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

Applicant:
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera (*cry1A.105,* Modified *cry2Ab2*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034, OECD UI: MON-89Ø34-3)
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 maize resistant to Lepidoptera (*cry1A.105,* Modified *cry2Ab2*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034, OECD UI: MON-89Ø34-3) (hereinafter referred to as "this recombinant maize") are shown in Figure 4 and Table 4.

2) Functions 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 the donor nucleic acid that was used for the development of this recombinant maize are shown in Table 4. The *cry1A.105* gene and the modified *cry2Ab2* gene, target genes in MON89034, are detailed below.

[*cry1A.105* gene]

The Cry1A.105 protein, which is encoded by the *cry1A.105* gene used for the development of this recombinant maize, is composed of Domains I and II of Cry1Ab protein, Domain III of Cry1F protein, and C-terminal Domain of Cry1Ac protein (Figure 1), and the Cry1Ac protein, Cry1Ab protein, and Cry1F protein possess the amino acid sequence homology of 93.6%, 90.0%, and 76.7% respectively with the Cry1A.105 protein (Table 1).

Three (3) types of Bt proteins, which make up the Cry1A.105 protein, are expressed in the cotton resistant to Lepidoptera (*cry1Ac, Gossypium hirsutum* L.)(531, OECD UI MON-ØØ531-6) (hereinafter referred to as "531"), the maize resistant to Lepidoptera (*cry1Ab*, *Zea mays* L.)(MON810, OECD UI: MON-ØØ81Ø-6) (hereinafter referred to as "MON810"), and the maize resistant to Lepidoptera and tolerant to glufosinate herbicide (*cry1F*, *pat, Zea mays* subsp. *mays* (L.) Iltis) (*B.t.* Cry1F maize line 1507, OECD UI: DAS-Ø15Ø7-1) (hereinafter referred to as "1507"), which have been all approved regarding Type I Use Regulation.

Bt proteins have been used safely as microbial pesticide over 40 years (Reference 15; Reference 16; Reference 17), and the mechanism of action against target insects has been already clarified (Reference 18; Reference 19). In addition, it has been revealed from previous studies that Bt proteins are composed of several domains, which possess different functions from each other, and what functions the domains can offer. For example, the Cry1A protein is composed of Domains I, II and III, and C-terminal Domain, and it has been proved that the Domain I takes part in

formation of cation selective pores, which lead to the inhibition of digestive process, Domain II takes part in recognition of specific receptors, Domain III takes part in binding to the receptors, and C-terminal Domain relates to crystalline structure of Bt proteins (Reference 20; Reference 21).

As mentioned above, the Cry1A.105 protein, which is encoded by the *cry1A.105* gene used for the development of this recombinant maize, is a synthetic Bt protein made up from Domains I and II of Cry1Ab protein, Domain III of Cry1F protein, and C-terminal Domain of Cry1Ac protein (Figure 1, p. 4), and it has been developed for the purposes of enhancing the insecticidal activity against target insects by combining the different Bt protein domains.

In recent years, Bt preparation has been developed featuring enhanced insecticidal activity against target insects by combining different Bt protein domains as described above (Reference 22; Reference 23; Reference 24) and in fact, a microbial pesticide, which incorporates the domains of Cry1Ac protein and Cry1F protein in conjunction, is commercially available (Lepinox WDG, Ecogen Inc.) (Reference 22; Reference 23).

In addition, the Cry1F protein, which is expressed in the cotton resistant to Lepidoptera and tolerant to glufosinate herbicide (*cry1F*, *cry1Ac*, *pat*, *Gossypium hirsutum* L.) (281×3006, OECD UI: DAS-24236-5×DAS-21023-5) (hereinafter referred to as "281×3006") granted an approval regarding Type I Use Regulation, is also a synthetic protein, which contains the domains or sequences of Cry1F protein, Cry1C protein and Cry1Ab protein in conjunction (Reference 25).

Moreover, it has been reported that the recombination of domains between Bt proteins has been taking place also in the nature in the course of evolution of Bt proteins in many years to acquire species diversity (Reference 20; Reference 21; Reference 26).

In order to investigate the insecticidal spectrum of Cry1A.105 protein, the Cry1A.105 protein was added to artificial feeds, which were given to 15 different kinds of insects including 5 species of insects of the order Lepidoptera.

As a result, the Cry1A.105 protein exhibited the insecticidal activity against the larvae of Corn earworm (CEW; *Helicoverpa zea*), Black cutworm (BCW; *Agrosis epsilon*), Fall armyworm (FAW; *Spodoptera frugiperda*), Southwestern corn borer (SWCB; *Diatraea grandiosella*), and European corn borer (ECB; *Ostrinia nubilalis*), which are the major pest insects for maize, though it did not exhibit any insecticidal activity against honey bee, ladybug and other beneficial insects except the insects of order Lepidoptera (Table 2).

Based on the above results, it was confirmed that the Cry1A.105 protein exhibits a selective insecticidal activity against only the insects of the order Lepidoptera similarly as the Cry1Ab protein, Cry1F protein and Cry1Ac protein, which are the component elements for the recombinant maize, and it does not possess any insecticidal activity against the other species of insects.

The DNA sequence of the *cry1A.105* gene is shown in Reference 1.

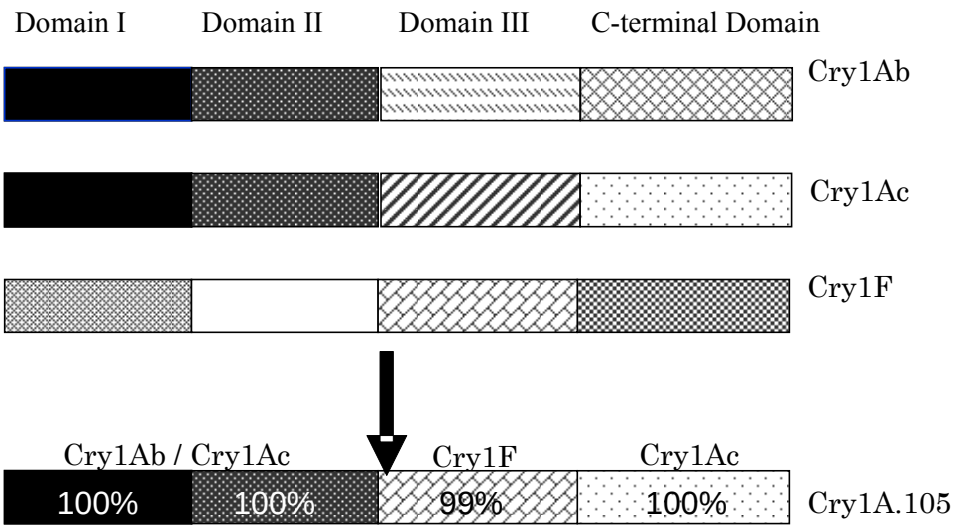

Figure 1 Structure of Cry1A.105 protein[1]

Different patterns refer to different origins of respective domains.

