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

Name: Du Pont Kabushiki Kaisha
Minoru Amoh, President
Address: 2-11-1, Nagata-chou, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides (modified *cry1F, cry1Ab, cry34Ab1, cry35Ab1, pat*, modified *cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (1507×59122×MON810×NK603, OECD UI: DAS-01507-1 × DAS-59122-7 × MON-00810-6 × MON-00603-6) ([including the progeny lines isolated from the maize lines, *B.t.* Cry1F maize line 1507, *B.t.* Cry34/35Ab1 Event DAS-59122-7, MON810 and NK603, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type 1 Use Regulation)]
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	-

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides (modified *cry1F, cry1Ab, cry34Ab1, cry35Ab1, pat*, modified *cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (1507×59122×MON810×NK603, OECD UI: DAS-01507-1×DAS-59122-7×MON-00810-6×MON-00603-6) (hereinafter referred to as "this stack maize line") is a cross progeny line developed by crossing the following four (4) recombinant maize lines, using the traditional crossbreeding method.

This stack maize line will be commercialized as a hybrid variety (F1), and the grain harvested from this stack maize line is composed of combinations of the transferred genes in the individual parent lines of this stack maize line due to the genetic segregation.

(a) 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-01507-1) (hereinafter referred to as "DAS-01507-1")
(b) Maize resistant to Coleoptera and tolerant to glufosinate herbicide (*cry34Ab1, cry35Ab1, pat, Zea mays* subsp. *mays* (L.) Iltis) (*B.t.* Cry34/35Ab1 Event DAS-59122-7, OECD UI: DAS-59122-7) (hereinafter referred to as "DAS-59122-7")
(c) Maize resistant to Lepidoptera (*cry1Ab, Zea mays* L.) (MON810, OECD UI: MON-00810-6) (hereinafter referred to as "MON-00810-6")
(d) Maize tolerant to glyphosate herbicide (*cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (NK603, OECD UI: MON-00603-6) (hereinafter referred to as "MON-00603-6")

For the parent lines of this stack maize line, the DAS-01507-1 (http://www.bch.biodic.go.jp/download/lmo/public_comment/1507ap.pdf) and the DAS-59122-7 (http://www.bch.biodic.go.jp/download/lmo/public_comment/DAS59122-7ap.pdf) were developed jointly by US Dow Agro-Science and US Pioneer Hybrid International, and the MON-00810-6 (http://www.bch.biodic.go.jp/download/lmo/public_comment/MON810ap.pdf and

USDA (1996)) and the MON-00603-6 (http://www.bch.biodic.go.jp/download/lmo/public_comment/NK603ap.pdf and USDA (2000)) were developed by US Monsanto. In the individual parent lines, the following genes are transferred.

DAS-01507-1:	The modified *cry1F* gene to confer resistance to the insects of order Lepidoptera and the *pat* gene to confer tolerance to glufosinate herbicide
DAS-59122-7:	The *cry34Ab1* gene and the *cry35Ab1* gene to confer the resistance to Coleoptera, and the *pat* gene to confer the tolerance to glufosinate herbicide
MON-00810-6:	The *cry1Ab* gene to confer resistance to the insects of order Lepidoptera
MON-00603-6:	The modified *cp4 epsps* gene to confer tolerance to glyphosate herbicide

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of parent lines are shown individually in Table 1 (p. 4) to Table 4 (p. 8).

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid are shown individually in Table 1 (p. 4) to Table 4 (p. 8).

Table 1 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of DAS-01507-1

Component elements	Size (kbp)	Origin and function
Modified *cry1F* gene expression cassette		
UBIZM1(2) Promoter	1.98	Ubitiquin constitutive promoter* derived from *Z. mays* (including intron and 5' untranslated region).
Modified *cry1F*	1.82	A gene that encodes the modified Cry1F protein derived from *Bacillus thuringiensis* var. *aizawai*. It has the nucleotide sequence modified to enhance its expression level in plants. The 604th phenylalanine amino acid sequence is substituted by leucine.
ORF25PolyA Terminator	0.72	A terminator from *Agrobacterium tumefaciens* pTi5955 to terminate transcription.
pat gene expression cassette		
CAMV35S Promoter	0.53	35S constitutive promoter from the cauliflower mosaic virus (CaMV).
pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *Streptomyces viridochromogenes*. It has the nucleotide sequence modified to enhance its expression level in plants. The amino acid sequence expressed by the modification remains unchanged.
CAMV35S Terminator	0.21	35S terminator to terminate transcription from cauliflower mosaic virus (CaMV).

* Constitutive promoter: A promoter that drives the expression of target genes in all sites in plant body

Table 2 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of DAS-59122-7

Component elements	Size (kbp)	Origin and function
cry34Ab1 gene expression cassette		
UBI1ZM PRO	1.98	Ubitiquin constitutive promoter derived from *Z. mays* (including intron and 5' untranslated region).
cry34Ab1	0.37	The gene that encodes the Cry34Ab1 protein derived from *B. thuringiensis* PS149B1 strain.
PIN II TERM	0.32	Protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum.*
cry35Ab1 gene expression cassette		
TA Peroxidase PRO	1.30	Peroxidase promoter (nucleotide sequence 45-1342 of GenBank X53675) derived from *Triticum aestivum*. Constitutive promoter.
cry35Ab1	1.15	A gene that encodes the Cry35Ab1 protein derived from *B. thuringiensis* PS149B1 strain.
PIN II TERM	0.32	Protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum.*
pat gene expression cassette		
35S PRO	0.53	35S constitutive promoter derived from cauliflower mosaic virus (CaMV).
pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *S. viridochromogenes.*
35S TERM	0.21	35S terminator to terminate transcription from cauliflower mosaic virus (CaMV).

Table 3 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of MON-00810-6

Component elements	Size (kbp)	Origin and function
cry1Ab gene expression cassette		
E35S	0.61	35S promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). This promoter makes the target gene expressed in all the tissues constitutively.
hsp70 intron	0.80	Intron of heat stress protein (heat shock protein) gene from maize. hsp70 intron is used to enhance the expression of foreign genes in plants.
cry1Ab	3.47	The gene that encodes the Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain existing in the soil.
NOS 3'	0.22	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
modified *cp4 epsps* gene expression cassette (This was not transfered to MON-00810-6 according to analysis on transferred genes.)		
E35S	0.61	35S promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). This promoter makes the target gene expressed in all the tissues constitutively.
hsp70 intron	0.8	Intron of heat stress protein (heat shock protein) gene from maize. hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 2	0.31	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
modified *cp4 epsps*	1.4	A synthetic sequence generated based on the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium*. It expresses the modified CP4 EPSPS protein that possesses high tolerance to glyphosate.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
gox gene expression cassette (This was not transferred to MON-00810-6 according to analysis on transferred genes.)		
E35S	0.61	35S promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). This promoter makes the target gene expressed in all the tissues constitutively.
hsp70 intron	0.80	Intron of heat stress protein (heat shock protein) gene from maize. hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 1	0.16	N-terminal chloroplast transit peptide sequence of the small subunit 1A of rubisco gene derived from *A. thaliana*. Transfers target proteins from cytoplasm to chloroplast.
gox	1.3	A synthetic sequence based on the glyphosate oxidoreductase

		(*gox*) of *Achromobacter* sp. strain LBAA. GOX protein degrades glyphosate.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene from *Agrobacterium tumefaciens*. Contains transcription terminator and polyadenylation signal for mRNA.

