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

Name: Bayer Crop Science K.K
John Gray, President (Seal)
Address: Marunouchi Kitaguchi Building,
1-6-5, Marunouchi, Chiyoda-ku,
Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Oilseed rape tolerant to bromoxynil herbicide (*oxy, Brassica napus* L.) (OXY-235, OECD UI: ACS-BNØ11-5)
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	—

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

Origins and functions of component elements of the donor nucleic acid used for the production of herbicide bromoxynil tolerant oilseed rape (*oxy*, *Brassica napus* L.) (OXY-235, OECD UI: ACS-BNØ11-5) (hereinafter referred to as "OXY-235") are shown in Table 1. In addition, the nucleotide sequence of *oxy* gene is shown in Annex 1.

Table 1 Name, sizes, origins and functions of component elements

Component elements	Size (kbp)	Origin and function
oxy gene expression cassette		
P35S	1.13	35S RNA promoter derived from cauliflower mosaic virus. It initiates the transcription (Reference 45).
5'ssuZm	0.04	The untranslated region of small sub-unit gene of ribulose bisphosphate carboxylase oxygenase (RuBisCO) derived from maize. It increases level of transcription (Reference 35).
oxy	1.00	Gene isolated from *Klebsiella pneumoniae* subsp.*ozaenae* encoding the enzyme nitrilase (Reference 70). It confers tolerance to the oxynil family of herbicides.
3'nos	0.68	The 3' untranslated region of the nopaline synthetase gene isolated from Ti plasmid (pTiT37). It contains signals for termination of transcription and directs 3' polyadenylation (Reference 12).
Additional information		
RB	0.02	The right border terminal region sequence derived from Ti plasmid pTiA6 of *Agrobacterium* (Reference 3). It transforms the T-DNA region into the plant genome.
LB	0.02	The left border terminal region sequence derived from Ti plasmid pTiA6 of *Agrobacterium* (Reference 3). It transforms the T-DNA region into the plant genome.

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

2) Function of component elements

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

Functions of component elements of donor nucleic acid transferred in OXY-235 are listed in Table 1 (p.9).

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

The oxynil family of herbicides comprises bromoxynil and ioxynil, which are classified into hydroxybenzonitrile. These herbicides act primarily to inhibit electron transport during the light reaction of photosynthesis by noncovalently binding to the QB plastoquinone-binding sub-unit (42 kDa) of photosystem II (References 48 and 77). In addition, at higher concentrations, they also act as uncoupling agents for photophosphorylation and oxidative phosphorylation (References 31 and 62).

The nitrilase protein, a product of the *oxy* gene, is an enzyme that hydrolyzes oxynil family of herbicides to non-phytotoxic compounds. When bromoxynil or ioxynil is sprayed to OXY-235 which contains the nitrilase protein, the herbicides produce herbicidal activity-free 3,5-dibromo-4-hydroxybenzoic acid or 3,5-diiodo-4-hydroxybenzoic acid and ammonia, respectively (Figure 1, p.11).

Spraying of the oxynil family of herbicides to OXY-235 causes production of ammonia in plant body due to the above-described mechanism of action. In practice, however, according to the results of investigations of possible herbicide injury of oxynil family of herbicides to OXY-235 based on spraying of herbicides in different concentrations (Bromoxynil: 450 and 1,200 g/ha, Ioxynil: 375 g/ha), no herbicide injury was observed even in the spraying of herbicides of higher concentrations (Annex 7, p.10, Figure B-1). In addition, as a result of comparison of amino-acid composition in the seeds of OXY-235 cultivated with the treatment of herbicide bromoxynil (450 g/ha) and the non-recombinant control oilseed rape, no statistically significant difference was observed (Annex 7, p.18). Consequently, it is considered unlikely that the ammonia produced by the mechanism of action could adversely affect the plant body.

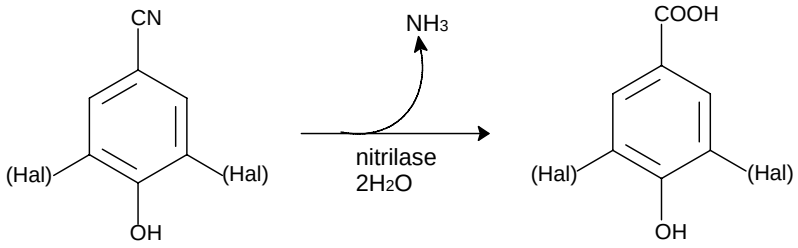

Figure 1 Degradation of oxynil family of herbicides by the nitrilase protein
(Hal) refers to halogen, in case of bromine (Br), it refers to bromoxynil, and in case of iodine (I), it refers to ioxynil.

