table 2, p. 12; Table 1 in Annex 3, p 7). Consequently, it was concluded that the transferred gene resides on the chromosome of this recombinant oilseed rape and is inherited consistent with Mendel's law of segregation.

Table 2 Segregation ratio of the modified *cp4 epsps* gene in the F2, F3, F4 generations of this recombinant oilseed rape[5]

Generation[1]	Number of individuals tested[2]	Observed value			Expected value based on 1:2:1 segregation ratio				
		Number of positive homozygotes	Number of positive heterozygotes	Number of negative homozygotes	Number of positive homozygotes	Number of positive heterozygotes	Number of negative homozygotes	χ^2	P value[3]
F_2	220	51	122	47	55.00	110.00	55.00	2.76	0.251
F_3	166	39	94	33	41.50	83.00	41.50	3.35	0.187
F_4	198	53	97	48	49.50	99.00	49.50	0.33	0.847

[1] The F2, F3 and F4 generations were produced by self-pollinating the respective parental generations (F1, F2 and F3 generations) that are heterozygous for the modified *cp4 epsps* gene expression cassette.

[2] The presence or absence of the modified *cp4 epsps* gene expression cassette was tested by real-time TaqMan PCR.

5 [3] The segregation ratios obtained for the F2, F3 and F4 generations were analyzed by Chi-square test ($p \leq 0.05$).

[5] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

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

5 As a result of Southern blot analysis for existence of the transgenes, it was confirmed that a single copy of the T-DNA region was transferred at a single site in the nuclear genome of this recombinant oilseed rape (Figure 4-5 in Annex 4, p 37-38). It was also confirmed that the vector backbone regions other than the T-DNA region were not transferred to this recombinant oilseed rape (Figure 6-8 in Annex 4, p. 39-41). In addition, Southern blot analysis of multiple generations (R2, R3, R4 and R5) showed that the 10 transgenes were stably inherited by the offspring (Figure 16 in Annex 4, p. 57). The map of the gene transferred in this recombinant oilseed rape is shown in Figure 3 (p. 14).