Table 1 Amino acid sequence homology between Cry1A.105 protein and Cry1Ac, Cry1Ab and Cry1F proteins[2]

Domain	Amino acid sequence homology with Cry1A.105 protein (%)		
	Cry1Ac	Cry1Ab	Cry1F
I	100	100	57
II	100	100	37
III	57	46	99
C-terminal	100	92	93
The entire protein	93.6	90	76.7

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

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

Table 2 Insecticidal spectrum of Cry1A.105 protein[3]

Order	Family	English name (Scientific name)	Insect Stage	LC_{50} (µg/mL or g diet)[a]	Reference
Lepidoptera	Noctuidae	Corn Earworm (*Helicoverpa zea*)	Larva	15	Reference 27
		Black Cutworm (*Agrotis ipsilon*)	Larva	33	Reference 28
		Fall Armyworm (*Spodoptera frugiperda*)	Larva	6.9	Reference 28
	Crambidae	Southwestern Corn Borer (*Diatraea grandiosella*)	Larva	37	Reference 28
		European Corn Borer (*Ostrinia nubilalis*)	Larva	0.43	Reference 29
Collembola	Isotomidae	Collembola *(Folsomia candida)*	Young adult	>80[b]	Reference 30
Coleoptera	Curculinoidae	Boll Weevil (*Anthonomus grandis grandis*)	Larva	>100	Reference 31
	Chrysomelidae	Southern Corn Rootworm (*Diabrotica unecimpunctata howardi*)	Larva	>100	Reference 31
	Coccinellidae	Spotted Lady Beetle *(Coleomegilla maculata)*	Larva	>240	Reference 32
Hymenoptera	Ichneumonidae	Parasitic wasp (*Ichneumon promissorius*)	Adult	>240	Reference 33
	Apidae	European Honey Bee (*Apis mellifera*)	Adult	>550	Reference 34
		European Honey Bee (*Apis mellifera*)	Larva	>11µg/cell	Reference 35
Hemiptera Sub-order: Homoptera	Aphididae	Green Peach Aphid (*Myzus persiscae*)	Adult/Young adult	>80	Reference 31
Hemiptera Sub-order: Heteroptera	Miridae	Western Tarnished Plant Bug (*Lygus hesperus*)	Young adult	>80	Reference 31
	Anthocoridae	Insidious Flower Bug (*Orius insidiosus*)	Young adult	>240	Reference 36

[a] The values with the sign ">" refer to the highest density among the samples used in testing.
[b] Testing was conducted using the freeze-dry leaves of this recombinant maize.

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

[Modified *cry2Ab2* gene]

The modified Cry2Ab2 protein, which is expressed in this recombinant maize, has the identical amino acid sequences as the modified Cry2Ab2 protein, which is expressed in the cotton resistant to Lepidoptera (*cry1Ac*, *cry2Ab*, *Gossypium hirsutum* L.)(15985, OECD UI: MON-15985-7) (hereinafter referred to as "15985") granted an approval regarding Type I Use Regulation.

Wild-type *cry2Ab2* gene is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium which universally exists in the soil, and it is also known as *cry2Ab*, *cryIIB*, *cryB2* or *cryIIAb* (Reference 37; Reference 38; Reference 39). The modified Cry2Ab2 protein, which is expressed in this recombinant maize, has the site broken by the restriction enzyme transferred during the cloning and then, a single aspartic acid (D in Figure 2) is transferred after the methionine (M in the wild type in Figure 2) at the N-terminal compared to the wild-type Cry2Ab2 protein, though the other amino acid sequences are same as those in the wild type.

In addition, the modified *cry2Ab2* gene transferred in this recombinant maize contains a nucleotide sequence added in the 5'-terminal region, which encodes the chloroplast transit peptide (CTP) to transfer the target proteins to the plastid and thus, the modified Cry2Ab2 protein is produced with the CTP connected to the N-terminal region. This CTP will be normally disconnected from the target proteins and broken down immediately by protease after the target proteins are transferred to the plastid (Reference 40). The deduced amino acid sequence of CTP contains recognition sites (methionine) estimated by protease in the 32nd and 3rd amino acids from C-terminal among the total length of 79 amino acid residues of CTP (Figure 2), and it has been expected that CTP would have been cleaved at either one of the sites. Actually an examination was conducted for this recombinant maize to identify if a cleavage is present between the CTP and the modified Cry2Ab2 protein. However, an analysis failed due to the possible cause that the N-terminal region of the modified Cry2Ab2 protein in this recombinant maize would be subjected to chemical modification. Consequently, it was impossible to locate the cleavage site in CTP.

Then, first, on the assumption that the CTP has been cleaved at the third methionine from the C-terminal in the CTP amino acid sequence, an attempt was made to make the modified Cry2Ab2 protein express in *Escherichia coli* with addition of 3 amino acids derived from CTP remaining in the N-terminal region of the modified Cry2Ab2 protein (Figure 2). This protein was compared based on the SDS-PAGE with the modified Cry2Ab2 protein which is expressed in this recombinant maize and as a result, the molecular weight was judged to be equivalent (Figure 3, p8).

Based on the above results, it was judged that in this recombinant maize, the modified Cry2Ab2 protein is functioning with 3 amino acids from CTP added to the N-terminal region and then, the bioassay on the insects described below was carried out using the CTP adhering modified Cry2Ab2 protein.

In order to investigate the insecticidal spectrum of modified Cry2Ab2 protein, modified Cry2Ab2 protein was added to artificial feeds, which were given to 15 different species of insects including 4 insects of the order Lepidoptera.

As a result, the modified Cry2Ab2 protein exhibited the insecticidal activity against the larvae of Corn earworm (CEW; *Helicoverpa zea*), Fall armyworm (FAW; *Spodoptera frugiperda*), and European corn borer (ECB; *Ostrinia nubilalis*) among the 4 species of major pest insects of the order Lepidoptera used in the investigation and not against Black cutworm (BCW; *Agrosis. epsilon*) (Table 3). Also, the modified Cry2Ab2 protein did not exhibit any insecticidal activity against honey bee, ladybug and other beneficial insects except the insects of the order Lepidoptera (Table 3); therefore, it was confirmed that the modified Cry2Ab2 protein offers specific insecticidal activity against only the insects of the order Lepidoptera and not against the other species of insects.

The DNA sequence of the modified *cry2Ab2* gene is shown in Reference 1.

