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

Name Du Pont Kabushiki Kaisha
Applicant 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, pat*, *cry34Ab1, cry35Ab1, cry1Ab,* modified *cp4 epsps,* modified *cry3Aa2, Zea mays* subsp. *mays* (L.) Iltis) (1507 × 59122 × MON810 × NK603 × MIR604, OECD UI: DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6 × SYN-IR6Ø4-5) (including the progeny lines isolated from the maize lines, *B.t.* Cry1F maize line 1507, *B.t.* Cry34/35Ab1 Event DAS-59122-7, MON810, NK603, and MIR604, that contain a combination of any of the transferred genes in the individual maize lines [except those already granted approval regarding Type 1 Use Regulation])
Content of the Type 1 Use of Living Modified Organisms	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, pat*, *cry34Ab1, cry35Ab1, cry1Ab,* modified *cp4 epsps,* modified *cry3Aa2, Zea mays* subsp. *mays* (L.) Iltis) (1507 × 59122 × MON810 × NK603 × MIR604, OECD UI: DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6 × SYN-IR6Ø4-5) (hereinafter referred to as “this stack maize line”) is a cross progeny line developed by crossing the following five recombinant maize lines, using the traditional crossbreeding method.

This stack maize line is 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-Ø15Ø7-1) (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-ØØ81Ø-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-ØØ6Ø3-6) (hereinafter referred to as “MON-00603-6”)
(e) Maize resistant to Coleoptera (modified *cry3Aa2, Zea mays* subsp. *mays* (L.) Iltis) (MIR604, OECD UI : SYN-IR6Ø4-5) (hereinafter referred to as “SYN-IR604-5”)

The parent lines of this stack maize line, DAS-01507-1 (Annex 1) and DAS-59122-7 (Annex 2) were jointly developed by Dow AgroSciences (USA) and Pioneer Hi-bred International (USA). The parent lines of this stack maize line, MON-00810-6 (Annex 3 and USDA [1996]) and MON-00603-6 (Annex 4 and USDA [2000]) were developed by the Monsanto Company (USA). The parent line of this stack maize line, SYN-IR604-5 (Annex 5 and USDA [2006]) was developed by Syngenta AG (Switzerland). The following genes are transferred to the individual parent lines.

DAS-01507-1: The modified *cry1F* gene to confer resistance to insects of the order Lepidoptera and the *pat* gene to confer tolerance to glufosinate herbicide

DAS-59122-7: The *cry34Ab1* gene and the *cry35Ab1* gene to confer resistance to insects of the order Coleoptera and the *pat* gene to confer tolerance to glufosinate herbicide

MON-00810-6: The *cry1Ab* gene to confer resistance to insects of the order Lepidoptera

MON-00603-6: The modified *cp4 epsps* gene to confer tolerance to glyphosate herbicide

SYN-IR604-5: The modified *cry3Aa2* gene to confer resistance to insects of the order Coleoptera and the *pmi* gene to confer the characteristic of selective marker

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of the parent lines are shown individually in Table 1 to Table 5 (p. 4–9).

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid are shown individually in Table 1 to Table 5 (p. 4–9).

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

Expression cassette	Component elements	Size (kbp)	Origin and function
Modified *cry1F* gene	*UBIZM1(2) Promoter*	1.98	Ubiquitin constitutive promoter derived from *Zea 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	*CAMV35S Promoter*	0.53	35S constitutive promoter derived from cauliflower mosaic virus.
	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.

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

Expression cassette	Component elements	Size (kbp)	Origin and function
cry34Ab1 gene	*UBI1ZM PRO*	1.98	Ubiquitin constitutive promoter derived from *Z. mays* (including intron and 5'-untranslated region).
	cry34Ab1	0.37	A gene that encodes the Cry34Ab1 protein derived from *B. thuringiensis* PS149B1 strain.
	PIN II TERM	0.32	A protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum*.
cry35Ab1 gene	*TA Peroxidase PRO*	1.30	Peroxidase promoter 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	A protease inhibitor II terminator to terminate transcription derived from *S. tuberosum*.
pat gene	*35S PRO*	0.53	35S constitutive promoter derived from cauliflower mosaic virus.
	pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein) derived from *S. viridochromogenes*. It has the nucleotide sequence modified to enhance its expression level in plants. The amino acid sequence expressed by the modification remains unchanged.
	35S TERM	0.21	35S terminator to terminate transcription from cauliflower mosaic virus.