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

The enzymatic properties of nitrilase, an *oxy* gene product, are described below (References 37 and 70).

- Nitrilase shows the optimal pH at 9.2. Nitrilase activity declines to about 15% at pH7.0, though it rapidly increases as pH value increases up to 9.2 and then it gradually decreases in the pH range of up to 10.6.
- Nitrilase show the optimal temperature at 35°C, and nitrilase activity declines to about 15% in temperatures of 10°C and 55°C.
- As a result of examination using several types of nitryl compounds, the nitrilase protein of *oxy* gene product exhibited higher substrate specificity for 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil). On the other hand, the nitrilase derived from the genus *Nocardia* of Actinomycetes exhibited higher substrate specificity for benzonitrile, and it hardly acted on bromoxynil (Table 2, p.12).
- Results of measurement of reaction velocity and *Km* value in the hydrolysis of nitrilase protein with several types of substrates are summarized in Table 3 (p.12). The nitrilase protein did not exhibit any enzyme activity against hydroxybenzonitrile, though it exhibited activity against the halogenated hydroxybenzonitrile, especially higher activity against the hydroxybenzonitrile halogenated at 3 and 5 positions. This suggests that the activity needs halogenated hydroxynil. At 3 and 5 positions, different *Km* values were identified depending on the type of halogen substituted in hydroxybenzonitrile; *Km* value was found lowest for the bromoxynil which substituted to the halogen type of bromine, showing high affinity, followed by ioxynil subsituted to iodine and chloroxynil substituted to chlorine. The reaction velocity was found highest for chloroxynil, followed by bromoxynil and ioxynil.

Based on the above understanding, the nitrilase protein of *oxy* gene product possesses high substrate specificity against halogenated 4-hydro-benzonitrile and thus, it is considered to show the highest affinity for bromoxynil.

Table 2 Substrate specificity of nitrilase protein

Substrate	NH_3 produced * (μmol)	
	Nitrilase of *oxy* gene product	Nitrilase derived from the genus *Nocardia*
3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil)	1.490	<0.005
3-bromo-4-hydroxybenzonitrile	0.250	0.045
4-hydroxybenzonitrile	0.021	0.166
Benzonitrile	<0.005	1.460
3,5-dibromo-4-hydroxybenzamide	<0.005	<0.005

*: For each assay enzyme reaction was conducted in 1mL phosphate buffer solution (pH7.5) containing individual substrates of 3 mM and nitrilase of about 400μg at 30℃ for one hour and the amount of ammonia produced (μmol) was measured.

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

Table 3 Analysis of reaction velocity with different substrates

Substrate	*Km* (mM)	*Vmax**
3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil)	0.31	15.0
3,5-diiodo-4-hydroxybenzonitrile (Ioxynil)	0.55	12.0
3,5-dichloro-4-hydroxybenzonitrile (Chloroxynil)	0.83	18.0
5-bromo-4-hydroxybenzonitrile	0.91	1.5
4-hydroxybenzonitrile	ND	0.23

*: Amount of NH_3 produced was shown in μmol/min/mg, ND: Not determined.

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

Furthermore, based on the amino acid sequence in *oxy* gene product, homology search with the sequence in known allergens proteins registered in Genbank database was conducted. As a result, the highest homology was observed in the allergen proteins PPPHLP5A {protein phlpVa in the pollens of timothy grass (*Phleum pratense*), a major pollen allergy-inducing substance} and PPHLPVB {protein phlpVb in the pollens of timothy grass (*Phleum pratense*), offering high homology with PPPHLP5A}, which exhibited a homology of 15% and 14%, respectively.