MON89034	**M-Q-A[1]-M-D[2] -** N-S-V-L-N
E. coli	**M-Q-A[1]-M-D[2] -** N-S-V-L-N
Wild type	**-M- -** N-S-V-L-N

1 M-Q-A –Deduced amino acid derived from chloroplast transit peptides (CTP)
2 D – Added amino acid for cloning

Figure 2 Deduced amino acid sequence in the N-terminal region of the modified Cry2Ab2 protein expressed in this recombinant maize, *E. coli*, and *B. thuringiensis*[4]

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

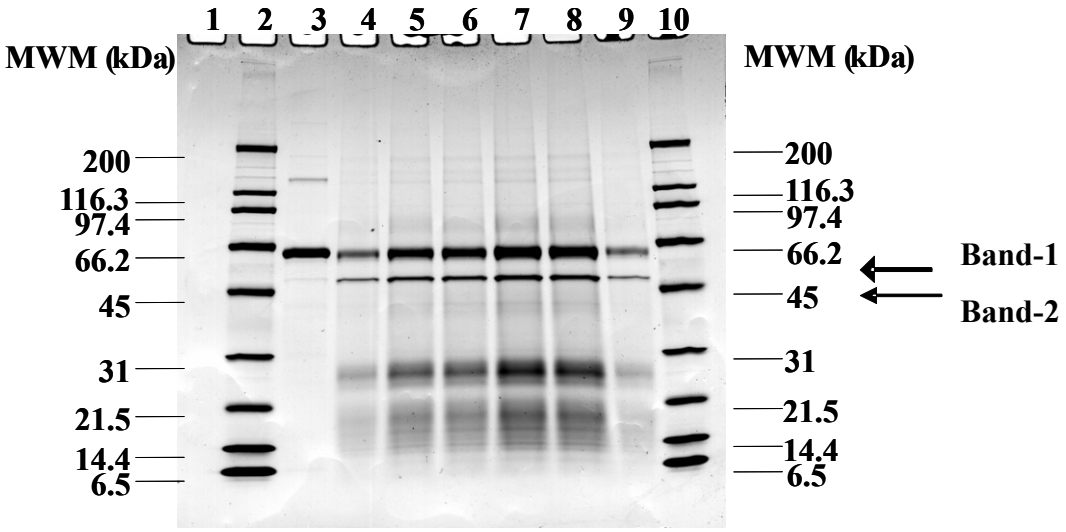

Figure 3 Comparison of molecular weight of modified Cry2Ab2 protein expressed in this recombinant maize and *E. coli* based on SDS-PAGE[5]

The modified Cry2Ab2 protein in this recombinant maize and *E. coli* was subjected to the polyacrylamide SDS gel electrophoresis and then stained with Brilliant Blue G-Colloidal stain. Band-1 refers to proteolytic fragment of the full length of modified Cry2Ab2 protein, and Band-2 refers to proteolytic fragment of modified Cry2Ab2 protein.

Lane 1	Blank (0 μg)
Lane 2	Molecular weight marker (4.5 μg)
Lane 3	Modified Cry2Ab2 protein with addition of 3 amino acids derived from CTP (1 μg)
Lane 4	Modified Cry2Ab2 protein of this recombinant maize (1 μg)
Lane 5	Modified Cry2Ab2 protein of this recombinant maize (2 μg)
Lane 6	Modified Cry2Ab2 protein of this recombinant maize (2 μg)
Lane 7	Modified Cry2Ab2 protein of this recombinant maize (3 μg)
Lane 8	Modified Cry2Ab2 protein of this recombinant maize (3 μg)
Lane 9	Modified Cry2Ab2 protein of this recombinant maize (1 μg)
Lane 10	Molecular weight marker (4.5 μg)

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

Table 3 Insecticidal spectrum of the modified Cry2Ab2 protein[6]

Order	Family	English name（Scientific name）	Insect Stage	LC_{50} (μg/mL or g diet)[a]	Reference
Lepidoptera	Noctuidae	Corn Earworm (*Helicoverpa zea*)	Larva	9.9	Reference 29
		Black Cutworm (*Agrotis ipsilon*)	Larva	>100[b]	Reference 41
		Fall Armyworm (*Spodoptera frugiperda*)	Larva	<50[c]	Reference 41
	Crambidae	European Corn Borer (***Ostrinia nubilalis***)	Larva	1.5	Reference 29
Collembola	Isotomidae	Collembola *(Folsomia candida)*	Young adult	>70[d]	Reference 30
Coleoptera	Curculinoidae	Boll Weevil (*Anthonomus grandis grandis*)	Larva	>100	Reference 41
	Chrysomelidae	Southern Corn Rootworm (*Diabrotica unecimpunctata howardi*)	Larva	>100	Reference 41
	Coccinellidae	Spotted Lady Beetle *(Coleomegilla maculata)*	Larva	>120	Reference 42
Hymenoptera	Ichneumonidae	Parasitic wasp (*Ichneumon promissorius*)	Adult	>100	Reference 43
		Parasitic wasp (*Nasonia vetripennis*)	Adult	>4500	Reference 44
	Apidae	European Honey Bee (*Apis mellifera*)	Adult	>68	Reference 45
		European Honey Bee (*Apis mellifera*)	Larva	>0.6 μg/cell	Reference 46
Hemiptera Sub-order: Homoptera	Aphididae	Green Peach Aphid (*Myzus persiscae*)	Adult/Young adult	>80	Reference 41
Hemiptera Sub-order: Heteroptera	Miridae	Western Tarnished Plant Bug (*Lygus hesperus*)	Young adult	>80	Reference 41
	Anthocoridae	Insidious Flower Bug (*Orius insidiosus*)	Young adult	>100	Reference 47

[a] The values with the sign ">" refer to the highest density among the samples used in testing. The values with the sign "<" refer to the lowest density among the samples used in testing.
[b] Mortality rate was 42% when the maximum dose of 100 μg/mL was given.
[c] Mortality rate was 61% when the minimum dose of 50 μg/mL was given.
[d] Testing was conducted using the freeze-dry leaves of this recombinant maize.

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

[*cry1A.105* gene ＋ modified *cry2Ab2* gene]

This recombinant maize is given the resistance to the target insects of the order Lepidoptera with simultaneous expression of both Cry1A.105 protein and modified Cry2Ab2 protein. Actually, as a result of tests of this recombinant maize for resistance to major pest insects of the order Lepidoptera (European corn borer, Southwestern corn borer, Corn earworm, Sugarcane borer (SCB; *Diatraea saccharalis*), Fall armyworm) conducted from 2003 to 2004 in the US, Puerto Rico and Argentine, it was confirmed that this recombinant maize exhibited resistance to all the Lepidopteran insects examined. In addition, as a result of comparison with MON810, the first-generation Lepidoptera-resistant maize, it was confirmed that this recombinant maize offered much more higher resistance to Fall armyworm and Corn earworm, which caused serious damage to cultivation of maize especially in the southern part of the US (Figures 1, 2, 3, 6, 7, 9, and 10 of Annex 2).