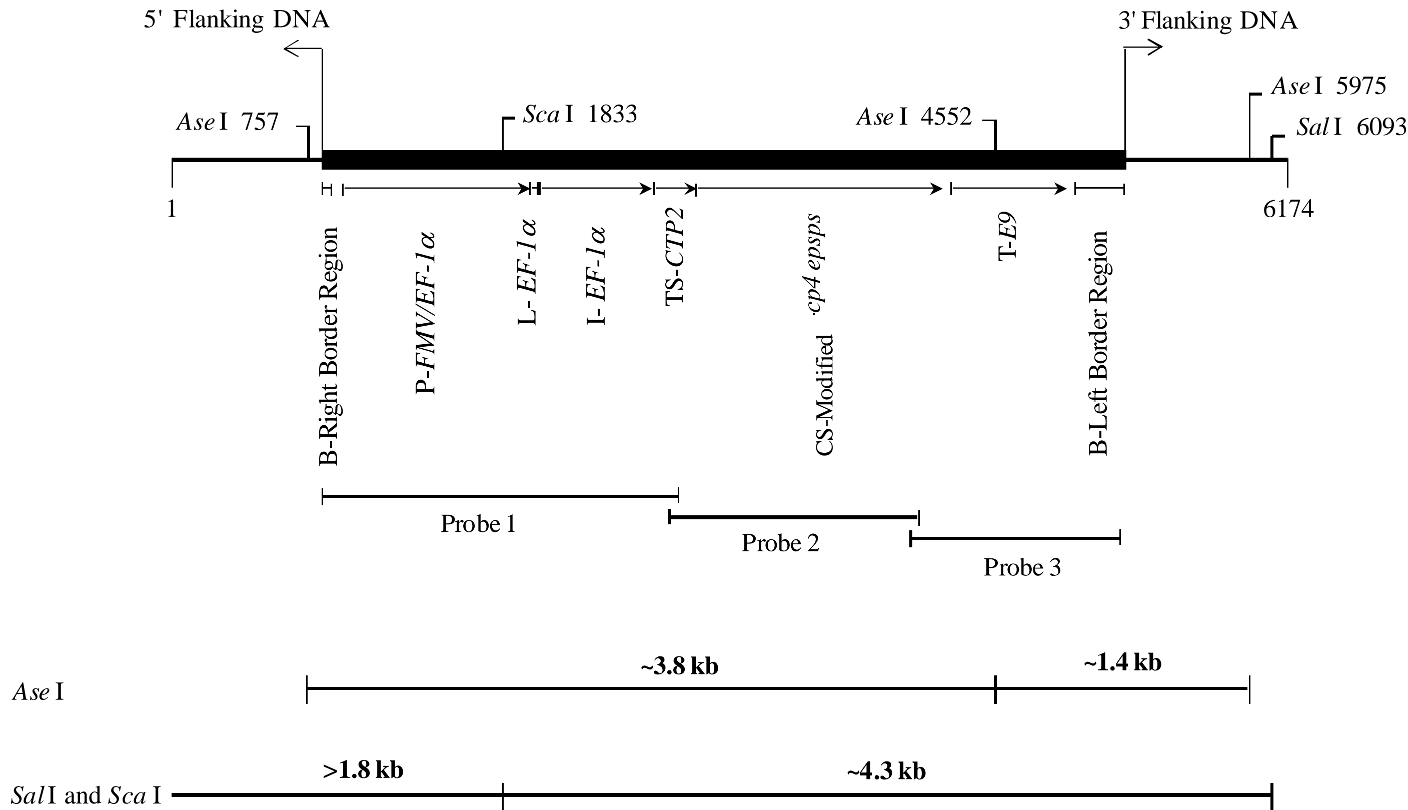


Figure 3 Map of the gene transferred in this recombinant oilseed rape[6]

The top diagram represents the map of the transferred gene and the flanking sequences in this recombinant oilseed rape. The map shows the relative positions of the component elements in the transferred gene and the restriction sites used in Southern blot analysis. The middle diagram shows the relative sizes and positions of the T-DNA probes shown in Figure 1 in Annex 4 (p. 33). The bottom two diagrams show the expected sizes of the DNA fragments produced after cleavage by the respective restriction enzymes. The arrows (→) indicate the 5′ and 3′ terminals of the transferred gene and the beginning of the flanking nuclear genomic DNA sequences at both terminals. The arrows (→) indicate the sequence orientation of the component elements in this recombinant oilseed rape.

[6] All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited.

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

This item is not applicable because there is only one copy (Figure 4-5 in Annex 4, p 37-38).

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

It was confirmed by Western blot analysis of leaves of multiple generations (R2, R3, R4 and R5) of this recombinant oilseed rape that the modified CP4 EPSPS protein is stably expressed (Figure 1 in Annex 5. p. 15).

This recombinant oilseed rape was cultivated in four replicated plots in three filed sites in the US (one each in Idaho, Minnesota, and North Dakota) and three in Canada (two in Manitoba and one in Saskatchewan). The samples were obtained from forages, seeds, leaves, and roots of the cultivated plants to analyze the expression levels of the modified CP4 EPSPS protein using ELISA (Table 3, p. 16; Annex 6). The results obtained from the ELISA demonstrated the expression of the modified CP4 EPSPS protein in the forages, seeds, leaves, and roots of this recombinant oilseed rape (Table 3, p. 16; Table 1 in Annex 6, p. 16-17).