However, also in the comparison with sequence of known allergen proteins using the ribulose-bis-phosphate carboxylase oxygenase small sub-unit (RuBisCO SSU) of rice, a very typical enzyme for plants not classified in allergen protein, DEPMAG29 {Mag29 protein of skin disease germ (*Dermatophagoides farinae*)} exhibited a homology of 14%, and CHCLAH5 {allergen in the hypha of (*Cladosporium herberum*)} exhibited a homology of 16%. As demonstrated by the above described findings, even the proteins not classified into allergen protein are considered to exhibit a homology of 14 to

15% with known allergen proteins; therefore, the homology of 14 to 15% of *oxy* gene product with PPPHLP5A or PPHLPVB is considered not to provide evidence of having the activity as an allergen. In addition, in the subactute oral toxicity test in mice, no toxicity was observed in the nitrilase protein (Annex 9).

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

Since the nitrilase protein of *oxy* gene product possesses high substrate specificity for halogenated hydroxybenzonitrile, it is considered that it does not cause any cyano group hydrolysis to the compounds other than the halogenated hydroxybenzonitrile of the substrate. Therefore, it is considered unlikely that the nitrilase protein would affect the metabolic pathway of plant body.

(2) Information concerning vectors

1) Name and origin

The vector used for the transformation is the plasmid pRPA-BL-235 including the replication origin (ORI ColE1) derived from pBR322 (Figure 2).

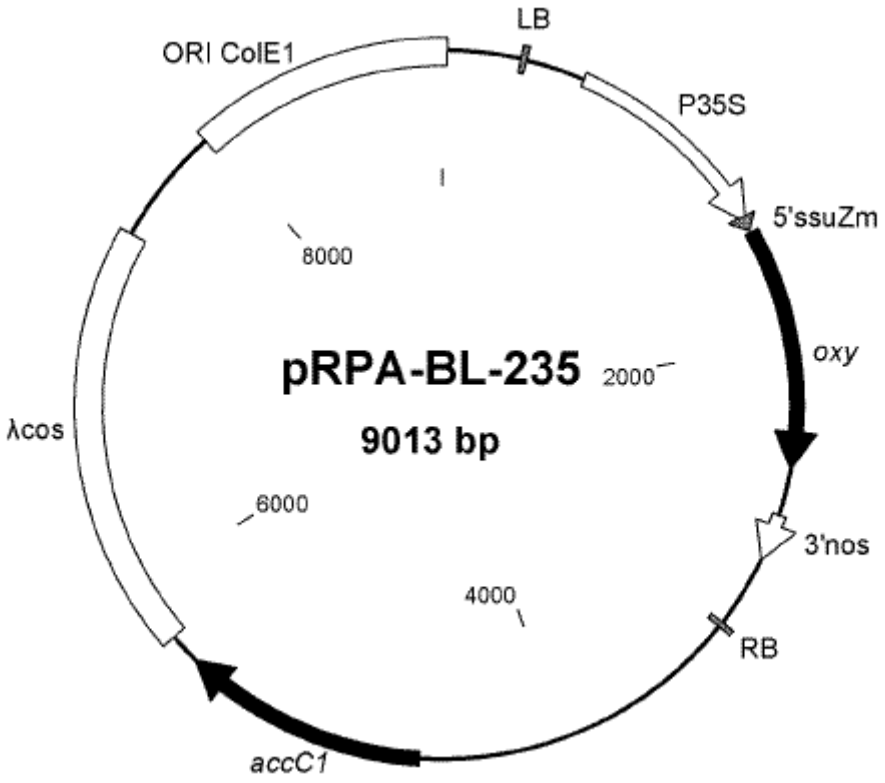

Figure 2 Physical Map of plasmid pRPA-BL-235

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

2) Properties

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

The total number of base pairs of the plasmid pRPA-BL-235 is 9,013bp. In addition, the entire nucleotide sequences of the plasmid are shown in Annex 2.

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

The plasmid pRPA-BL-235 possesses the gentamycin resistance gene (*accC1*) derived from the plasmid pPH1J1 outside the T-DNA region (Reference 82). This gene was used as selectable marker for *Escherichia coli* (*E. coli*) and *Agrobacterium*. In addition, it also contains the COS site (λcos) which is derived from λ phage and confers the packaging ability to λ phage particle (Reference 23) and the replication origin derived from pBR322 (ORI ColE1) (Reference 7). These sequences are all located outside the T-DNA region and thus, they are not transferred into OXY-235. As a result of Southern blotting analysis for the genome DNA extracted from OXY-235 using the region containing ORI ColE1 as a probe, it was confirmed that the region is not transferred into the genome DNA (Figure 3).