In addition, it has been also confirmed that Cry1A.105 protein and modified Cry2Ab2 protein both possess insecticidal activity against Corn earworm, Fall armyworm and European corn borer (Table 2 and Table 3). However, with simultaneous expression of two proteins offering insecticidal spectrum which overlaps to some extent with each other, the target Lepidopteran insects, which exhibit sensitivity to this recombinant maize, could not acquire any insusceptibility to this recombinant maize unless they become insensitive to two kinds of Bt proteins. This raises expectations that this recombinant maize would be able to substantially reduce the probability of occurrence of insensitive pest insects compared to the Bt maize in which only one kind of Bt protein is independently expressed.

It has been confirmed that Cry1A.105 protein and modified Cry2Ab2 protein do not offer any synergistic insecticidal activity against the target insects of the order Lepidoptera which show sensitivity to the both Bt proteins (Table 1 and Table 2 of Annex 3).

Furthermore, although mycotoxin generically refers to fungal toxins including aflatoxin and ochratoxin A, which are known as carcinogen and occurs in the parts damaged by Lepidopteran insects, it is expected that this recombinant maize would reduce the occurrence of mycotoxin due to the Lepidoptera resistance and enhance the safety for use as food and feed of maize.

(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 (excluding allergenicity as food)

In order to investigate whether the Cry1A.105 protein and modified Cry2Ab2 protein share functionally important amino acid sequences with known allergens, the Cry1A.105 protein and modified Cry2Ab2 protein were compared with known allergens in the database (including GenBank, EMBL, PIR, PBD, and SwissProt). As a result, the Cry1A.105 protein and modified Cry2Ab2 protein did not share structurally related homologous sequences with any of the known allergens examined.

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

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(2) Information concerning vector

1) Name and origin

The plasmid vector PV-ZMIR245 used to develop this recombinant maize is assembled from plasmids including the vector pBR322 derived from *E. coli* (Reference 48).

2) Properties

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

The total number of base pairs of PV-ZMIR245 used to develop this recombinant maize is 17,600 bp. The nucleotide sequence of PV-ZMIR245 is provided in Annex 1.

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

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

(c) Presence or absence of infectivity 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 in the recipient organism

The component elements of this plasmid vector transferred in the recipient organism are listed in Table 4. In addition, the location and section broken by restriction enzyme of the component elements of the donor nucleic acid in the vector are shown in Figure 4.

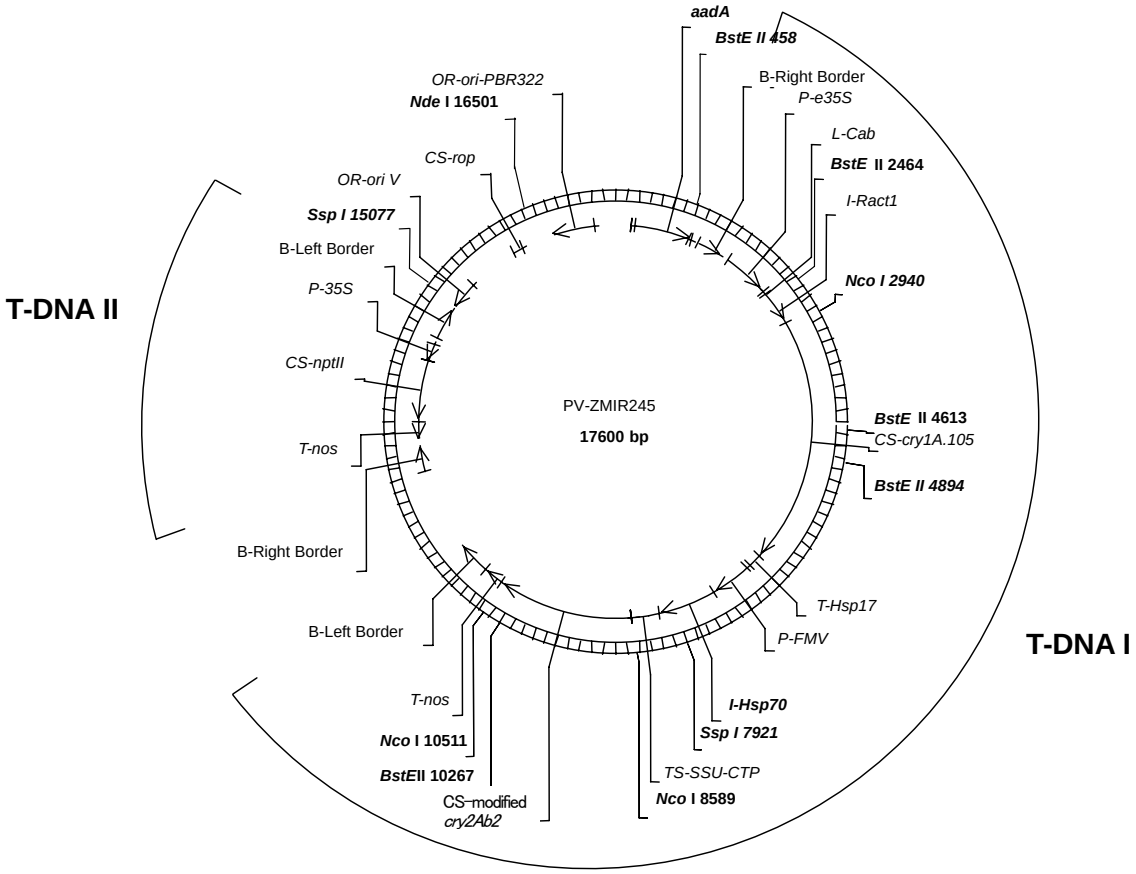

Figure 4 Map of the plasmid PV-ZMIR245 used to develop this recombinant maize[7]

In the process of rearing of this recombinant maize, those individuals were selected that contain the T-DNA I region shown above but not contain T-DNA II region.