Table 3 Expression level of the modified CP4 EPSPS protein in the respective sites of this recombinant oilseed rape in the fields in the US and Canada[1] (2009)[7]

Sites tested [2]	Mean of fresh weight (SD) Range (μg/g FW)	Mean of dry weight (SD) Range (μg/g DW)
Forage	18 (4.4) 14-28	170 (22) 120-210
Seed	25 (5.2) 21-43	27 (5.6) 22-46
Leaf (OSL-1)	23 (10) 10-45	180 (40) 110-250
Leaf (OSL-2)	22 (5.9) 18-37	180 (41) 120-250
Leaf (OSL-3)	31 (6.3) 20-41	230 (50) 130-300
Leaf (OSL-4)	36 (14) 20-85	210 (80) 110-500
Root (Root-1)	19 (4.1) 11-25	82 (17) 46-100
Root (Root-2)	10 (3.3) 7.0-17	38 (14) 24-62

[1.] This recombinant oilseed rape was cultivated in four replicated plots in three filed sites in the US (one each in Idaho, Minnesota, and North Dakota) and three in Canada (two in Manitoba and one in Saskatchewan). The samples were
5 obtained from forages, seeds, leaves, and roots of the cultivated plants to analyze the expression levels of the modified CP4 EPSPS protein using ELISA.

[2] Sampling time of respective sites and the numbers of the samples are as follows.

Forage (n=20): Beginning of elongation of main stem, Seed (n=16): Harvest, Leaf (OSL-1) (n=16): The third- and fourth- leaf development stage, Leaf (OSL-2) (n=9):The seventh- to ninth-leaf development stage, Leaf (OSL-3)
10 (n=20): Beginning of elongation of main stem, Leaf (OSL-4) (n=20): Flower initiation to 20% of flowering time, Root (Root-1) (n=19): Beginning of elongation of main stem, Root (Root-2) (n=11): When 10-30% of pods have reached the end of the full pod stage

[7] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

(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

The transferred nucleic acid does not contain any sequence allowing transmission. Therefore, it is considered unlikely that it could be transmitted through virus infection and/or other routes to any other wild animals and wild plants.

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

This recombinant oilseed rape can be specifically detected and identified by End-Point TaqMan PCR using a primer set specifically binding to this recombinant oilseed rape (Annex 7). The recommended DNA concentration for this assay is 5-10 ng per PCR reaction, so the assay can be performed using a single seed.

The reproducibility and reliability of this method was verified using 89 seeds from this recombinant oilseed rape and 180 seeds from the non-recombinant oilseed rape (Annex 7).

(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

The modified *cp4 epsps* gene transferred into this recombinant oilseed rape expresses the modified CP4 EPSPS protein to confer tolerance to glyphosate herbicide.

In order to confirm that this recombinant oilseed rape had tolerance to glyphosate herbicide, glyphosate herbicide of 1,500 g a.e.[8]/ha was applied to this recombinant oilseed rape and the control non-recombinant oilseed rape in a climate chamber. As a result, all the individuals of the control non-recombinant oilseed rape were dead on days 5-6 after the application, while all the individuals of this recombinant oilseed rape survived (Table 4, p. 17). Therefore, it was confirmed that this recombinant oilseed rape is tolerant to glyphosate herbicide.

Table 4 Results of the evaluation of glyphosate herbicide tolerance of this recombinant oilseed rape

[8] a.e.; acid equivalent. An active ingredient in herbicide formulations is contained as a salt form or as itself in the formulations. When an active ingredient exits itself, the active ingredient is acid and the base moieties vary with the formulation. When the content of salt of the active ingredient in the formulation is used as the application amount, the content of the active ingredient cannot be accurately compared among formulations whose base moieties are different. Therefore, acid equivalent of the active ingredient was used as a unit.

(1) (2) 9

	Individuals that survived	Individuals that died
This recombinant oilseed rape	24	0
Control non-recombinant oilseed rape	0	24

(1) Glyphosate herbicide was applied to this recombinant oilseed rape and the control non-recombinant oilseed rape on the days 5 to 7 after sowing (primary leaf stage). On the days 5-6 after the application, survival/death was determined by the appearance.

5 (2) Twenty-four individuals each of this recombinant oilseed rape and the control non-recombinant oilseed rape were tested.