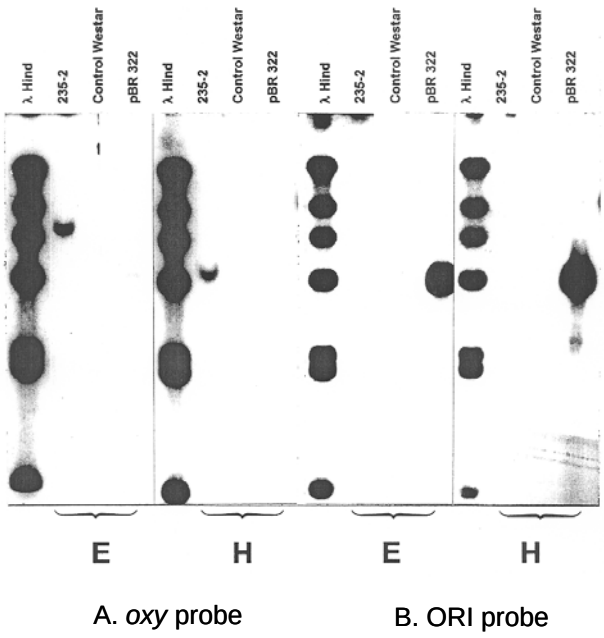

Figure 3 Identification of the sequences outside the T-DNA region in OXY-235

235-2=OXY-235, E=EcoR I, H=Hind III
A: oxy probe of 850bp
(Band size detected in 235-2 E: 7.2kbp, H: 5.1kbp)
B: ORI ColE1 probe of 820bp derived from pBR322
(Band size detected in pBR322 E: 4.3kbp, H: 4.4kbp)

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

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

The range of recipient organisms for the autonomous replication of plasmid pRPA-BL-235 is limited to *Escherichia coli* and *Agrobacterium tumefaciens*, and the plasmid does not possess any infectious characteristics in plants.

(3) Method of preparing living modified organisms

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

The T-DNA region transferred into OXY-235 is located between LB and RB on the plasmid pRPA-BL-235 (Figure 2, p.14). The restriction enzyme cleavage site in this region is shown in Annex 3 (p.14, Figure 2).

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

The *Agrobacterium* method was used for transferring the nucleic acid to the recipient organism. From the *Escherichia coli* (*E. coli*) which possesses the plasmid pRPA-BL-235, the plasmid was transferred to the disarmed strain of *Agrobacterium tumefaciens* EHA101 which possesses the pTVK291 cosmid (Reference 33) (Reference 13). Transformation to the recipient cultivar Westar (hereinafter referred to as "Westar") was conducted through the mixed culture of *Agrobacterium tumefaciens* EHA101 strain containing pRPA-BL-235 and stem explants (Reference 36).

3) Processes of rearing of living modified organisms

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

Stem explants of Westar were mixed-cultured with *Agrobacterium tumefaciens* EHA101 strain containing pRPA-BL-235 for 72 hours and then cultured on a regeneration medium. To the regeneration medium, an antibiotic (Augmentin) for prevention of possible bacterial growth, and bromoxynil for selection of transformed cells were added (Reference 18).

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

Due to the culture on the regeneration medium containing the antibiotic (Augmentin), the *Agrobacterium* used for transformation was removed.

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

Seedlings generated from the regeneration medium were transferred to growth medium for culture on the medium containing bromoxynil, and the seedlings exhibiting bromoxynil tolerance were transplanted to hormone-free growth medium. Then the plants were transplanted in pots in a greenhouse and sprayed with herbicide bromoxynil to select the herbicide tolerant individuals. The commercial lines were produced by selective breeding of T_3 and subsequent generations. The pedigree tree of OXY-235 is shown in Figure 4.

The approvals received from organizations in Japan are as follows.

- In April 1997, based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", conducting isolated field test was approved by the Ministry of Agriculture, Forestry and Fisheries. In addition, in July 1998, based on the Guideline, the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

- In November 1999, the compatibility to the "Guideline for food safety assessment of food and food additives derived from Recombinant-DNA technology" was confirmed by the Ministry of Health and Welfare (the Ministry of Health, Labour and Welfare, currently). In addition, along with legislating, passing through the "Procedures for food safety assessment of food and food additives derived from recombinant-DNA technology", safety of use for food was approved by the Ministry of Health, Labour and Welfare in March 2001.