Table 4 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of MON-00603-6

Component elements	Size (kbp)	Origin and function
modified *cp4 epsps* gene expression cassette (1)		
P-ract1	0.9	Promoter region of actin 1 gene derived from rice. It makes target genes expressed constitutively.
ract1 intron	0.5	Rice actin gene intron. Activates the expression of target genes by enhancing the splicing efficiency.
CTP 2	0.2	N-terminal chloroplast transit peptide sequence in the EPSPS protein, derived from the *Arabidopsis thaliana epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
modified *cp4 epsps*	1.4	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived from *Agrobacterium* strain CP4.
NOS 3'	0.3	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
modified *cp4 epsps* gene expression cassette (2)		
E35S	0.6	35S promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). This promoter makes the target gene expressed in all the tissues constitutively.
ZmHsp70 Intron	0.8	Intron of heat stress protein (heat shock protein) gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
CTP2	0.23	N-terminal chloroplast transit peptide sequence in the EPSPS protein, derived from the *Arabidopsis thaliana epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
modified *cp4 epsps*	1.37	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived from *Agrobacterium* strain CP4.
NOS 3'	0.25	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.

(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

a. Functions of proteins produced by the expression of target genes

Bt protein:
The Bt protein including the modified Cry1F protein, the Cry1Ab protein, the Cry34Ab1 protein and the Cry35Ab1 protein generally binds to specific receptors in the midgut cells of pest insects to form pores in the cells, thereby destructing the midgut cells and resultantly exhibiting insecticidal activity (Schnepf *et al*., 1998). The Bt protein possesses specificity against the target insect fauna for insecticide (Shirai, 2003).

Modified Cry1F protein:
The modified Cry1F protein is a kind of insecticidal crystal protein (Bt protein) known as a δ-endotoxin produced by *B. thuringiensis* var. *aizawai*.

The modified Cry1F protein exhibits a high insecticidal activity against European corn borer (*Ostrinia nubilalis*), Fall armyworm (*Spodoptera frugiperda*), Beet armyworm (*Spodoptera exigua*) and other Lepidopteran insects, but it has been confirmed to exhibit no toxicity against any non-target organisms other than the Lepidopteran insects, including the mammals, birds, fishes, and Coleopteran, Hymenopteran, Neuropteran, Collembolan and other insects (EPA, 2005).

Cry1Ab protein:
The Cry1Ab protein is a kind of Bt proteins, derived from *B. thuringiensis* subsp. *kurstaki*. This protein functions similarly as the modified Cry1F protein, except that it binds to different receptors in the midgut cells of pest insects from those to which the modified Cry1F protein binds (Hua *et al.,* 2001).

The Cry1Ab protein shows an insecticidal activity against European corn borer, Southwestern corn borer (*Diatraea grandiosella*), Southern cornstalk borer (*Diatraea crambidoides*), Sugarcane cornstalk borer (*Diatraea saccharalis*), Corn earworm (*Helicoverpa zea*), Fall armyworm, and Stalk borer (*Papaipema nebris*), the pest insects of order Lepidoptera, but it has been confirmed to exhibit no toxicity against any non-target organisms other than the Lepidopteran insects, including the mammals, birds, and Coleopteran, Hymenopteran, Neuropteran and other insects (USDA, 1995).

Insecticidal effects of simultaneous expression of the modified Cry1F protein and the Cry1Ab protein on the pest insects of order Lepidoptera:
The modified Cry1F protein and the Cry1Ab protein both possess the insecticidal activity against pest insects of the order Lepidoptera. It is generally known that continual use of any one kind of insecticide can cause occurrence of insecticide-resistant insect pests. However, the receptors in the midgut cells, to which the modified Cry1F protein and the Cry1Ab protein expressed in this stack maize line bind, vary (Hua *et al.,* 2001). Therefore, it is considered low that pest insects of the order Lepidoptera acquire the resistance to these two proteins at the same time. This raises expectations that possible occurrence of insecticide-resistant insect pests would be suppressed compared to the case in which insect pests resistant to only one kind of protein could occur.

Cry34Ab1 protein and Cry35Ab1 protein:
The Cry34Ab1 protein and the Cry35Ab1 protein are a kind of Bt protein derived from *B. thuringiensis* PS149B1 strain. The Cry34Ab1 protein possesses the insecticidal activity against Corn rootworm (*Diabrotica spp.*), though the Cry35Ab1 protein does not exhibit any insecticidal activity by itself. When the both proteins are set to work in concert with each other, a maximum of about 8-times higher insecticidal acidity compared to single use of the Cry34Ab1 protein is attained. The mechanism of action is considered such that the Cry34Ab1 protein acts as the pore-forming protein against the phospholipid membrane, and the Cry35Ab1 protein enlarges the pores and increases the permeability through the membrane.

The Cry34Ab1 protein and the Cry35Ab1 protein specifically exhibit the insecticidal activity against the larvae of two kinds of insect pests, Northern corn rootworm (*Diabrotica barberi*) and Western corn rootworm (*Diabrotica virgifera virgifera*) classified as insects of the order Coleoptera, and they exhibit no toxicity against any non-target organisms other than the Coleopteran insects, including the mammals, birds, fishes, and Lepidopteran, Hymenopteran, Neuropteran, Hemiptera and other insects (EPA, 2005b).

PAT protein:
The glufosinate herbicide inhibits the activity of glutamine synthase due to the *L*-glufosinate, an active ingredient, which causes the ammonia, the substrate, to be accumulated in the plant body and causes the plant to die. The PAT protein acetylates the *L*-glufosinate to make it nontoxic, thereby conferring glufosinate herbicide tolerance to the plant.

Modified CP4 EPSPS protein:
The glyphosate herbicide inhibits the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19) in the shikimate pathway, an aromatic amino acid biosynthesis pathway in plant body, which interrupts the synthesis of aromatic amino acids in plants, causing the plant to die. The modified CP4 EPSPS protein exhibits the activity even in the presence of glyphosate, preventing the shikimate pathway from being inhibited, thereby conferring glyphosate herbicide tolerance to the plant.

b. The fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

As a result of the amino acid sequence homology search in 2010 using the database (AD_2010, TOX_2010 and PRT_2010 for the modified CP4 EPSPS protein, and FARRP10 for the other proteins) to investigate whether the modified Cry1F protein, the Cry1Ab protein, the Cry34Ab1 protein, the Cry35Ab1 protein, the PAT protein and the modified CP4 EPSPS protein share any structurally related homologous sequences with any of the known allergenic proteins, it has been confirmed that the proteins do not share structurally related homologous sequences with any of the known allergenic proteins.

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

For the possibility of changing the metabolic system of recipient organism, individual proteins expressed in this stack maize line were examined.

<u>Cry protein</u>
There is no report that the modified Cry1F protein, the Cry1Ab protein, the Cry34Ab1 protein and the Cry35Ab1 protein possess any enzyme activity.

<u>PAT protein</u>
The PAT protein possesses substrate specificity and catalyzes the reaction to acetylate free amino groups of *L*-glufosinate, an active ingredient of glufosinate herbicide, though there are no other amino acids and *D*-glufosinate reported for the substrate of the PAT protein (OECD, 1999).

<u>Modified CP4 EPSPS protein</u>
The EPSPS is not a rate-determining enzyme in the shikimate pathway for the biosynthesis of aromatic amino acids, and as such it is not considered that any enhanced EPSPS activity due to the production of the modified CP4 EPSPS protein will increase the concentration of aromatic amino acids, the end products of this pathway. In fact, it has been confirmed that there is no

difference regarding the aromatic amino acid content between the genetically modified crops for tolerance to glyphosate herbicide (soybean, oilseed rape, cotton, and maize) and the original non-recombinant crops (http://www.bch.biodic.go.jp/download/lmo/public_comment/NK603ap.pdf) .