(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

10 the recipient organism belongs, and the degree of difference, if present

In 2011 and 2012, isolated field tests were carried out in Kawachi Research Farm, Monsanto Japan Limited, using this recombinant oilseed rape. The tests were conducted using the R5 generation of this recombinant oilseed rape (Figure 2, p. 10). As the control non-recombinant oilseed rape, Ebony,

15 the host plant of this recombinant oilseed rape for gene transfer was used. In addition, a heat tolerance test in the early growth stage (item b, p. 18) was performed in a climate chamber in the US. A test for evaluation of crossing rate (item f. p. 19) was conducted in a field in the US (California).

a. Morphological and growth characteristics

20

The differences in morphological and growth characteristics were evaluated for 12 items (initiation of germination, uniformity of germination, time of flower initiation, flowering time, time of flower completion, plant type, plant height, number of primary branches, maturation period, weight of forage, and appearance of harvested seed (color and uniformity of grains)).

25

Statistical analyses were conducted on the data of plant height, number of primary branches, and weight of forage, but not on the data of initiation of germination, uniformity of germination, time of flower initiation, flowering time, time of flower completion, plant type, maturation period, and appearance of harvested seed (color and uniformity of grains). As a result, no statistically significant

30 difference was observed in the items analyzed statistically between this recombinant oilseed rape and the control non-recombinant oilseed rape. As for the items not subjected to statistical analysis, there were differences in the data of time of flower initiation and flowering time between this recombinant oilseed rape and the control non-recombinant oilseed rape (Table 4 in Annex 8, p. 13).

35 The time of flower initiation and flowering time are March 11 and April 9 and March 9 and April 7

[9] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

for this recombinant oilseed rape and the control non-recombinant oilseed rape, respectively (Table 4 in Annex 8, p. 13).

b. Heat tolerance at the early stage of growth

Heat tolerance at the early stage of growth was evaluated in a climate chamber of Monsanto Company in the US. In this evaluation, this recombinant oilseed rape, the control non-recombinant oilseed rape Ebony, and four conventional commercial cultivars were grown in a greenhouse, and 20 days after sowing, the seedlings were transferred to and grown in a climate chamber at 35°C (day)/30°C (night) for 21 days to examine and compare growth stage, plant vigor, fresh weight, and dry weight. As a result, out of the items subjected to statistical analysis (plant vigor, fresh weight, and dry weight), a statistically significant difference was observed in plant vigor between this recombinant oilseed rape and the control non-recombinant oilseed rape. As for the item not subjected to statistical analysis (growth stage), there was no difference between this recombinant oilseed rape and the control non-recombinant oilseed rape (Table 5 in Annex 9, p. 21).

The mean of plant vigor[10] was 5.9 and 5.1 for this recombinant oilseed rape and the control non-recombinant oilseed rape, respectively. The plant vigor of this recombinant oilseed rape is lesser than the control (Table 5 in Annex 9, p. 21).

c. Summer survival of the mature plant

This recombinant oilseed rape and the control non-recombinant oilseed rape raised in an isolated field were left to grow after the maturation period to observe the growth conditions in summer in Japan. As a result of observation made on July 24 2012, in the plot for investigation of surviving summer, this recombinant oilseed rape and the control non-recombinant oilseed rape were both found dead (Figure 6 in Annex 8, p. 15).

d. Fertility and size of the pollen

Pollens were sampled from this recombinant oilseed rape and the control non-recombinant oilseed rape grown in an isolated field, and the samples were stained with Alexander solution to observe their fertility and size. The results of the statistical analysis of these items showed that no statistically significant difference was observed in pollen fertility or size between this recombinant oilseed rape and the control non-recombinant oilseed rape (Table 5 in Annex 8, p. 16).