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

Table 4[8] Origins and functions of component elements of PV-ZMIR245 used for the development of this recombinant maize

Component elements	Origin and function
T-DNA I region	
B[a]-Right Border	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 49).
P[b]-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Reference 51) promoter and 9bp leader sequence, containing double enhancer regions (Reference 50). Involved in the constant expression of the target gene in the entire tissue of plant body.
L[c]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Reference 52).
I[d]-*Ract1*	Rice actin gene intron (Reference 53). Activates the expression of target gene.
CS[e]-*cry1A.105*	A gene that encodes the Cry1A.105 protein. Details are described in I-2-(1)-ii-(a).
T[f]-*Hsp17*	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (Reference 54).
P[b]-*FMV*	35S promoter derived from Figwort Mosaic Virus (Reference 55). Involved in the constant expression of the target gene in the entire tissue of plant body.
I[d]-*Hsp70*	First intron of maize heat shock protein 70 gene (Reference 56). Activates the expression of target gene.
TS[g]-*SSU-CTP*	Transit peptide of small subunit of ribulose 1,5-carboxylase diphosphate of maize, including the first intron sequence (Reference 57). Transfers downstream-connected protein to plastid.
CS[e]-modified *cry2Ab2*	A gene that encodes the modified Cry2Ab2 protein derived from *B. thuringiensis* (Reference 58). Details are described in I-2-(1)-ii-(a).
T[f]-*nos*	3' untranscribed region of nopaline synthase (*nos*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 59).
B[a]-Left Border	A DNA fragment containing the left border sequence (25bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 60).

[a]B – border
[b]P – promoter
[c]L – leader
[d]I – intron
[e]CS – coding sequence
[f]T – transcript termination sequence
[g]TS – targeting sequence

Table 4[8] Origins and functions of component elements of PV-ZMIR245 used for the development of this recombinant maize (continued)

Component elements	Origin and function
T-DNA II region	
B-Right Border	A DNA fragment containing the right border sequence (24 bp) of nopaline type T-DNA, derived from *A. tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 49).
T-*nos*	3' transcription region of nopaline synthase (*nos*) gene derived from *A. tumefaciens*. Terminates transcription of mRNA and induces polyadenylation (Reference 59).
CS-*nptII*	A gene derived from *E. coli* transposon Tn5 (Reference 61). Encodes the neomycin phosphotransferase II and confers kanamycin resistance to plants. Used as marker to select the transgenic plant during the gene transfer (Reference 62).
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 51). Involved in the constant expression of the target gene in the entire tissue of plant body.
B-Left Border	A DNA fragment containing the left border sequence (25 bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome (Reference 60).
Plasmid backbone region	
OR[a]-*ori V*	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *A. tumefaciens* (Reference 63).
CS-*rop*	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Reference 64).
OR[a]-*ori-PBR322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Reference 48).
aadA	Bacteria promoter, code region and terminator for the 3'(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Reference 65).

[a]OR – Origin of Replication

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

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

The expression vector PV-ZMIR245, which has two independent T-DNA regions (T-DNA I region and T-DNA II region), was transferred into the immature germ cell of LH172, a conventional cultivar of maize which is classified into dent type, by the *Agrobacterium* method.

3) Processes of rearing of living modified organisms

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

R_0 individuals obtained by transferring of expression vector PV-ZMIR245, which has two independent T-DNA regions (T-DNA I region and T-DNA II region), were transferred to the medium containing paromomycin and the individuals which contains both regions T-DNA I and T-DNA II transferred or the individuals (R_0) which contain only T-DNA II region were selected (Figure 5).

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

Agrobacterium was removed by adding carbenicillin to the medium (Reference 66).

(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 LH172BC0F_1 generation, which was obtained by crossing of regenerated individuals of R_0 generation with other conventional cultivar of maize LH172, those individuals were selected based on the PCR method that contain only T-DNA I region with T-DNA II region separated. The individuals that contain T-DNA II region were discarded (Figure 5).

Regarding the selected individuals, further selection was carried out based on the analysis of transferred genes and the expression level of the Cry1A.105 protein and modified Cry2Ab2 protein. Tests in climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters (morphological and growth characteristics, yield and productivity, pest insect sensitivity, etc.) were examined in outdoor field tests. This recombinant maize was selected upon the comprehensive evaluation of these results (The generations used in the tests are shown in Figure 6). The term "this recombinant maize MON89034" in this Biological Diversity Risk Assessment Report refers to all the individuals that have been confirmed in the LH172BC0F_1 generation based on the PCR analysis to contain only the T-DNA I region and eliminate the T-DNA II region and their posterity.

The following shows the approvals received from organizations in Japan.

May, 2006: The Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment granted the approval of Type I Use Regulations (Cultivation in isolated field, storage, transportation, disposal, and acts incidental to them) in accordance with the "Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms."

February, 2007: An application was filed to the Ministry of Health, Labour and Welfare for approval of the safety of use of the cultivar as food based on the "Safety Evaluation Criteria for Food derived from Recombinant-DNA Techniques."

February, 2007: An application was filed to the Ministry of Agriculture, Forestry and Fisheries for approval of the safety of use of the cultivar as feed based on the “Safety Evaluation Criteria for Feed and Additives Produced by Recombinant-DNA Techniques”.