EPSPS specifically reacts with the substrates, phosphoenolpyruvic acid (PEP) and shikimate-3-phosphate (S3P). It is also known to react with the shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living body (http://www.bch.biodic.go.jp/download/lmo/public_comment/NK603ap.pdf) .

(2) Information concerning vectors

1) Name and origin

The plasmid vectors used for the development of the parent lines are as follows.

DAS-01507-1: Plasmid PHP8999 constructed based on the plasmid pUC19 derived from *Escherichia coli* (*E. coli*) (Figure 1, p.14).

DAS-59122-7: Plasmid PHP17662 constructed based on the plasmid pSB1 of *Agrobacterium* (*A. tumefaciens*) (Figure 2, p15).

MON-00810-6: Plasmids PV-ZMBK07 and PV-ZMGT10 constructed based on the plasmid pUC119 derived from *Escherichia coli* (*E. coli*) (Figure 3, p.16).

MON-00603-6: Plasmid PV-ZMGT32 constructed based on the plasmid pUC119 derived from *Escherichia coli* (*E. coli*) (Figure 4, p.17).

2) Properties

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

The total number of base pairs in the plasmid vectors used for the development of parent lines is as follows.

DAS-01507-1(PHP8999):	9,504 bp
DAS-59122-7 (PHP17662):	50,321 bp
MON-00810-6:	7,800 bp for PV-ZMBK07, 9,447 bp for PV-ZMGT10
MON-00603-6(PV-ZMGT32):	9,308 bp

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

The following genes were used as selectable markers for selection of parent lines. None of these marker genes have been transferred in the parent lines.

DAS-01507-1:	The *nptII* gene to confer the resistance to kanamycin and neomycin
DAS-59122-7:	The gene (*tet* gene) to confer the resistance to tetracycline, and the gene (*spc* gene) to confer the resistance to spectinomycin
MON-00810-6:	Partial coding sequence (*LacZ* gene) for β-D-galactosidase (LacZ protein) and the *nptII* gene to confer the resistance to kanamycin and neomycin
MON-00603-6:	The *nptII* gene to confer the resistance to kanamycin and neomycin

(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

Neither these vectors is known to be infectious.

(3) Method of preparing living modified organisms

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

Composition of the donor nucleic acid used for the development of the parent lines DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, and the positions and sites cleaved by restriction enzymes are shown in Figure 1 to Figure 4 (p.14 – p.17).

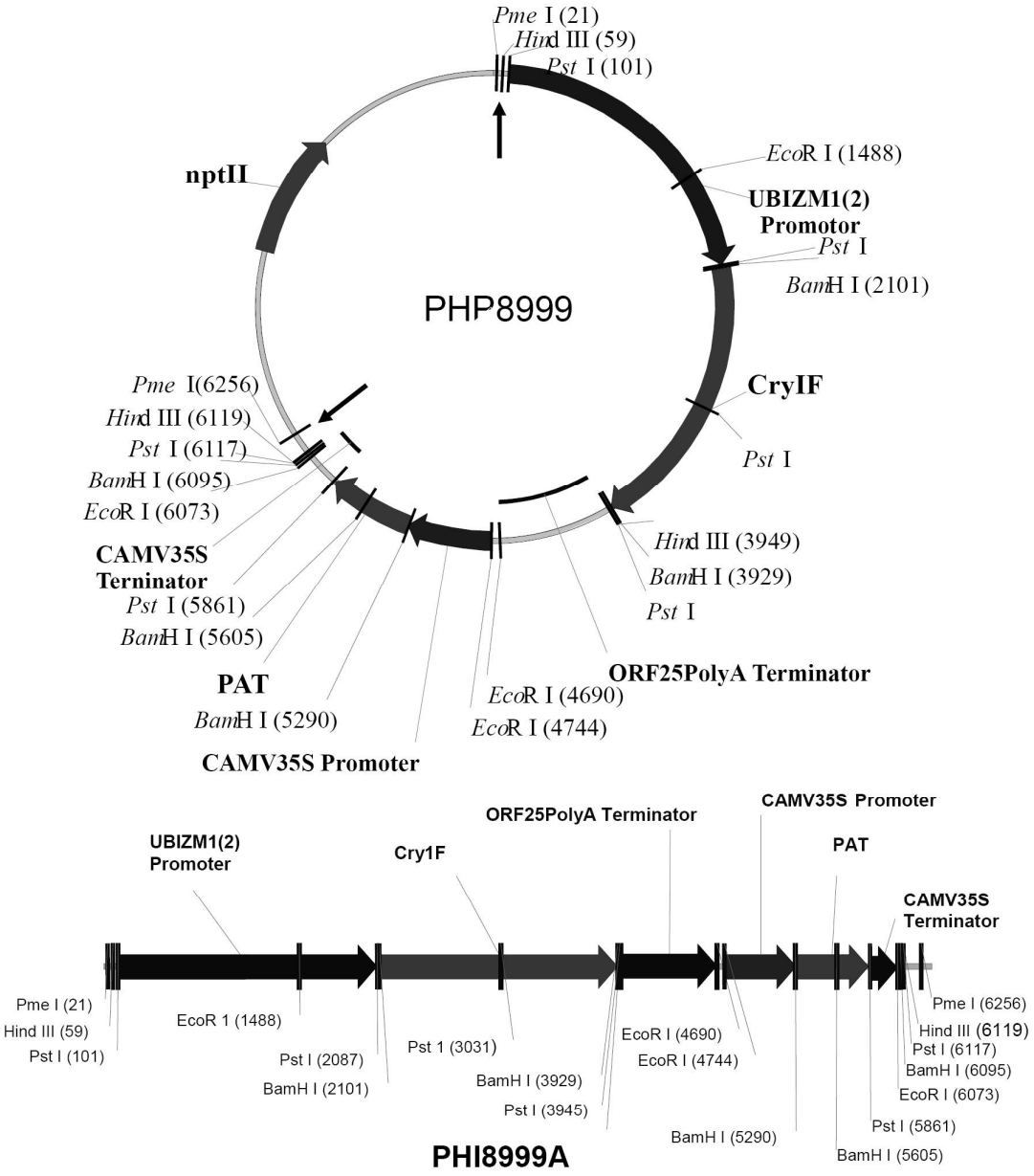

Figure 1 Composition of the plasmid PHP8999* (top) and the transferred DNA region PHI8999A (bottom)

* The vector used for the development of DAS-01507-1

The plasmid PHP8999 was treated by the restriction enzyme *Pme* I (cleaved at the two sites indicated by arrows in the top diagram) to prepare the linear DNA fragment PHI8999A (bottom diagram), which was in turn used for transferring genes into the recipient organism.