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

Items related to seed production (number of ripe pods, number of seeds per rod, and thousand kernel weight) were examined in this recombinant oilseed rape and the control non-recombinant oilseed rape grown in an isolated field, and the obtained data was subjected to statistical analyses. As a result,

[10] Plant vigor was evaluated by a trained measurer by visual observation. Each individual was relatively evaluated on a scale of 1 to 9. One means very good growth and nine is death or near death. The bigger the number, the lesser the growth.

statistically significant differences were observed in thousand kernel weight between this recombinant oilseed rape and the control non-recombinant oilseed rape (Table 6 in Annex 8, p. 17). The mean of thousand kernel weight was smaller in this recombinant oilseed rape (3.30 g) than in the control non-recombinant oilseed rape (4.61 g) (Table 6 in Annex 8, p. 17). However, the former mean (3.30 g) was within the previously reported range of thousand kernel weight (approximately 2.5-6 g) (CCC, 2012).

Regarding the shattering habit, this recombinant oilseed rape and the control non-recombinant oilseed rape grown in an isolated field were harvested during the maturation period, and the harvested plants were left to air-dry in a vinyl house before examining the pod shattering rate. The statistical analysis of the pod shattering rate showed a statistically significant difference between this recombinant oilseed rape and the control non-recombinant oilseed rape. The mean of pod shattering rate of this recombinant oilseed rape (1.8%) was smaller than that of the control non-recombinant oilseed rape (3.2%) (Table 6 in Annex 8, p. 17).

Regarding dormancy and germination rate, seeds were collected after harvesting this recombinant oilseed rape and the control non-recombinant oilseed rape grown in an isolated field, and the seeds were germinated on a Petri dish under the temperature condition of 25ºC to examine the number of germinated plants over time. As a result, the germination rates of this recombinant oilseed rape and the control non-recombinant oilseed rape were both high, 100%, and dormancy was not observed (Table 8 in Annex 8, p. 18).

f. Crossability

The crossability was evaluated in a field in the US (California) from 2009 to 2010. In the evaluation test, this recombinant oilseed rape and the control non-recombinant oilseed rape were used as pollen parents to examine the frequency of occurrence of hybrids in the harvested seeds of the conventional oilseed rape, which was grown at a distance of 2 m from those pollen parent individuals. This test was performed under the conditions that the flowering time of this recombinant oilseed rape and the control non-recombinant oilseed rape as the pollen parents matched that of the conventional oilseed rape as the seed parent (Table 2 in Annex 10a, p. 6; Table 3 in Annex 10b, p. 7). The hybrid was identified by detecting the transgene using PCR when this recombinant oilseed rape was used as the pollen parent, and by detecting the eight SNP markers specific to the control non-recombinant oilseed rape using DNA microarray when the control non-recombinant oilseed rape was used as the pollen parent.

One thousand of the harvested seeds of the conventional oilseed rape were examined for the frequency of occurrence of hybrids when each this recombinant oilseed rape the control non-recombinant oilseed rape was used as the pollen parent. As a result, the crossability was 1.3% and 1.4% when the pollen parent was this recombinant oilseed rape and the control non-recombinant oilseed rape, respectively (Table 3 in Annex 10a, p. 6; Table 4 in Annex 10b, p. 7). Both values were comparable to the previously reported crossability of oilseed rape (0.0121-14.5% when the distance between pollen and seed parents was within 5 m) (Beckie et al., 2003; Ramsay et al., 2003; Hüsken

and Dietz-Pfeilstetter, 2007; Cai et al., 2008).

g. Productivity of harmful substances

5 To confirm whether or not this recombinant oilseed rape produces any substances affecting soil microbes and other plants, a soil microflora test, a plow-in test and a succeeding crop test were performed. As a result, no statistically significant difference was observed between this recombinant oilseed rape and the control non-recombinant oilseed rape regarding the number soil microbes and the germination rate and dry weight of radish (Tables 9-11 in Annex 8, p. 20).