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

Expression cassette	Component elements	Size (kbp)	Origin and function
cry1Ab gene	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	A gene that encodes the Cry1Ab protein of *B. thuringiensis* subsp. *krustaki* HD-1 strain existing in the soil.
	NOS 3'	0.26	3'-untranslated region of nopaline synthase (NOS) gene derived from *A. tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
Modified *cp4 epsps* gene*	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	0.22	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 *A. tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
gox 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.
	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 oxidoreducase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein degrades glyphosate.
	NOS 3'	0.26	3'-untranslated region of nopaline synthase gene derived from *A. tumefaciens*. Contains transcription terminator and polyadenylation signal for mRNA.

* The analysis of the inserted gene revealed that these expression cassettes were not inserted into MON-00810-6.

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

Expression cassette	Component elements	Size (kbp)	Origin and function
Modified *cp4 epsps* gene (i)	P-ract1	0.9	Promoter region of actin 1 gene derived from rice. This promoter makes the target gene expressed constitutively.
	ract1 intron	0.5	Rice actin gene intron. Activates the expression of target gene.
	CTP2	0.2	A sequence encoding the N-terminal chloroplast transit peptide of EPSPS protein in 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* CP4 strain. To enhance the expression in plants, the second amino acid from the N-terminal in the wild-type CP4 EPSPS protein is modified to leucine instead of serine.
	NOS 3’	0.3	3'-untranslated region of nopaline synthase (NOS) gene derived from *A. tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
Modified *cp4 epsps* gene (ii)	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	A sequence encoding the N-terminal chloroplast transit peptide of EPSPS protein in 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* CP4 strain. To enhance the expression in plants, the second amino acid from the N-terminal in the wild-type CP4 EPSPS protein is modified to leucine instead of serine.
	NOS 3’	0.25	3'-untranslated region of nopaline synthase (NOS) gene derived from *A. tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.

Table 5 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of SYN-IR604-5

Expression cassette	Component elements	Size (kbp)	Origin and function
Insect pest-resistance gene	*MTL*	2.56	A promoter derived from the *metallothionein* gene of maize. Since Corn rootworm, the target insect of the order Coleoptera, eats and damages the roots of maize, *MTL* promoter is used to induce the start of transcription of target genes in the roots.
	Modified *cry3Aa2*	1.80	The gene derived from *Bacillus thuringiensis* subsp. *tenebrionis*, which encodes the modified Cry3Aa2 protein. In order to enhance insecticidal activity against Corn rootworm, the nucleotide sequence was modified as follows: the 108th to 110th amino acid sequence (valine-serine-serine) of the Cry3Aa2 protein was changed into four amino acids (alanine-alanine-proline-phenylalanine), the cathepsin G protease recognition sequence. In addition, in order to enhance its expression level in plants, the contents of GC have been changed.
	Nos	0.25	The terminator region of the nopaline synthase gene of *Agrobacterium tumefaciens*, which terminates transcription and induces polyadenylation.
Selective marker gene	*ZmUbiInt*	1.99	A promoter derived from the *polyubiquitin* gene of maize to induce the start of transcription of target genes throughout the entire plant body of monocotyledon.
	pmi	1.18	The gene derived from *Escherichia coli* (*E. coli*), which encodes the PMI protein (phosphomannose isomerase). The PMI protein is an enzyme that has the capability of catalyzing the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. Transferring of this enzyme allows utilization of mannose as a carbon source. The *pmi* gene was used for selection of transformed cells.
	Nos	0.25	The terminator region of the nopaline synthase gene of *Agrobacterium tumefaciens*, which terminates transcription and induces polyadenylation.
Other regions	*Spec*	0.79	The streptomycin adenyltransferase gene *aadA*, derived from the transposon Tn7 of *Escherichia coli* (*E. coli*). This gene is used as a bacteria selective marker to confer the resistance to erythromycin, streptomycin, and spectinomycin.
	VS1 ori	0.41	The replication origin consensus sequence derived from the plasmid pVS1 of *Pseudomonas* bacteria. Functions as the replication origin of plasmid in *A. tumefaciens*.
	ColE1 ori	0.81	The replication origin that permits replication of plasmid in bacteria.
	LB	0.03	T-DNA left border region derived from *A. tumefaciens* nopaline Ti-plasmid.
	RB	0.03	T-DNA right border region derived from *A. tumefaciens* nopaline Ti-plasmid.
	VirG	0.73	A region involved in transfer of T-DNA, derived from *A. tumefaciens*.
	RepA	1.07	The pVS1 replication protein derived from *Pseudomonas* bacteria, taking on part of the responsibility for replication of pVS1 in the gram-negative bacteria living parasitically in plants.