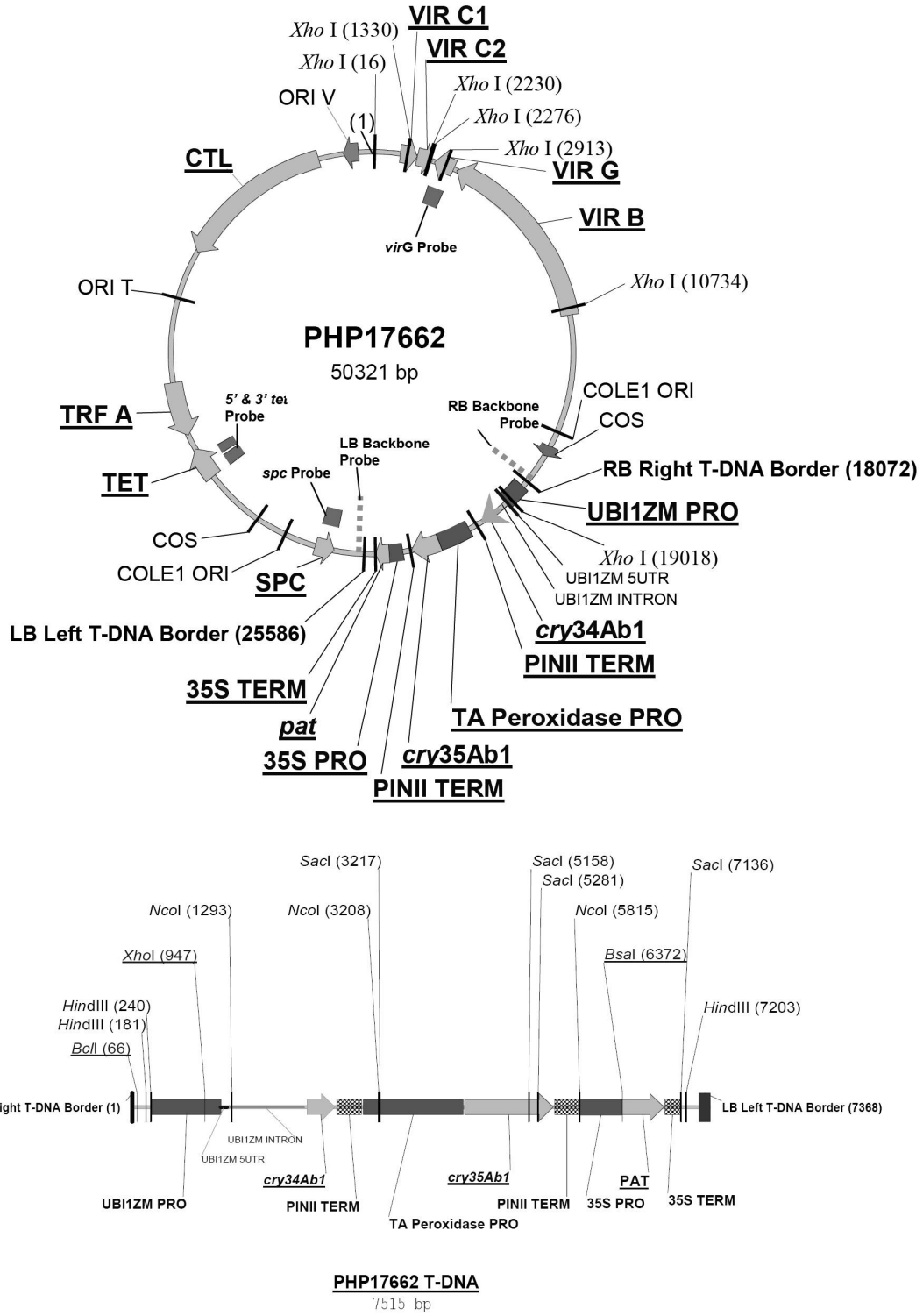

Figure 2 Composition of the plasmid PHP17662* and T-DNA region

* The vector used for the development of DAS-59122-7

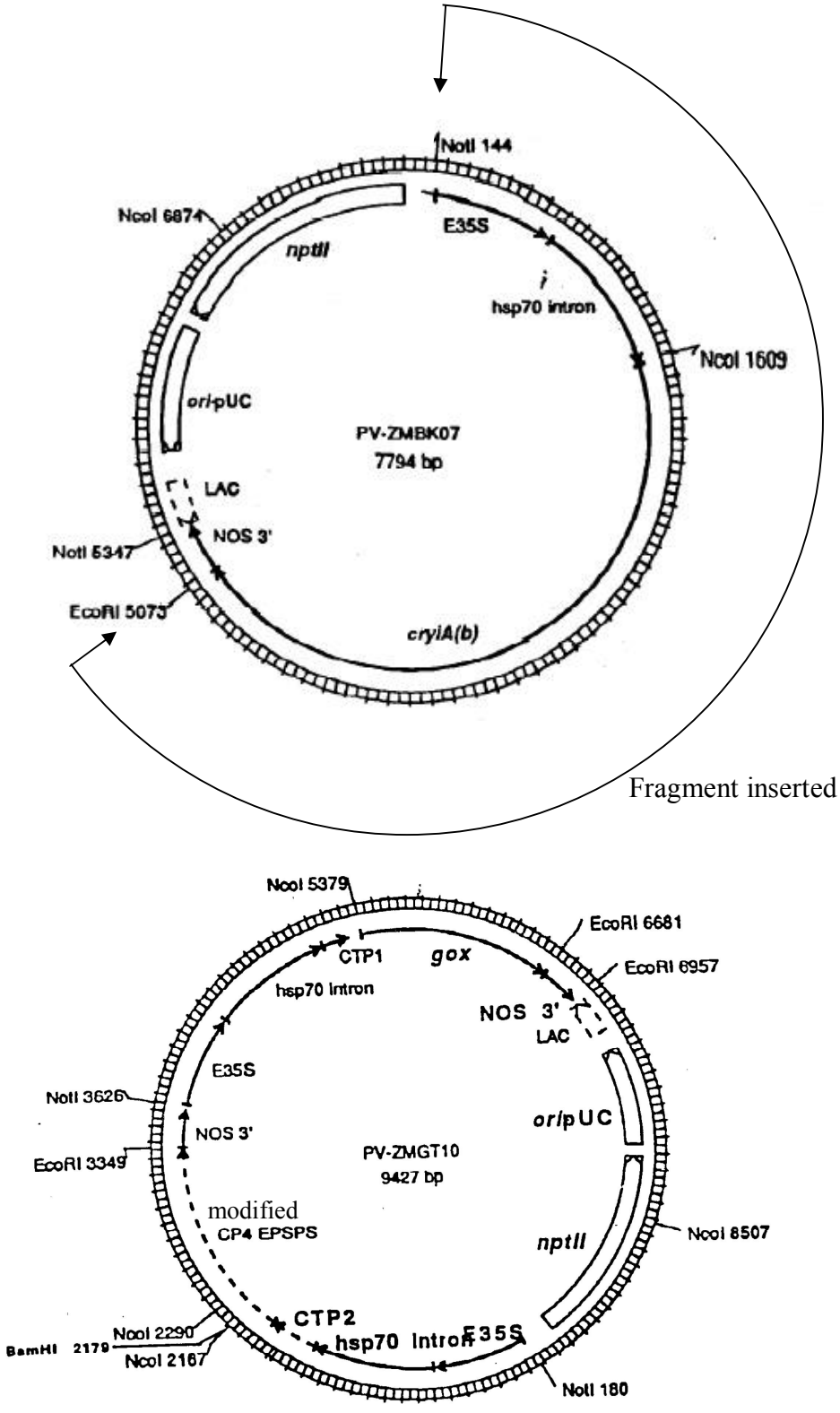

Figure 3 Composition of the plasmids PV-ZMBK07 and PV-ZMGT10*

* The vectors used for the development of MON-00810-6
Actually transferred in the recipient organism is the "Fragment inserted" region.