- In December 1999, the compatibility to the "Guideline for feed safety assessment of recombinant feed" was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, along with legislating, passing through the "Procedures for feed safety assessment of feed and feed additives derived from recombinant-DNA technology", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in March 2003.

Confidential: Not made available or disclosed to unauthorized person

Figure 4 Pedigree tree of OXY-235

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

1) Place where the replication product of transferred nucleic acid exists

Since it is expected that the original gene transformant (T_0) of OXY-235 is heterozygote for the transferred gene locus, a segregation ratio of 3:1 would be obtained in theory between herbicide bromoxynil-tolerant and herbicide bromoxynil-sensitive individuals in the T_1 generation obtained from self-fertilization of the original gene transformant. In 1991, the seeds of T_1

generation obtained from self-fertilization of the original gene transformant (T_0) were grown in a field in France and sprayed with herbicide bromoxynil. As a result, the segregation ratio showed a good agreement with the theoretical segregation ratio (Annex 4, Table in p.1). Consequently, it is considered that the tolerance to herbicide bromoxynil is transferred onto the chromosome at one site.

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

As a result of Southern blotting analysis for the genome DNA extracted from self-fertilized progenies of OXY-235 using 5'ssuZm, *oxy* gene, 3'nos and the entire T-DNA region as probes, it was confirmed that one copy of T-DNA region was transferred (Annex 3, p.15 Figure 3 to p.18 Figure 6).

In addition, as a result of Southern blotting analysis for the genome DNA of T_3 and BC3F1 generations of OXY-235 with cleavage made by the restriction enzyme EcoR I, Hind III and the both, the identical band patterns were identified and thus, it was confirmed that the transferred nucleic acid was stably inherited (Figure 5).

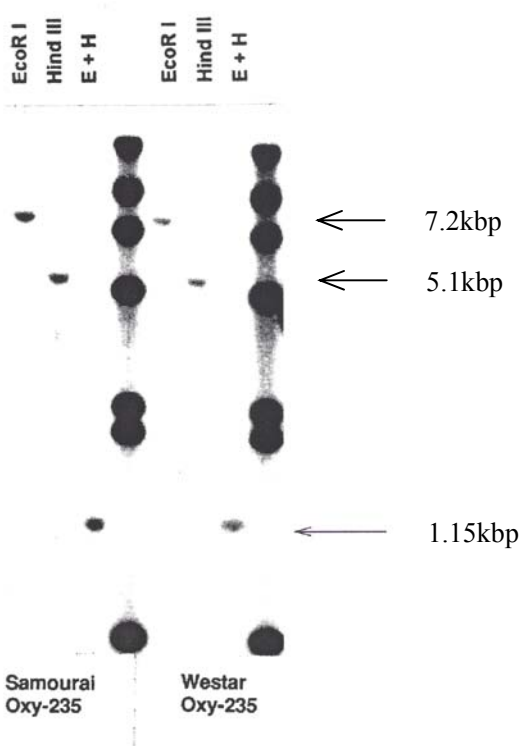

Figure 5 Southern blotting analysis to show the stability of inheritance of transferred nucleic acid

Left: BC3F1 generation, Right: T_3 generation

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

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

As a result of the isolated field test conducted in FY 1997 at the Hokkaido Agricultural Research Center of the Ministry of Agriculture, Forestry and Fisheries, in which the seeds of OXY-235 and Westar were sown in vats in an isolated greenhouse and the germinated 100 or more seedlings were sprayed with herbicide bromoxynil, the individuals of OXY-235 all survived while the individuals of Westar all died (Annex 5, p.3). Moreover, as a result of the special screened greenhouse test conducted in 2007, in which the seeds of self-fertilized progenies of OXY-235 and the non-recombinant oilseed rape cultivar Drakkar (hereinafter referred to as "Drakkar") were sown and the germinated seedlings were sprayed with herbicide ioxynil, one of the oxynil family of herbicides, the individuals of Drakkar all died while the individuals of OXY-235 all exhibited the tolerance to ioxynil (Annex 6, p.15, Table 18). Consequently, it was confirmed that the tolerance to oxynil family of herbicides is stably expressed in the multiple generations of OXY-235.