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

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

[The insecticidal protein]

The insecticidal crystalline proteins (Bt proteins) including the modified Cry1F protein, the Cry34Ab1/Cry35Ab1 protein, the Cry1Ab protein, and the modified Cry3Aa2 protein generally bind to specific receptors in the midgut cells of pest insects to form pores in the cells, thereby destroying the midgut cells and resultantly exhibiting insecticidal activity (Schnepf *et al.*, 1998). The Bt proteins exhibit insecticidal specificity against the target fauna (Shirai, 2003).

Modified Cry1F protein:

The modified Cry1F protein is the δ-endotoxin that was derived from *B. thuringiensis* var. *aizawai*.

It exhibits high insecticidal activity against European corn borer (*Ostrinia nubilalis*), Fall armyworm (*Spodoptera frugiperda*), Beet armyworm (*Spodoptera exigua*), and other order Lepidoptera insects. On the other hand, it was confirmed that the modified Cry1F protein exhibits no toxicity against any insects other than the order Lepidoptera, such as the insects of the orders Coleoptera, Hymenoptera, Neuroptera, and Collembola, and mammals, birds, fish, and non-target organisms (EPA, 2005a).

Cry1Ab protein:

The Cry1Ab protein is the δ-endotoxin derived from *B. thuringiensis* subsp. *kurstaki*. This protein functions similarly to 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 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 insects other than the order Lepidoptera, such as the insects of the orders Coleoptera, Hymenoptera and Neuroptera, and mammals, birds, fish, and non-target organisms (USDA, 1995).

Cry34Ab1/Cry35Ab1proteins:

The Cry34Ab1 protein and the Cry35Ab1 protein are the δ-endotoxins that were derived from *B. thuringiensis* PS149B1 strain. The Cry34Ab1 protein possesses

insecticidal activity against Corn rootworm (*Diabrotica spp.*), though the Cry35Ab1 protein dose not exhibit any insecticidal activity by itself. When both proteins are set to work in concert with each other, a maximum of about 8-times higher insecticidal activity 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/Cry35Ab1 proteins exhibit insecticidal activities 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, but they exhibit no toxicity against any insects other than the order Coleoptera, such as the insects of the orders Lepidoptera, Hymenoptera, Neuroptera, Hemiptera, and mammals, birds, fish, and non-target organisms (EPA, 2005b).

Modified Cry3Aa2 protein:

The modified Cry3Aa2 protein is the δ-endotoxin that was derived from *B. thuringiensis* subsp. *tenebrionis*.

The modified Cry3Aa2 protein exhibits high insecticidal activity against the following four Coleopteran insects: Western corn rootworm, Northern corn rootworm, Colorado Potato Beetle (*Leptinotarsa decemlineata*), and Banded cucumber beetle (*Diabrotica balteata*). On the other hand, it was confirmed that the modified Cry3Aa2 protein exhibits no toxicity against any insects other than the order Coleoptera, such as the insects of the order Lepidoptera and Diptera, and mammals, birds, fish, and non-target organisms (Outline of the Biological Diversity Risk Assessment Report, 2009; USDA, 2006).