10

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

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

(1) Item-by-item assessment of Adverse Effects on Biological Diversity

This recombinant oilseed rape was developed by transferring the T-DNA region of the plasmid PV-BNHT2672, constructed based on the plasmid pBR322, etc., derived from *E. coli*, by the *Agrobacterium* method.

Based on the segregation form of the transferred gene and the Southern blot analysis, it has been confirmed that a single copy of the T-DNA region, which contains the modified *cp4 epsps* gene encoding the modified CP4 EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase) derived from *Agrobacterium tumefaciens* resides on the chromosome of this recombinant oilseed rape and is stably inherited across multiple generations. In addition, it has been confirmed by Western blot analysis and ELISA that the target gene is stably expressed across multiple generations.

1) Competitiveness

It has been reported that oilseed rape, the taxonomical species to which the recipient organism belongs, can grow voluntarily in the areas with periodic human intervention, such as roadsides and old factory sites, but not in the natural environment.

The various characteristics related to competitiveness of this recombinant oilseed rape and the control non-recombinant oilseed rape were evaluated in isolated fields in Japan. As a result, statistically significant differences were observed in thousand kernel weight, an item related to seed production, and pod shattering rate, an item related to shedding habit. There were differences in time of flower initiation and flowering time, which were items without statistical analysis. The results of the evaluation of heat tolerance at the early stage of growth in a climate chamber in the US showed statistically significant difference in the plant vigor. The above-mentioned significant differences and differences were determined not to improve competitiveness, based on the results that the thousand kernal weight of this oilseed rape was within the range of the previously reported thousand kernal weight of oilseed rape, that for this recombinant oilseed rape the pod shattering rate was lower, the time of flower initiation and flowering time were delayed for two days, and the plant vigor was lesser in the test of the heat tolerance at the early stage, compared with the control non-recombinant oilseed

rape.

This recombinant oilseed rape is given the trait to be tolerant to glyphosate herbicide due to the expression of the modified CP4 EPSPS protein. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of glyphosate herbicide, the tolerance to glyphosate herbicide would increase the competitiveness of this recombinant oilseed rape.

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

2) Productivity of harmful substances

Seeds of the conventional oilseed rape contain erucic acid and glucosinolate, which is considered to be harmful to animals. On the other hand, the line used as the recipient organism of this recombinant oilseed rape is so-called canola, in which contents of both substances are decreased by breeding, and therefore, it is considered not to affect wildlife inhabiting.

As result of soil microflora tests, plow-in tests and succeeding crop tests carried out in isolated fields in Japan to examine the production of harmful substances by this recombinant oilseed rape (the substances secreted from the roots, which can affect other plants and microorganisms in soil; the substances existing in the plant body, which can affect other plants after dying), no difference was observed between this recombinant oilseed rape and the control non-recombinant oilseed rape.

In this recombinant oilseed rape, the CP4 EPSPS protein is expressed by the transgene, and the protein has been confirmed to have no amino acid sequence homology with any known allergens. It has also been confirmed that the modified CP4 EPSPS protein is not a rate-limiting enzyme in the shikimic acid pathway, and even with increased activity of the EPSPS protein the concentration of the aromatic amino acids, the end products of this pathway, is not raised. Therefore, it is unlikely that unintended harmful substances are produced by the expression of the modified CP4 EPSPS protein.

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

3) Crossability

No affected wild animals and plants were identified, because there are no wild relatives which can be

crossed with oilseed rape in Japan.

Based on the above understanding, it was judged that the conclusion made by the applicant that the use of this recombinant canola poses no risk of Adverse Effects on Biological Diversity attributable to crossability is reasonable.