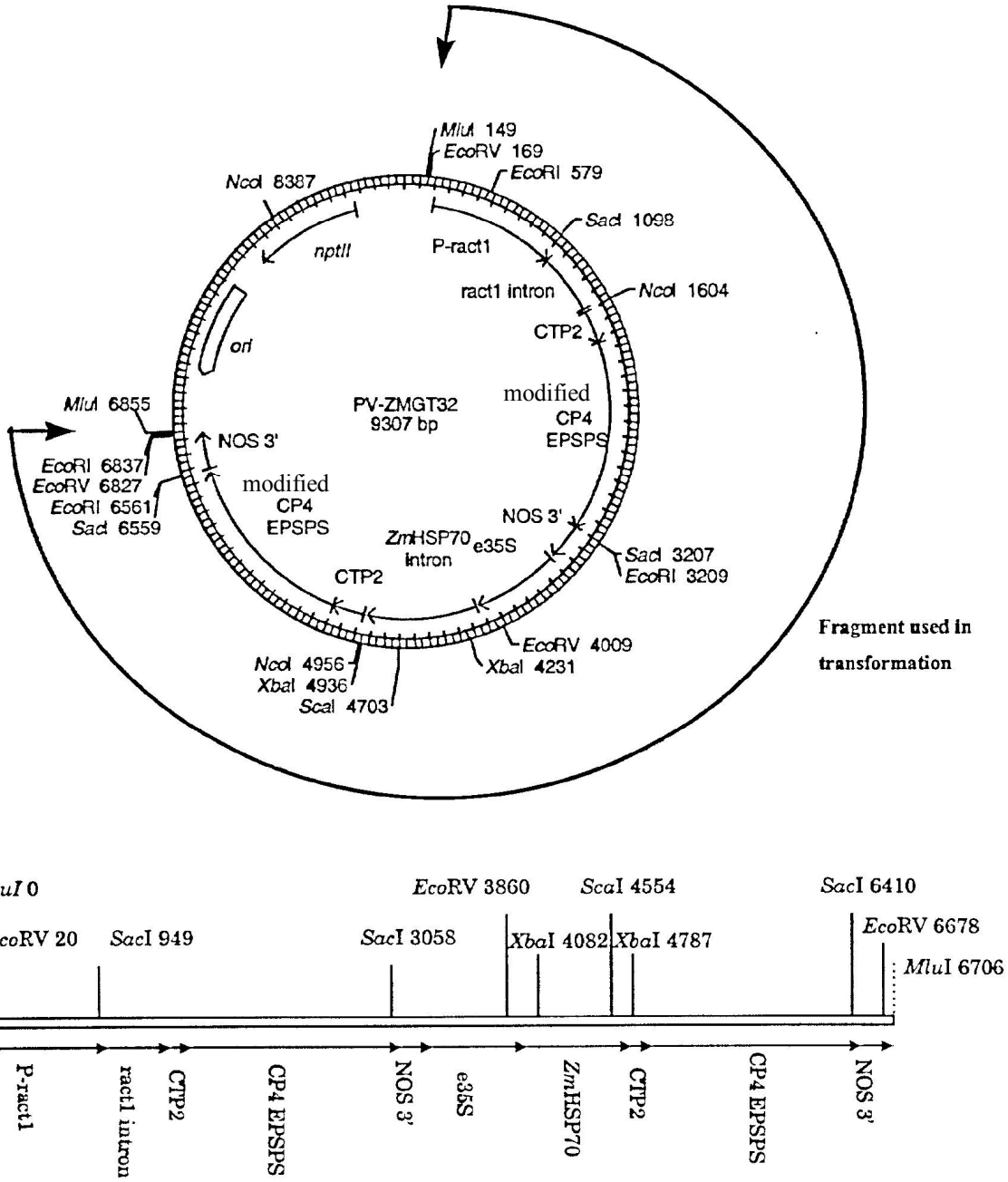

Figure 4 Composition of the plasmid PV-ZMGT32* (top) and the transferred DNA region PV-ZMGT32L (bottom)

* The vector used for the development of MON-00603-6

The plasmid PV-ZMGT32 was treated by the restriction enzyme *Mlu*I (cleaved at the two sites indicated by arrows in the top diagram) to prepare the linear DNA fragment PV-ZMGT32L (bottom diagram), which was in turn used for transferring genes into the recipient organism.

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

Transferring nucleic acid into the recipient organism was based on the particle gun bombardment for DAS-01507-1, MON-00810-6 and MON-00603-6, and the *Agrobacterium* method for DAS-59122-7.

3) Processes of rearing of living modified organisms

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

Selection of nucleic acid-transferred cells was made based on the culture on the medium containing the substances listed below.
DAS-01507-1 and DAS-59122-7: Glufosinate
MON-00810-6 and MON-00603-6: Glyphosate

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

For DAS-59122-7 developed based on the *Agrobacterium* method, *Agrobacterium* was removed by addition of Carbenicillin to the culture cell medium described in the above (a).

(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

This stack maize line was developed by cross-breeding with use of DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6. The process is shown in Figure 5 (p.19; Confidential: Not disclosed to unauthorized person). In addition, the status of approval of these parent lines in Japan is listed in Table 5 (p.19).

(Not made available or disclosed to unauthorized person)

Figure 5 Process of rearing of this stack maize line

5

Table 5 Status of approval of the parent lines and this stack maize line in Japan

Line	Food	Feed	Environment
DAS-01507-1	2002	2003	2005
DAS-59122-7	2005	2006	2006
MON-00810-6	2001	2003	2004
MON-00603-6	2001	2003	2004
This stack maize line	Pending application	2009	2010: Submitted

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

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

It has been confirmed that the traits from DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6 are transferred in accordance with the Mendel's law and that the replication product of transferred nucleic acid exists on the maize genome chromosome.

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

DAS-01507-1:
As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy each of the modified *cry1F* gene expression cassette and the *pat* gene expression cassette is transferred in the maize genome and that the transferred genes are inherited stably in offspring.

As a result of the nucleotide sequence analysis of transferred DNA, it was confirmed that the transferred DNA contained a part of the modified *cry1F* gene sequence in the 5'-terminal region, a part of the *pat* gene sequence in the 5'-terminal and 3'-terminal regions, and a part of the *ORF25PolyA Terminator* sequence in the 3'-terminal region. However, Northern blotting analysis confirmed that these gene fragments were not transcribed into mRNA, thereby not functioning.

DAS-59122-7:
As a result of Southern blotting analysis, it was confirmed that one copy of each of the *cry34Ab1* gene expression cassette, the *cry35Ab1* gene expression cassette, and the *pat* gene expression cassette are transferred in the maize genome and then it was confirmed that respective genes are stably inherited in offspring.

MON-00810-6:
As a result of Southern blotting analysis of transferred genes, it was confirmed that the genome of the maize contained a transferred copy of a DNA fragment essential for the expression of the *cry1Ab* gene derived from PV-ZMBK07 and that the transferred genes are inherited stably in offspring.

Southern blotting analysis indicated that only a region essential for the expression of the Cry1Ab protein derived from PV-ZMBK07 was transferred into the maize genome, and the *nptII* gene and the modified *CP4 EPSPS* gene derived from PV-ZMGT10 as well as the *gox* gene expression cassette were not.

For the reasons why the modified *CP4 EPSPS* gene was found not transferred based on the Southern blotting analysis in spite of the fact that the nucleic acid-transferred cells were selected on the medium containing glyphosate (I. 2. (3). 3) (a), p.18), it was considered likely that segregation of the transferred genes might take place in the following generation of regenerated individuals (Outline of the Biological Diversity Risk Assessment Report, 2004).

MON-00603-6:
As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy of PV-ZMGT32L (composed of two (2) modified *cp4 epsps* gene expression cassettes) is transferred in the maize genome and that the transferred genes are inherited stably in offspring.

It was found that a 217bp fragment of *P-ract1* was transferred in the 3'-terminal neighborhood of the transferred gene in the reverse direction, though this fragment was confirmed based on the Western blotting analysis not to take part in the production of any new protein. In addition, the base in the modified *cp4 epsps* gene induced by the *E35S* was changed during the development of MON-00603-6, and an amino acid forming the modified CP4 EPSPS protein was changed. However, based on the findings that this amino acid is not included in the seven amino acids essential for activating the EPSPS protein family, this change of amino acid does not affect the active site of the protein and three-dimensional structure, and the traits of enzyme activity and immune response are substantially comparable to those of the original protein, the structure and function of the protein is considered to remain unchanged (to be substantially comparable).