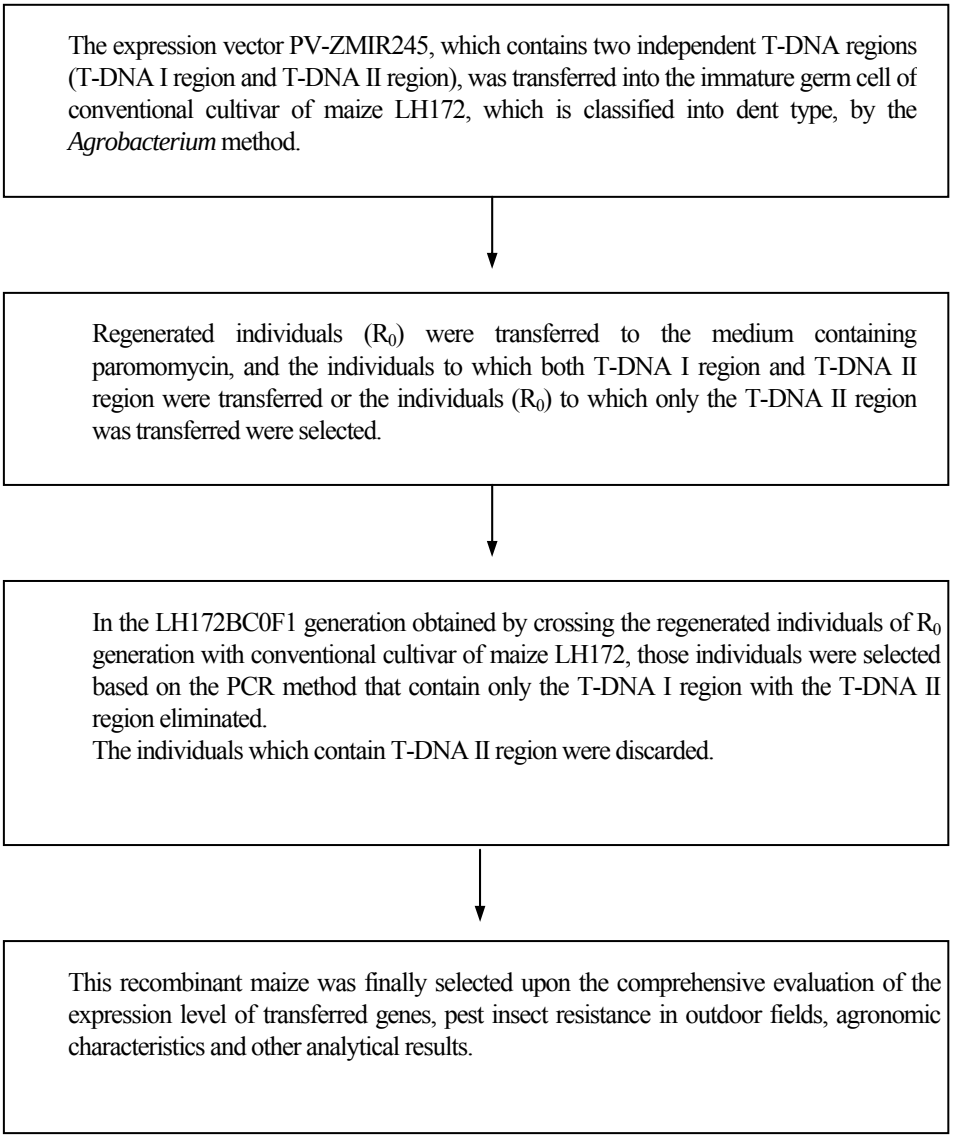

Figure 5 Method for selection of this recombinant maize[9]

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

Figure 6　Process of rearing of this recombinant maize

(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

Using a total of 5 generations including 3 generations ($LH172BC0F_2$ generation, $LH172BC0F_3$ generation, and $LH172BC0F_4$ generation) obtained by repeated inbreeding of $LH172BC0F_1$ generation of this recombinant maize, the $LH172BC1F_1$ generation obtained by crossing of $LH172BC0F_1$ generation with the commercial cultivar of maize LH172, and the $LH172BC1F_2$ generation obtained by inbreeding of the $LH172BC1F_1$ generation, examination was conducted to identify the expression of two Bt proteins and the segregation pattern.

As a result, in all the generations examined, no statistically significant difference was observed between the actually measured values and expected values based on the chi square test (Table 5).

Consequently, it was concluded that the transferred genes in this recombinant maize are inherited to the following generations in accordance with the Mendel's law and then, the transferred genes were confirmed to exist on the chromosome.

Table 5 Segregation ratio in the posterity of this recombinant maize[10]

Generation	No. of plants tested	Measured value		Expected value		X^2
		＋	－	＋	－	
$LH172BC0F_2$	11	7	4	8.25	2.75	0.2727
$LH172BC0F_3$	24	24	0	24	0	Fixed +
$LH172BC0F_4$	30	30	0	30	0	Fixed +
$LH172BC1F_1$	28	13	15	14	14	0.0357
$LH172BC1F_2$[a]	24	20	4	18	6	0.5
$LH172BC1F_2$[a]	24	17	7	18	6	0.0556

＋; Expression of proteins observed
－; Expression of protein not observed
[a]; Samples were taken from different populations in the same $LH172BC1F_2$ generation.

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

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, it was found that one copy of T-DNA I region, which is composed of *cry1A.105* gene expression cassette and modified *cry2Ab2* gene expression cassette, is transferred into the genomic DNA of this recombinant maize at one site (Figure 7 ; for details see Annex 4). In addition, it was also confirmed that there were no other unintended fragments transferred into this recombinant maize, including backbone region and T-DNA II region (Figure 7 ; for details see Annex 4).

In addition, as a result of analysis on the nucleotide sequence of transferred genes, it is found that the 5'-terminal region of P-e35S to control the expression of *cry1A.105* gene and the neighboring right border region have been replaced by the left border region in the T-DNA II region and the 5'-terminal region of P-35S to control the expression of *nptII* gene due to the homologous recombination (Figure 8). However, this homologous recombination is found not to take place in the protein encoding regions, and it has been confirmed that even in the Cry1A.105 protein encoding region, the nearest open reading frame, Cry1A.105 protein is expressed normally in individual tissues (Figure 2 of Annex 5). Consequently, it was concluded that this homologous recombination could not cause formation of any new open reading frame.

Furthermore, it was revealed based on the Southern blotting analysis for multiple generations that the transferred genes are stably inherited in offspring (Figure 17 of Annex).

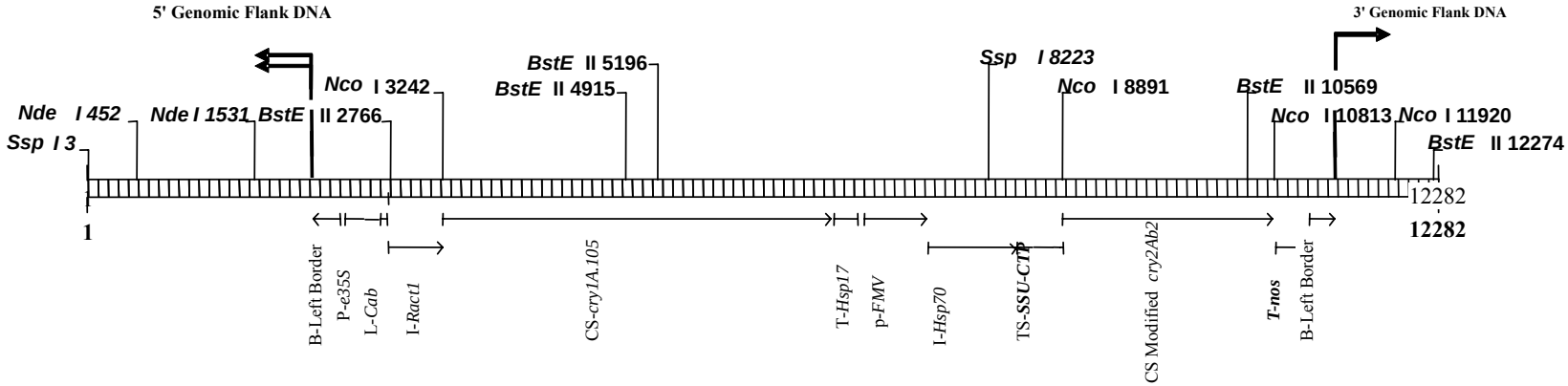

Figure 7 Map of transferred genes in this recombinant maize[11]