Furthermore, as a result of Northern blotting analysis for the RNA extracted from leaves, seeds and roots of OXY-235 using the *oxy* gene as a probe, the band of 1.3kb was detected in all the tissues and then, it was confirmed that the *oxy* gene is expressed in the tissues (Annex 7, p.19, Figure II-3).

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

OXY-235 contains no DNA sequence that possesses transferring factor and therefore, it is considered unlikely that the transferred nucleic acid would be transmitted to wild animals and wild plants under a natural environment.

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

Specific identification of this recombinant is available by PCR method using the flanking sequences of DNA transferred in OXY-235. Identification of higher sensitivity is attained using 20 to 50 ng of DNA. This PCR method is utilized effectively for cultivation management of OXY-235 (Annex 8).

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

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

OXY-235 exhibits the trait to be tolerant to bromoxynil and ioxynil in the oxynil family of herbicides due to the nitrilase protein produced by the expression of the

transferred *oxy* gene.

2) 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

In FY 1997, the isolated field tests were conducted at the Hokkaido Agricultural Research Center of the Ministry of Agriculture, Forestry and Fisheries to make comparison between OXY-235 and Westar (Annex 5). In addition, for references of evaluation, Karafuto (oilseed rape) and leaf mustard (*B. juncea*) were also cultivated at the same time. Moreover, in 2007, in the special screened greenhouse in Japan, comparison was made between OXY-235 and Drakkar regarding the heat-tolerance at the early stage of growth, fertility and size of the pollen, dormancy and germination rate of the seed, and productivity of harmful substances (Annex 6). The Drakkar is a spring sowing variety of oilseed rape same as the recipient cultivar Westar.

(a) Morphological and growth characteristics

Comparison was made for the items regarding morphological and growth characteristics; plant height, the number of primary branches, dry weight of aerial parts (stems and leaves), plant shape, color of leaves, time of bolting, flowering period, maturation period, rate of pods formation, length of pod, the number of seed setting, property of open pods, color of seed, seed yield and 1,000-seeds weight. As a result, for the plant height, length of pod, the number of seed setting, seed yield and 1,000-seeds weight, no significant difference was observed between OXY-235 and Westar. In addition, for the weight of aerial parts (stems and leaves) and rate of pods formation, the average values of OXY-235 were all found within the range of average values of the oilseed rape cultivars Westar and Karafuto, though statistical treatment was not completed, so therefore, it is considered that the characteristics fall within the range of differences between cultivars. Moreover, in all the other items examined, no difference was observed (Annex 5, p.4).

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

In the isolated field tests, seedlings of OXY-235 and Westar were exposed to low temperatures, an average temperature of around 12°C from May to the early June which correspond to the early stage of growth of OXY-235 and Westar, and a minimum temperature of around 5 to 8°C (Annex 5, p.10, Meteorological data), though OXY-235 grew same as Westar and no difference was observed between the both plants.

In addition, in the special screened greenhouse tests conducted to evaluate the heat-tolerance of young plant body, changes in the degree of yellowing over time was visually evaluated in a long period of time under the conditions (35°C and 12-hour day length and 12-hour night length). As a result, it was found that yellowing was rapidly accelerated in the both lines up to five weeks from the start of cultivation under high-temperature conditions, and the both lines

were turned yellow completely. After that, yellowing advanced gradually in the both lines, and a statistically significant difference was observed between the lines after 5 weeks to 11 weeks, though the both lines did not show any recovery from yellowing, and it was confirmed that all the individuals died after 12 weeks (Annex 6, p.13, Table 17).

For the cold-tolerance at the early stage of growth, the oilseed rape varieties sown in autumn in Japan are generally known to grow even in winter in both warm and cold districts, though the rate of growth varies (Reference 67).

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

As a result of observation for the summer survival in the isolated field tests, no difference was observed between OXY-235 and Westar that had been sown in spring (Annex 5, p.3).

It is generally known that oilseed rape shows high cold tolerance and high snow endurance (Reference 67).

(d) Fertility and size of the pollen

Pollens were collected from OXY-235 and Drakkar cultivated in the special screened greenhouse, and stained with acetocarmine solution and observed under a microscope. As a result, 97.3% of the pollens from OXY235 and 96.9% of the pollens from Drakkar were found stained, showing a high fertility of the pollens (Annex 6, p.4). In addition, as a result of comparison of size of pollen, no statistically significant difference was observed between the both lines (Annex 6, p.5, Table 2).