4) Others

Of the relatives, for which the crossing with oilseed rape cannot be denied in Japan, oilseed rape and native rapeseed (*Brassica rapa*) are cultivated species, and black mustard (*B. nigra*), wild radish (*Raphanus raphanistrum*), charlock (*Sinapis arvensis*), mustard (*B. juncea*) and Daikonmodoki (*Hirschfeldia incana*) are naturalized plants. Therefore, no Japanese native wild animals and plants which are likely to be affected by the biological diversity attributed to the crossing were identified. However, as for the possibilities of indirect effects when oilseed rape is crossed with these relatives ((1) possibility that the hybrid progeny produced by crossing will become dominant and invade populations of other wild plant species, (2) possibility that the population of the relatives crossed decreases due to the burden of the transferred genes by the crossing, resulting in those populations of wildlife, such as insects dependent to those relatives, will be affected), the effects were examined.

Consequently, it was judged that the conclusion made by the applicant that the use of this recombinant oilseed rape poses no risk of indirect Adverse Effects on Biological Diversity attributable to crossing this recombinant oilseed rape with relatives is reasonable, based on the following.

The possibility (1) that the hybrid progeny produce will become dominant and invade populations of other wild plant species under natural conditions is very low, because various reproductive isolation barriers exist.
The possibility (2) that the decreased population of the relatives crossed will affect populations of wildlife, such as insects dependent to those relatives is very low, because it has been reported that transferring the trait of herbicide tolerance to the genome of relative species is not a burden and it is unlikely that the modified *cp4 epsps* gene becomes a burden to affect maintaining the population of the hybrid relatives.

(2) Conclusion based on the Biological Diversity Risk Assessment Report

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

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List of Annexes for oilseed rape tolerant to glyphosate herbicide (Modified *cp4 epsps, Brassica napus* L.) (MON88302, OECD UI: MON-883Ø2-9)

Annex 1 Amino Acid Sequence of the Modified CP4 EPSPS Protein Deduced from the Modified *cp4 epsps* Gene Used for Developing this Recombinant Oilseed rape (Confidential)

Annex 2 Summary of PCR Analysis to Confirm the Absence of Agrobacterium Used To Produce Glyphosate-Tolerant (Roundup Ready®) RR2 Canola MON 88302 (Confidential)

Annex 3 Segregation of the *cp4 epsps* Coding Sequence in MON 88302 in the F_2, F_3 and F_4 Populations (RPN-10-085) (Confidential)

Annex 4 Molecular Analysis of Glyphosate-Tolerant Roundup Ready®2 (RR2) Canola MON 88302 (MSL0022523) (Confidential)

Annex 5 Demonstration of the Presence of CP4 EPSPS Protein in Canola Leaf Tissue of MON 88302 Across Multiple Generations by Western Blot Analysis Produced in U.S. Greenhouse during 2009-2010 (MSL0022592) (Confidential)

Annex 6 Amended Report for MSL 0022681: Assessment of CP4 EPSPS Protein Levels in Canola Tissues Collected from MON 88302 Produced in United States and Canadian Field Trials during 2009 (MSL0023090) (Confidential)

Annex 7 a) EndPoint TaqMan PCR with *FatA* Internal Control for Single Seed (BQ-QC-10760-03) (Confidential)
b) Supplemental File for BQ-QC-10760-03 Canola MON88302 EndPoint TaqMan PCR with *FatA* Internal Control for Single Seed (Confidential)

Annex 8 Biological Diversity Risk Assessment Report of Oilseed Rape Tolerant to Glyphosate Herbicide (Modified *cp4 epsps, Brassica napus* L.) (MON88302, OECD UI: MON-883Ø2-9) in an Isolated Field (Confidential)

Annex 9 Assessment of the Effect of Heat Stress on Glyphosate Tolerant Canola MON 88302 under Growth Chamber Conditions (MSL0023177) (Confidential)

Annex 10 a) Assessment of the Outcrossing Rate of Roundup Ready 2 Canola MON 88302 in 2009-2010 Field Trial in the US (PLC-09-422) (Confidential)
b) Assessment of the Outcrossing Rate of Ebony Canola in 2009-2010 Field Trial in

the US (PLC-09-422) (Confidential)

Annex 11 Report on Monitoring Results (Confidential)