[11] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

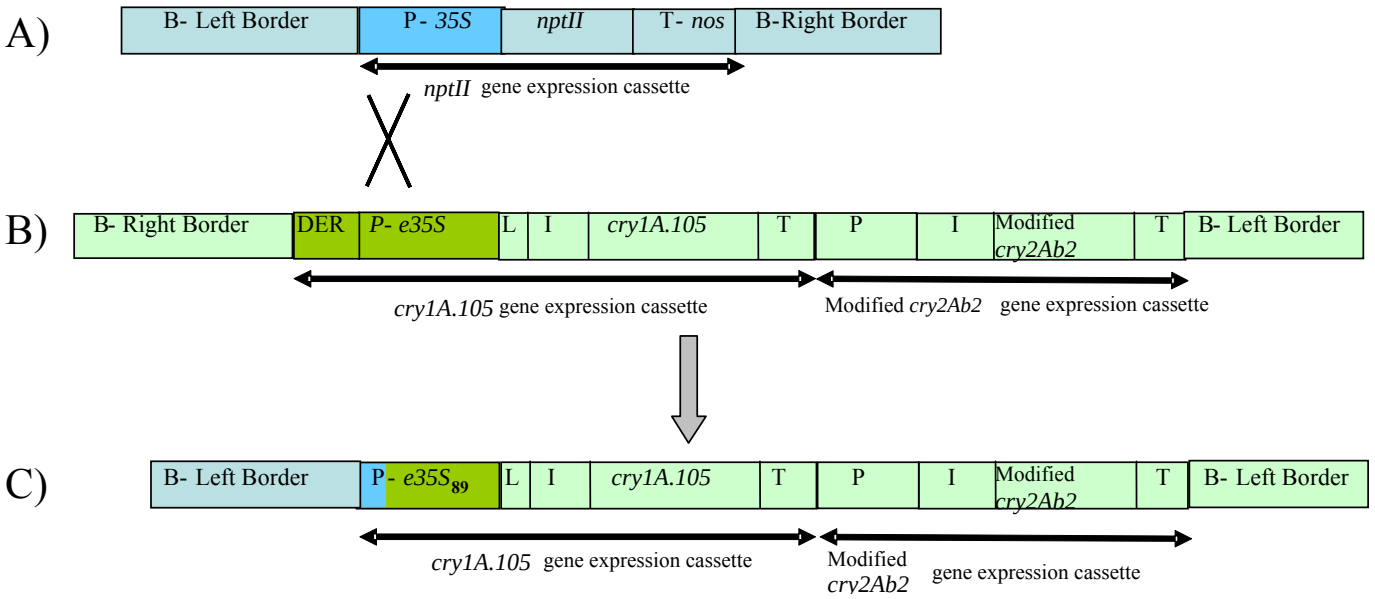

Figure 8 Schematic of homologous recombination in the 5'-terminal region of transferred genes[12]

A) T-DNA II region in the plasmid vector PV-ZMIR245

B) T-DNA I region in the plasmid vector PV-ZMIR245

C) T-DNA I region in this recombinant maize

DER = double enhancer region; L = leader sequence; I = intron sequence; P = promoter; T = terminator.

The diagrams refer to the homologous recombination estimated to have occurred between P-e35S and P-35S in the T-DNA I region and T-DNA II region of the plasmid vector PV-ZMIR245. Due to the homologous recombination, the modified P-*e35S* (P-*e35S*$_{89}$) produced in this recombination has lost the double enhancer region (DER).

12 All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

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

This item is not applicable because of one copy.

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

In order to identify the stability of expression of Cry1A.105 protein and modified Cry2Ab2 protein in multiple generations of this recombinant maize, Western blotting analysis was conducted in 6 generations (LH172BC0F_3, LH172BC0F_4, LH172BC0F_5, LH172BC0F_6, [LH172BC0F_7 x LH198]F_{1H}, and TI:BC1:F_1xRP) of this recombinant maize. As a result, it was confirmed that the Cry1A.105 protein and modified Cry2Ab2 protein expressed in all the generations examined (Figures 2 and 3 of Annex 5, p17, 18). In the individual Western blotting analyses, in addition to the full length of Cry1A.105 protein and the modified Cry2Ab2 protein, the bands of different sizes were detected from this recombinant maize, though these had been all detected also from the non-recombinant control maize. Consequently, it was concluded that these bands were all detected as a result of cross reaction with maize intrinsic proteins.

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

Regarding the plasmid PV-ZMIR245, the region of recipient organism, which allows autonomous replication, is limited to gram-negative bacteria such as *E. coli* and *A. tumefaciens*. Therefore, there is no possibility that the plasmid might be transmitted to any wild animals and wild plants under natural environment.

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

This recombinant maize can be specifically detected by using the DNA sequences of the transferred genes and the nearby regions of the plant genome as primers (Figure 20 of Annex 4).

(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

With the expression of Cry1A.105 protein and modified Cry2Ab2 protein due to the transferred *cry1A.105* gene and modified *cry2Ab2* gene respectively, this recombinant maize is given resistance to the insects of the order Lepidoptera (Figures 1 to 10 of Annex 2).

2) [13]Differences between the recombinant plant and the taxonomic species to which the recipient organism belongs

Isolated field tests were carried out in Kawachi Research Farm (KRF), Monsanto Japan Limited, in 2006 using this recombinant maize and the non-recombinant control maize (Annex 6). The maize lines under test included [LH172BC0F_7 x LH198]F_{1H} generation of this recombinant maize and the non-recombinant control maize line LH172 x LH198 which has the similar genetic backgrounds as the recombinant maize line tested (Figure 6).

(a) Morphological and growth characteristics

For the morphological and growth characteristics, evaluation was conducted on a total of 19 items (uniformity of germination, number of germinated plants, germination rate, time of tasseling, time of silking, flowering time, culm length, culm diameter, plant shape, tiller number, height of ear, maturation time, number of ears, number of productive ears, weight of above-ground parts at the harvest time, ear length, ear diameter, grain color, and grain shape). As a result, in the ear diameter, a statistically significant difference was observed between this recombinant maize and the non-recombinant control maize (P=0.02), though no difference was observed in the other items examined. The average value of ear diameter, in which significant difference was observed between this recombinant maize and the non-recombinant control maize, was 5.1 cm for this recombinant maize and 5.0 cm for the non-recombinant control maize, respectively (Table 2 of Annex 6).

As a result of comparison made between the minimum and maximum mean values of the non-recombinant maize used as control for the recombinant maize (MON863 line, MON810 line, NK603 line, DLL25 line, MON88001 line, MON88012 line, MON88017 line and LY038 line) subjected to isolated field tests and the variable ranges for conventional maize, the average value of ear diameter for this recombinant maize (5.1 cm), in which significant difference was observed, was found to fall within the variable ranges (3.6 to 5.8 cm) for conventional maize (Table 2 of Annex 6).

[13] All the rights pertinent to the information in the paragraphs (a) through (g) following this section and the responsibility for the content rest upon Monsanto Japan Limited.