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

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

The stability of expression of the parent lines of this stack maize line was identified as follows.

DAS-01507-1: Confirming the expression of the modified Cry1F protein and the PAT protein by ELISA method, the bioassay for resistance to pest insects of the order Lepidoptera, and glufosinate herbicide-spraying test

DAS-59122-7: Confirming the expression of the Cry34Ab1 protein, the Cry35Ab1 protein and the PAT protein by ELISA method, the bioassay for resistance to pest insects of the order Coleoptera, and glufosinate herbicide-spraying test

MON-00810-6: Bioassay for resistance to pest insects of the order Lepidoptera
MON-00603-6 Glyphosate herbicide-spraying test

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

The transferred nucleic acid does not contain any sequence allowing transmission and thus, there is no possibility that the nucleic acid transferred to the maize lines could be transmitted to any other wild animals and wild plants through virus infection and/or other routes.

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

Method of detection:
Specific detection method for individual parent lines (DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6) based on the real-time quantitative PCR analysis is available from the Web site of European Commission (Joint Research Centre, 2005 to 2007).

Sensitivity:
The detection sensitivity is 0.1% for all of the parent lines DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6.

Reliability:
For the method for detection of DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, the reliability has been verified as a result of tests in 2, 4, 3 and 2 repeats at 14, 14, 14 and 12 member test laboratories of the European Network of GMO Laboratories, respectively.

For the detection and identification of this stack maize line, the above-mentioned methods must be applied to each grain of maize seeds or each individual of plant body.

(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

This stack maize line is given the traits to be resistant to Lepidoptera due to the modified *cry1F* gene derived from DAS-01507-1 and the *cry1Ab* gene derived from MON-00810-6, resistant to Coleoptera due to the *cry34/35Ab1* gene derived

from DAS-59122-7, tolerant to glufosinate herbicide due to the *pat* gene derived from DAS-01507-1 and DAS-59122-7, and tolerant to glyphosate herbicide due to the modified *cp4 epsps* gene derived from MON-00603-6.

For the possibility of functional interaction between the proteins produced by the transferred genes, examination was conducted from the three (3) viewpoints, between the pest insects-resistant proteins, between the herbicide-tolerant proteins, and between the pest insect-resistant protein and the herbicide-tolerant protein.

Functional interaction between the pest insect-resistant proteins

The modified Cry1F protein and the Cry1Ab protein exhibit insecticidal activity against several kinds of Lepidopteran insects, including European corn borer (EPA, 2005a ; USDA, 1996), and the Cry34Ab1/Cry35Ab1 protein exhibits insecticidal activity against several kinds of Coleopteran insects, including Western corn rootworm (EPA, 2005b). The modified Cry1F protein and the Cry1Ab protein exhibit no insecticidal activity against pest insects of the order Coleoptera against which the Cry34Ab1/Cry35Ab1 protein exhibit the insecticidal activity (EPA, 2005b). Similarly, the Cry34Ab1/Cry35Ab1 protein exhibits no insecticidal activity against pest insects of the Lepidoptera against which the modified Cry1F protein and the Cry1Ab protein exhibit the insecticidal activity (Ellis *et al.,* 2002).

The modified Cry1F protein and the Cry1Ab protein both exhibit insecticidal activity against pest insects of the order Lepidoptera, though they specifically bind to different receptors in the midgut cells of the pest insects (Hua *et al.,* 2001). This suggests that the specificity of insecticidal activity given to the pest insect-resistant proteins depends on the structure of protein and thus the insecticidal effects on the target insects remain unaffected unless there is any change to the regions associated with the specificity.

In addition, there is no report that in the previously approved stack lines, any pest insect-resistant proteins have exhibited synergy effects. Consequently, it is considered unlikely that synergy effect or antagonism due to the interaction in terms of function would take place in this stack maize line even though the insecticidal activity of individual parent lines could be additively enhanced.

Functional interaction between the herbicide-tolerant proteins

The *L*-glufosinate, an active ingredient of glufosinate herbicide, inhibits the activity of glutamine synthase. The PAT protein possesses high substrate specificity and the *L*-glufosinate acts as the substrate, though *D*-glufosinate and

other amino acids do not act as the substrate (OECD, 1999). On the other hand, the modified CP4 EPSPS protein possesses the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) in the shikimate pathway for biosynthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine). Possible substrates for the EPSPS are limited to phosphoenolpyruvate (PEP), shikimate-3-phosphate (S3P) and shikimate. As mentioned above, the PAT protein and the modified CP4 EPSPS protein differ from each other in the substrate and the mechanism of action, and their involved metabolic pathways are also independent from each other; therefore it is considered unlikely that any unexpected metabolites would be produced.

Functional interaction between the pest insect-resistant proteins and herbicide-tolerant proteins

The pest insect-resistant proteins and the herbicide-tolerant proteins differ from each other in the available function and then, they are considered unlikely to interact each other. In addition, there is no report that in the previously approved stack lines, the pest insect-resistant proteins and the herbicide-tolerant proteins have interacted with each other.

In practice, in order to confirm whether the proteins expressed in this stack maize line from individual parent lines interact with each other in terms of the resistance to European corn borer, the pest insect of the order Lepidoptera, resistance to Western corn rootworm, the pest insect of the order Coleoptera, and tolerance to glufosinate and glyphosate herbicides, examination was conducted using this stack maize line, the parent lines and the non-recombinant control maize.

Resistance to European corn borer (*Ostrinia nubilalis*)

On the leaves of this stack maize line, parent lines (DAS-01507-1 and MON-00810-6) and the non-recombinant control maize, larvae of European corn borer (*Ostrinia nubilalis*) were raised to observe the levels of feeding damage (Annex; Confidential: Not disclosed to unauthorized person). As a result, regarding the levels of feeding damage to leaves of this stack maize line by the larvae of European corn borer, no statistically significant difference from the two parent lines examined was observed (Table 6, p.24).

Table 6 Levels of feeding damage to leaves of this stack maize line by the larvae of European corn borer (*Ostrinia nubilalis*)

Samples tested	Levels of feeding damage
This stack maize line	0.0029 ± 0.0066 a
Control; Parent line DAS-01507-1	0.0046 ± 0.0081 a
Control; Parent line MON-00810-6	0.0097 ± 0.0162 a
(Reference) Non-recombinant control maize	0.3841 ± 0.1286 b

n=20, mean value ± standard deviation

Test conditions: Individual lines were cultivated in a greenhouse in the US from 2008 to 2009, and at the 4-leaf stage (V4 stage), leaves were collected and the larvae of European corn borer (*Ostrinia nubilalis*) immediately after hatching on the blades were raised for 48 hours. Tests were conducted for a total of 10 repeats with 2 samples per repeat.

Evaluation method: Levels of feeding damage were determined based on the creation of digital images by blade. The feeding damage-free tissue was represented by the number of pixels, and the number of pixels was divided by the mean number of pixels over all control blades (left out from feeding by larvae) to obtain the values. The values were subtracted from unity (1) to determine the levels of feeding damage (0 to 1). The inverse sine transformed values of the determined levels of feeding damage were used for statistical analysis.

Different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (analysis of variance using the inverse sine transformed values and paired comparison based on the Tukey's Test, $P<0.05$).