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

Regarding seed production, comparison was made for the rate of pods formation, the number of seed setting, seed yield and 1,000-seeds weight between OXY-235 and Westar. As a result, in the number of seed setting, seed yield and 1,000-seeds weight, no significant difference was observed. In addition, the rate of pods formation was found 60.6% for OXY-235 and 59.0% for Westar, showing that the both lines are equivalent to each other (Annex 5, p.4, Table 2).

For the property of open pods, comparison was made between OXY-235 and Westar based on the five-scale evaluation (Scale 1 for difficult opening of pods to Scale 5 for easy opening of pods) and as a result, the both lines showed the relatively easy property of open pods (Scale 4), showing the equivalence between the both plants (Annex 5, p.4, Table 2).

Forty (40) seeds each obtained from OXY-235 and Drakkar harvested in the special screened greenhouse were sown to evaluate the changes in germination rate from the second day to eighth day of sowing. As a result, in all the days examined, no statistically significant difference was observed between the both lines, and the both lines showed a high germination rate of 95% or more on the

fourth day of sowing. In addition, on the eighth day of sowing, one seed each from the both lines did not germinate, the seeds were found died, and it was confirmed that the failure of germination did not result from dormancy. Based on the above understanding, the dormancy of the seed of OXY-235 is considered very low similarly to the non-recombinant plant (Annex 6, p.17, Table 19).

(f) Crossability

In the isolated field test, possible crossing of OXY-235 with the oilseed rape Westar and Karafuto cultivated adjacent to OXY-235 at each furrow distance of 80 cm was investigated. In the flowering period, about 3,000 honeybees were released. The seedlings derived from the seeds harvested from two individuals each of Westar and Karafuto were sprayed with herbicide bromoxynil and as a result, the herbicide bromoxynil tolerance was observed in 10.6% and 13.8% of individuals of Westar, and in none of seedlings of Karafuto (Annex 5, p.11).

It is generally known that the out-crossing rate of oilseed rape is 5 to 30% (References 24, 46, 55), and the crossability of OXY-235 with the adjacent oilseed rape was found not exceeding the above described rate.

(g) Productivity of harmful substances

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

Succeeding crop test:
In the remaining soil after cultivating OXY-235 and Drakkar, radishes were sown as test plants, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight of radishes. As a result, in germination rate, plant height, root length and fresh weight, no statistically significant difference was observed between the lines (Annex 6, pp.7-8, Tables 4,6, and 8). On the other hand, the dry weight of radishes in the soil after cultivation of OXY-235 was found heavier than that in the soil after cultivation of Drakkar, showing a statistically significant difference (Annex 6, p.8, Table 8).

Plow-in test:
In the soil mixed with 1% each of plant body samples from OXY-235 and Drakkar, radishes were sown, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed (Annex 6, pp.9-10, Tables 10,12, and 14).

Soil microflora test:
The soil was obtained after cultivating OXY-235 and Drakkar, and was diluted

as appropriate by adding sterilized phosphate buffer solution. Bacteria and Actinomycetes were incubated in PTYG medium, and filamentous fungi were incubated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, there was no statistically significant difference in any items (Annex 6, p.12, Table 16).

In addition, in the investigations conducted in foreign countries, it was confirmed that the contents of erucic acid and glucosinolate in the seed of OXY-235 fall within the range specified for canola cultivars (Annex 7, p.16 Table 2.3.2.1, p.17 Table 2.3.2.2).

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex List

Annex 1: Nucleotide sequence of *oxy* gene

Confidential: Not made available or disclosed to unauthorized person

Annex 2: Nucleotide sequence of plasmid pRPA-BL-235

Confidential: Not made available or disclosed to unauthorized person

Annex 3: Identification of transferred gene in OXY-235

Confidential: Not made available or disclosed to unauthorized person

Annex 4: Reference data for segregation ratio in OXY-235 (T_1 generation)

Confidential: Not made available or disclosed to unauthorized person

Annex 5: FY 1997 isolated field test report

Confidential: Not made available or disclosed to unauthorized person

Annex 6: 2007 special screened greenhouse test report

Confidential: Not made available or disclosed to unauthorized person

Annex 7: Biological characteristics of OXY-235 and evaluation of possible effects

Confidential: Not made available or disclosed to unauthorized person

Annex 8: Event identification method

Confidential: Not made available or disclosed to unauthorized person

Annex 9: Subacute oral toxicity test using mice

Confidential: Not made available or disclosed to unauthorized person

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

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

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

(1) Competitiveness

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

This recombinant oilseed rape is given a trait to be tolerant to oxynil family of herbicides, though it is generally considered that the oxynil family of herbicides does not exert selective pressure under a natural environment. Therefore, it is considered unlikely that the trait can cause the recombinant oilseed rape to become competitive under a natural environment.