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

This recombinant maize and the non-recombinant control maize were grown to the four-leaf stage (Figure 6-1 of Annex 6), and they were transferred to a climate chamber maintained at 5℃ (12-hour day length) to observe the growing condition.

As a result, this recombinant maize and the non-recombinant control maize both withered and died 35 days after transfer to the climate chamber, and no difference was observed between this recombinant maize and the non-recombinant control maize in the withering and death (Figure 6-2 of Annex 6).

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not re-grow and propagate vegetatively, or produce seeds. Actually, the growing condition was observed November 7, 2006, and this recombinant maize and the control maize were both found dead and no difference was observed in the death (Figure 7 of Annex 6).

(d) Fertility and size of the pollen

This recombinant maize and the non-recombinant control maize both exhibited high fertility of the pollen and no significant difference was observed between the both plants. In addition, also regarding the shape and size of pollen, no difference was observed between this recombinant maize and the non-recombinant control maize (Figures 8-1 and 8-2 of Annex 6).

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

Regarding seed production (grain number per ear, row number per ear, grain number per row, and 100-kernel weight), the differences between this recombinant maize and the non-recombinant control maize were examined and as a result, a statistically significant difference was observed in grain number per ear ($P=0.03$). However, no difference was observed in other items (Table 6 of Annex 6). The average value of grain number per ear, in which a significant difference was observed between this recombinant maize and the non-recombinant control maize, was 663.6 grains in this recombinant maize and 592.1 grains in the non-recombinant control maize (Table 6 of Annex 6).

As a result of comparison made between the minimum and maximum mean values of the non-recombinant maize used as control for the recombinant maize (MON863 line, MON810 line, NK603 line, DLL25 line, MON88001 line, MON88012 line, MON88017 line and LY038 line) subjected to isolated field tests and the variable ranges for conventional maize, the average value of grain number per ear in this recombinant maize, in which significant difference was observed, was found to fall within the variable ranges (549.2 to 728.6 grains) for conventional maize (Table 6 of Annex 6).

In both this recombinant maize and the non-recombinant control maize, seeds are covered with bracts at harvest time; therefore, shedding habits of the seed were not

observed under natural conditions. In addition, even after removal of bracts, the seeds from both this recombinant maize and the non-recombinant control maize were found hard to shed.

In order to identify the dormancy of harvested seeds, germination test was carried out for the seeds harvested from this recombinant maize and the non-recombinant control maize, which were sown in a total of 3 repeats with 60 grains per repeat. As a result, nearly all the seeds of this recombinant maize and the non-recombinant control maize became germinated within 5 days after sowing (Table 3 of Annex 6), and no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize in the final number of germinated plants (Table 4 of Annex 6). In this recombinant maize, only one grain failed to germinate, though this seed was found decayed due to fungul infection.

(f) Crossability

Crossability test was not performed for this recombinant maize since there is no report that any wild relatives that can be crossed with maize are growing voluntarily in Japan.

(g) Productivity of harmful substances

In order to investigate whether this recombinant maize might produce any harmful substances that could affect soil microflora, a soil microflora test was carried out.

As a result, no statistically significant difference was observed in the number of bacteria, actinomyces and filamentous fungi in the soil used for cultivation of this recombinant maize or the non-recombinant control maize (Table 7 of Annex 6).

In order to investigate whether the above-ground parts of this recombinant maize might produce any harmful substances that could affect the neighboring flora of plants, a plow-in test was carried out using the above-ground parts of the mature plants of this recombinant maize and the non-recombinant control maize.

As a result, no statistically significant difference was observed in the number of germinated plants, plant height and fresh weight of radish (test plant) sown in the soil into which the mature plants of this recombinant maize or the non-recombinant control maize were plowed (Table 8 of Annex 6).

In order to investigate whether the underground parts of this recombinant maize might produce any harmful substances that could affect the neighboring flora of plants, succeeding crop test was carried out.

As a result, no statistically significant difference was observed in the number of germinated plants, plant height and fresh weight of radish (test plant) sown in the soil collected at the time of harvesting of this recombinant maize and the non-recombinant control maize (Table 9 of Annex 6).

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

Maize (*Zea mays* subsp. *mays* (L.) Iltis), the species to which the recipient organism belongs, has been long cultivated and used in Japan, though there is no report that it has become self-seeding in a natural environment in Japan.

In the isolated field tests in Japan, regarding this recombinant maize, morphological and growth characteristics (19 items) and productivity of seeds (4 items) have been investigated. As a result, a significant difference from the non-recombinant control maize was observed only in the ear diameter for the morphological and growth characteristics and only in the grain number per ear for the productivity of seeds. However, the average values of these items were found to fall within the variable ranges for conventional maize as a result of comparison made between the minimum and maximum mean values of the non-recombinant maize used as a control in the past isolated field tests and the variable ranges for conventional maize; therefore it is considered unlikely that these differences could cause this recombinant maize to become competitive.

This recombinant maize is given the traits to be resistant to the insects of the order Lepidoptera due to the transferred *cry1A.105* gene and modified *cry2Ab2* gene. However, it is not generally considered that the insect damage by Lepidopteran insects is the major factor to inhibit the growth of maize under the natural environment in Japan; therefore it is hard to consider that these characteristics could enhance the competitiveness of this recombinant maize.

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 maize poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

2) Productivity of harmful substances

There has been no report that maize, the species to which the recipient organism belongs, produces any harmful substances that could affect wild animals and wild plants.

In the isolated field tests in Japan, this recombinant maize has been investigated for productivity of any harmful substances (the substances secreted from the roots which can affect other plants, the substances secreted from the roots which can affect

microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying) with the result that there is no significant difference observed from the non-recombinant control maize.

This recombinant maize produces the Cry1A.105 protein and the modified Cry2Ab2 protein that possess the insecticidal activity against the insects of the order Lepidoptera. This suggests that there is a possibility that the both proteins expressed in the pollens of this recombinant maize when cultivated could affect the Lepidopteran insects listed in the Red Data Book published by the Ministry of the Environment (2006 edition). However, based on the investigation on the extent of pollen dispersion around the cultivation fields, the degree of the effects, if present, is limited; therefore it is considered extremely low that the pollens dispersed from this recombinant maize could affect wild animals and wild plants at levels of their individuals.

As a result of searching for homology with amino acid sequences, it has been confirmed that the Cry1A.105 protein and the modified Cry2Ab2 protein have no sequence which is structurally homologous with any known allergens.

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 maize poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

3) Crossability

In the Japanese natural environment, there are no wild plants which can cross with maize. Therefore, it was judged that there are no specific wild plants that are possibly affected by this recombinant maize, and that the use of such maize poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by applicant 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 recombinant maize 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.

[Bibliography]