Resistance to Western corn rootworm

For this stack maize line, parent line DAS-59122-7 and the non-recombinant control maize, the levels of feeding damage to roots by Western corn rootworm was observed (Annex; Confidential: Not disclosed to unauthorized person). As a result, regarding the levels of feeding damage to roots of this stack maize line by Western corn rootworm, no statistically significant difference from the parent line examined was observed (Table 7, p25).

Table 7 Levels of feeding damage to root of this stack maize line by Western corn rootworm

Samples tested	Nodal Injury score (NIS)
This stack maize line	0.0295 ± 0.0231 a
Control: Parent line DAS-59122-7	0.0360 ± 0.0196 a
(Reference) Non-recombinant control maize	1.5960 ± 0.5347 b

n=20, mean value ± standard deviation

Test conditions: Individual lines were cultivated in greenhouse in the US from 2008 to 2009, and at the 3- to 4-leaf stage (V3 to V4 stage), the eggs of Western corn rootworm were inoculated to the roots. After hatching, the severity of feeding damage at the roots was observed. Tests were conducted for a total of 10 repeats with 2 samples per repeat.

Evaluation method: Score (0 to 3) was evaluated based on the Node Injury Scale (Oleson *et al.*, 2005). Scale 0: No damage, Scale 1: The roots for a node were damaged up to 5 cm from the stem, Scale 2: Two nodes were damaged, Scale 3: Three or more nodes were damaged.

Different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean vales (analysis of variance and paired comparison based on the Tukey's Test, $P<0.05$).

Tolerance to glufosinate herbicide and glyphosate herbicide

Glufosinate herbicide was sprayed to this stack maize line, parent lines (DAS-01507-1 and DAS-59122-7) and the non-recombinant control maize to observe the severity of herbicide injury (Annex; Confidential: Not disclosed to unauthorized person). In addition, glyphosate herbicide was sprayed to this stack maize line, parent line MON-00603-6 and the non-recombinant control maize to observe the severity of herbicide injury (Annex; Confidential: Not disclosed to unauthorized person). As a result, regarding the severity of herbicide injury to this stack maize line, no statistically significant difference from individual control parent lines was observed (Table 8 and Table 9, p.26 – p.27).

Table 8 Levels of herbicide injury to this stack maize line and parent lines by spraying of glufosinate herbicide

Samples tested	Levels of herbicide injury (%)			
	Not sprayed	Normal dosage[1)]	16-times higher dosage	32-times higher dosage
This stack maize line	0 ± 0	0 ± 0 a[2)]	3.89 ± 2.36 a	4.11 ± 3.42 a
Control; Parent line DAS-01507-1	0 ± 0	0 ± 0 a	3.44 ± 2.57 a	7.89 ± 5.28 a
Control: Parent line DAS-59122-7	0 ± 0	0 ± 0 a	2.77 ± 2.52 a	6.56 ± 6.81 a
(Reference) Non-recombinant control maize	0 ± 0	7.56 ± 4.48 b		

n=15, mean value ± standard deviation

Test conditions: Five (5) plants for each line were cultivated in a greenhouse in the US from 2008 to 2009 and at the 3-leaf stage (V3 stage) the herbicide was spayed. Tests were conducted for 3 repeats.

Evaluation method: On the 7th, 14th and 21st days after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis or bleaching) was visually evaluated based on the scale from 0% (intact) to 100% (complete death). Multiple tests were carried out based on the analysis of variance and Sidak method (Westfall *et al.,* 2006) by individual herbicide dosages (normal dosage, 16-times higher dosage and 32-times higher dosage).

1) Dosage tested includes normal dosage of 0.468 kg active ingredient (a.i.)/ha, 16-times higher dosage of 6.96 kg a.i./ha, and 32-times higher dosage of 15.5 kg a.i./ha. The spraying at the concentration of 16-times and 32-times higher dosages was intended for evaluation of levels of herbicide tolerance, and spraying of herbicides at such concentrations is not intended in the commercial cultivation.
2) Different alphabetical letters in a given column indicate that statistically significant difference ($P<0.05$) was observed between the relevant mean values.

Table 9 Levels of herbicide injury to this stack maize line and parent lines by spraying of glyphosate herbicide

Samples tested	Levels of herbicide injury (%)			
	Not sprayed	Normal dosage[1)]	16-times higher dosage	32-times higher dosage
This stack maize line	0 ± 0	0 ± 0 a[2)]	3.33 ± 2.38 a	4.56 ± 1.79 a
Control; Parent line MON-00603-6	0 ± 0	0.111±0.745a	4.11 ± 1.93 a	4.56 ± 1.79 a
(Reference) Non-recombinant control maize	0 ± 0	72.9 ±18.0 b		

n=15, mean value ± standard deviation

Test conditions: Five (5) plants for each line were cultivated in a greenhouse in the US from 2008 to 2009 and at the 3-leaf stage (V3 stage) the herbicide was spayed. Tests were conducted for 3 repeats.

Evaluation method: On the 7th, 14th and 21st days after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis or bleaching) was visually evaluated based on the scale from 0% (intact) to 100% (complete death). Multiple tests were carried out based on the analysis of variance and Sidak method (Westfall *et al.,* 2006) by individual herbicide dosages (normal dosage, 16-times higher dosage and 32-times higher dosage).

1) Dosage tested includes normal dosage of 1.143 kg acid equivalent (a.e.)/ha, 16-times higher dosage of 20.18 kg a.e./ha, and 32-times higher dosage of 36.4 kg a.e./ha. The spraying at the concentration of 16-times and 32-times higher dosages was intended for evaluation of levels of herbicide tolerance, and spraying of herbicides at such concentrations is not intended in the commercial cultivation.
2) Different alphabetical letters in a given column indicate that statistically significant difference ($P<0.05$) was observed between the relevant mean values.

Based on the above results, it was found that the traits given to the parent lines remain unchanged in this stack maize line and thus, it was considered unlikely that any interaction would take place due to the expression of the genes from parent lines in this stack maize line. Consequently, with regard to the differences in physiological or morphological characteristics between this stack maize line and maize, the taxonomic species to which the recipient organism belongs, the evaluation was conducted based on the results of the isolated field tests of the parent lines conducted in Japan (http://www.bch.biodic.go.jp/download/lmo/public_comment/1507ap.pdf, http://www.bch.biodic.go.jp/download/lmo/public_comment/DAS59122-7ap.pdf, http://www.bch.biodic.go.jp/download/lmo/public_comment/MON810ap.pdf and http://www.bch.biodic.go.jp/download/lmo/public_comment/NK603ap.pdf).

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

(a) Morphological and growth characteristics

For the morphological and growth characteristics, examination was conducted for the items listed in Table 10 (p.29), using the parent lines (DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6) and their non-recombinant control maize. As a result, regarding the germination rate and ear diameter of DAS-01507-1, culm length of DAS-59122-7, culm length of MON-00810-6, and 100-kernel weight of MON-00603-6, a statistically significant difference from the non-recombinant control maize was observed for one variety among the two varieties of recombinant maize examined, though no significant difference was observed for the rest one variety.

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

It has been confirmed that DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6 withered, wilted or died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control maize.

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it is not known to survive the winter. In addition, it does not re-grow and propagate vegetatively nor produce seeds after harvesting. Actually, DAS-01507-1 and DAS-59122-7 plant death was confirmed by observation at the time of harvesting after cultivation in a field in the US. Furthermore, also for MON-00810-6 and MON-00603-6, at the end of isolated field tests, the start of withering and death after ripening was observed.

(d) Fertility and size of the pollen

As a result of the observation under a microscope with stained pollens, no difference was observed in fertility, size or shape of the pollen between DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, and their non-recombinant control maize.