In the isolated field in Japan, the traits relating to the competitiveness of this recombinant oilseed rape were examined based on the comparison with the non-recombinant oilseed rape of recipient cultivar (Westar) for morphological and growth characteristics, summer survival of the matured plant, and production and shedding habit of the seed. As a result, there was no statistically significant difference or difference observed between the both plants. In addition, in the special screened greenhouse in Japan, the comparison was made between this recombinant oilseed rape and the non-recombinant oilseed rape (Drakkar) regarding heat-tolerance at the early stage of growth, fertility and size of the pollen, and germination rate and dormancy of the seed. As a result, in all the items examined but the heat-tolerance at the early stage of growth, no statistically significant difference or difference was observed between OXY-235 and the control cultivar. Moreover, for the heat-tolerance at the early stage of growth, a statistically significant difference was observed between OXY-235 and Drakkar regarding the degree of yellowing in the process of investigations on the changes in yellowing over time. However, the both lines did not exhibit possible recovery from yellowing, and the individuals investigated were all confirmed to die after 12 weeks; therefore it was considered unlikely that this recombinant oilseed rape could survive under high temperature conditions. Based on the above understanding, it is considered unlikely that OXY-235 has newly acquired any traits to enhance the competitiveness.

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

Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

Conventional oilseed rape is known to produce erucic acid and glucosinolate in the seeds recognized as harmful substances to human and other mammals. The cultivar of the recipient organism of this recombinant oilseed rape (Westar) is one of the canola cultivar, in which the erucic acid and glucosinolate content was reduced by breeding. In the componential analysis of this recombinant oilseed rape, it was confirmed that the erucic acid and glucosinolate content is within the range of canola cultivar.

This recombinant oilseed rape produces the nitrilase protein which confers the trait to be tolerant to oxynil family of herbicides. There is no report that the nitrilase protein possesses adverse effect on wild animals and wild plants. In addition, it is suggested that the nitrilase protein possesses high substrate specificity. Therefore, it is considered that the nitrilase protein does not affect the metabolic pathway of the recipient organism. Moreover, based on the amino acid sequence of nitrilase protein, homology search was conducted and as a result, no homology with any known allergen was observed.

In order to check for possible productivity of harmful substances of this recombinant oilseed rape (the substances excreted from the roots which can affect other plants, the substances existing in the plant body which affect other plants after dying, and the substances excreted from the roots which can affect microorganisms in soil), succeeding crop test, soil microflora test and plow-in test were conducted in the special screened greenhouse in Japan. As a result, in all the tests conducted, no significant difference was observed in comparison with the test plots for the non-recombinant oilseed rape (Drakkar).

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

(3) Crossability

In a natural environment in Japan, a number of plants of the family *Brassicaceae* are growing, though known species that can be crossed with oilseed rape (*Brassica napus*) include *B. rapa* (turnip, Komatsuna, conventional rapeseed, etc.) of the genus *Brassica*, *B. juncea* (mustard, leaf mustard, etc.), *B. nigra* (black mustard) and *Raphanus raphanistrum* (wild radish) in addition to oilseed rape itself. However, they are all regarded as foreign species and are not specified as wild species as to be possibly affected.

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

(4) Additional information

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

(i) It was confirmed that the crossability of this recombinant oilseed rape with the non-recombinant oilseed rape does not exceed any existing findings on the crossability between oilseed rape varieties.

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

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

In addition, it has been confirmed that this recombinant oilseed rape does not differ from the non-recombinant oilseed rape regarding the competitiveness, the productivity of harmful substances and the crossability and thus, it is considered unlikely that the transferred gene to this recombinant oilseed rape would affect the maintenance of the related species population.

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

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