Table 10 Investigational results of morphological and growth characteristics

	DAS-01507-1	DAS-59122-7	MON-00810-6	MON-00603-6
Germination rate	○*	○	○	○
Uniformity of germination	○	○	○	○
Time of tasseling	○	○	○	○
Time of silking	○	○	○	○
Time of flower initiation	○	○	○	-
Time of flower completion	○	○	○	-
Flowering period	○	○	○	-
Maturation time	○	○	○	○
Plant shape or Plant type	○	○	○	○
Tiller number	○	○	○	○
Number of ears (Total number of ears)	○	-	○	○
Number of productive ears	○	○	○	-
Grain color and shape	○	○	○	○
Culm length	○	○*	○*	○
Height of ear	○	○	○	○
Ear length	○	○	○	○
Ear diameter	○*	○	○	○
Row number per ear	○	○	○	○
Grain number per row	○	○	○	○
100-kernel weight	○	○	○	○*
Weight of above-ground parts (Fresh weight, Plant weight)	○	○	○	○
Flower shape	-	○	-	-

○: Examined
-: Not examined
*: For one of the two varieties for a given event tested, a statistically significant difference from the relevant non-recombinant control maize was observed.

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

Production of the seed: In the investigation discussed in 2)-(a) (p.28), ear length, ear diameter, row number per ear, grain number per row and 100-kernel weight were examined. As a result, regarding the ear diameter of DAS-01507-1 and the 100-kernel weight of MON-00603-6, a statistically significant difference from the relevant non-recombinant control maize was observed for one of the two recombinant varieties examined, though no significant difference was observed for the rest one variety.

Shedding habit of the seed: There is a low possibility that maize seeds are spontaneously shed and dispersed, since the ears are covered with husks

(OECD, 2003). In DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, shedding habit in the natural conditions has not been observed.

Germination rate: As a result of examination on the germination rate of the seeds harvested from DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, no difference from their non-recombinant control maize has been confirmed.

Dormancy of the seed: Maize seeds reportedly show almost no dormancy (CFIA, 1994). In the germination rate tests referred to above, DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6 showed high germination rates and no difference from their non-recombinant control maize.

(f) Crossability

Crossability test was not performed since no wild relatives (Teosinte (*Euchlaena mexicana*)) that can be naturally crossed with maize of the recipient organism are growing voluntarily in Japan.

(g) Production of harmful substances

For DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, plow-in tests, succeeding crop tests and soil microflora tests were carried out. As a result, regarding the fresh weight of lettuce in the plow-in tests and succeeding crop tests of DAS-01507-1, a statistically significant difference from the non-recombinant control maize was observed for one of the two recombinant varieties tested, though no significant difference was observed regarding the germination rate of lettuce. For the rest one variety, no significant difference was observed from the relevant non-recombinant control plant.

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

This stack maize line was developed by crossing maize resistant to Lepidoptera and tolerant to glufosinate herbicide (DAS-01507-1), maize resistant to Coleoptera and tolerant to glufosinate herbicide (DAS-59122-7), maize resistant to Lepidoptera (MON-00810-6) and maize tolerant to glyphosate herbicide (MON-00603-6) through the traditional crossing method. These parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stack line maize.

It was considered that the specificity of the Bt protein might be governed by the structure of protein and then the protein would bind to different receptors in the midgut cells of pest insects. In addition, there is no report that in the stack lines granted approvals to date, the Bt protein has exhibited synergy effect. Consequently, it was considered unlikely that the individual Bt proteins (modified Cry1F protein, Cry1Ab protein, Cry34Ab1 protein and Cry35Ab1 protein) would interact with each other in this stack maize line and affect the specificity of the Bt proteins. Furthermore, the PAT protein and the modified CP4 EPSPS protein differ from each other in their substrate and mechanism of action, and their involved metabolic pathways are also independent from each other. There is no report that the Bt proteins possess any enzyme activity. Therefore, it was considered unlikely that these proteins, even if expressed in this stack maize line, would interact with each other to affect the metabolic system of their recipient organisms and produce any unexpected metabolites. Consequently, it was considered unlikely that the proteins expressed in this stack maize line would exhibit functional interaction with each other.

In addition, the resistance to Lepidoptera and Coleoptera and the tolerance to glufosinate and glyphosate herbicides in this stack maize line were found at similar levels as exhibited by the individual parent lines. Consequently, it is considered low that the proteins expressed in this stack maize line derived from the parent lines would cause functional interaction in the plant body of this stack maize line, and it is considered unlikely that notable changes in traits have occurred in this stack maize line except for the traits that it received from the parent lines.

(1) Competitiveness

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

For various characteristics referring to competitiveness of DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, the parent lines of this stack maize line, the investigation was conducted by parent line, using two kinds of recombinant maize derived from the parent lines. As a result, in all the tests conducted, a statistically significant difference was observed between one of the two kinds of recombinant maize tested and their non-recombinant control maize regarding some

traits examined. However, the differences were judged not to be so large as enhancing the competitiveness.

This stack maize line is given traits to be resistant to pest insects of the order Lepidoptera due to the modified *cry1F* gene and the *cry1Ab* gene and to be resistant to pest insects of the order Coleoptera due to the *cry34Ab1* gene and the *cry35Ab1* gene. However, the insect damage by Lepidopteran and Coleopteran insect pests is not the major factor to inhibit the growth of maize under the natural environment in Japan. Therefore, it is considered unlikely that these traits cause maize, a crop plant, to become self-seeding in the natural environment and enhance the competitiveness. In addition, this stack maize line is given traits to be tolerant to glufosinate and glyphosate herbicides due to the *pat* gene and the modified *cp4 epsps* gene. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of these herbicides, the tolerances to glufosinate and glyphosate would increase the competitiveness of this stack maize line.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and the progeny lines of stack maize line isolated from the parent lines, DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

(2) Productivity of harmful substances

Maize (*Zea mays* subsp. *mays* (L) Iltis), the taxonomical species to which the recipient organism belongs, has been long used in Japan, though it is not generally known that the maize produces any harmful substances that could affect wild animals and wild plants.

It has been confirmed that the proteins expressed in this stack maize line (the modified Cry1F protein, the Cry34Ab1 protein, the Cry35Ab1 protein, the Cry1Ab protein, the PAT protein and the modified CP4 EPSPS protein) do not have any homology with any of the known allergens. In addition, for the ability of the parent lines of this stack maize line to produce any harmful substances (the substances secreted from the roots which can affect other plants and microorganisms in soil, the substances existing in the plant body which can affect other plants after dying), plow-in tests, succeeding crop tests and soil microflora tests were conducted. As a result, there was no difference observed in all tests suggesting that the productivity of harmful substances of the parent lines might have increased. Consequently, it is considered unlikely that this stack maize line possesses productivity of unintended harmful substances.

On the other hand, this stack maize line is given the traits to be tolerant to pest insects

of the order Lepidoptera due to the modified *cry1F* gene and the *cry1Ab* gene and to be resistant to pest insects of the order Coleoptera due to the *cry34Ab1* gene and the *cry35Ab1* gene. Then there is a concern about possible effects on the non-target species of Lepidopteran insects and Coleopteran insects which could eat directly this stack maize line or eat pollens dispersed from this stack maize line by eating with dietary plants. However, it is considered unlikely that these species of insects inhabit locally near the fields for cultivation of this stack maize line and then, it is considered extremely low that they could be affected in the level of population.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and the progeny lines of stack maize line isolated from the parent lines of this stack maize line, DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances.

(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 stack maize line and the progeny lines of stack maize line isolated from the parent lines of this stack maize line, DAS-01507-1, DAS-59122-7, MON-00810-6 and MON-00603-6, that contain a combination of any of the transferred genes in the individual parent lines, in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.