[Herbicide tolerant protein]

PAT protein:

The glufosinate herbicide inhibits glutamine synthase in plants by its active ingredient *L*-glufosinate, causing the plants to die due to accumulation of ammonia in the cells, one of the enzyme's substrates. However, the PAT protein acetylates and inactivates *L*-glufosinate, thereby conferring tolerance to glufosinate herbicide to the plants (OECD, 2002).

Modified CP4 EPSPS protein:

The glyphosate herbicide inhibits the activity of 5-enol-pyruvyl-shikimate-3-phosphatesynthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis in plants, and interrupts the aromatic amino acid biosynthesis, thereby causing the plants to die. The modified CP4 EPSPS protein exhibits activity even in the presence of glyphosate herbicide, and the shikimate pathway is not interrupted, thereby conferring tolerance to

glyphosate herbicide to the plants.

[Selective marker]

PMI protein:

Generally, maize cannot utilize mannose as a carbon source. The PMI protein catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate, enabling maize to utilize mannose as a carbon source. Therefore, the PMI protein was used as a selective marker. The PMI protein exists widely in nature, including the human digestive system and in fact, is present in soybean and other plants, though it has not been identified in maize.

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

Amino acid sequence homology search was conducted using the database.[1)] The result indicated that the modified Cry1F protein, the PAT protein, the Cry34Ab1 protein, the Cry35Ab1 protein, the Cry1Ab protein, the modified CP4 EPSPS protein, the modified Cry3Aa2 protein, and the PMI protein do not share structural homology with any of the known allergens.

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

Bt protein:

There is no report that the modified Cry1F protein, the Cry34Ab1 protein, the Cry35Ab1 protein, the Cry1Ab protein, and the modified Cry3Aa2 protein possess enzymatic activity.

PAT protein:

The PAT protein exhibits substrate specificity and catalyzes the reaction to acetylate free amino groups of *L*-glufosinate, an active ingredient of the glufosinate herbicide, although it does not use other amino acids or *D*-glufosinate as its substrate (OECD, 1999).

Modified CP4 EPSPS protein:

The EPSPS protein, functionally identical to the modified CP4 EPSPS protein, is not a rate-determining enzyme in the shikimate pathway for the biosynthesis of aromatic amino

---

[1)] Modified Cry1F protein, PAT protein, Cry34Ab1 protein, and Cry35Ab1 protein: Search conducted using the database NCBI Release 177.0 in June 2010.
Cry1Ab protein: Search conducted using the database NCBI Release 181.0 in February 2011.
Modified CP4 EPSPS protein: Search conducted using the databases AD_2010, TOX_2010, and PRT_2010 in February 2010.
Modified Cry3Aa2 protein and PMI protein: Search conducted using the database FARRP 10.0 in March 2010.

acids. As such, the enhanced EPSPS activity due to the production of the modified CP4 EPSPS protein would not increase the concentration of aromatic amino acids, the end products of this pathway (Annex 4; Ridley *et al.*, 2002; Padgette *et al.*, 1996). In fact, it has been confirmed that there is no difference in the aromatic amino acid content between the glyphosate-tolerant recombinant crops (soybean, oilseed rape, cotton, and maize) and the original non-recombinant crops.

The EPSPS protein specifically reacts with its substrates, phosphoenolpyruvic acid (PEP) and shikimate-3-phosphate (S3P) (Gruys *et al.*, 1992). It is also known to react with shikimate, an analogue of S3P, but the reactivity with shikimate is only one–two millionth of the reactivity with S3P, when compared in terms of specificity constant k_{cat}/K_m. Thus, it is unlikely that shikimate reacts as the substrate of EPSPS in the living organisms (Annex 4; Biological Diversity Risk Assessment Report, 2011).

PMI protein:

The PMI protein catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. The PMI protein specifically reacts with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein (Freeze, 2002).

Consequently, it is very unlikely that these proteins affect the metabolic pathway of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The 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* (Figure , p. 14).

DAS-59122-7: Plasmid PHP17662 constructed based on the plasmid pSB1 derived from *A. tumefaciens* (Figure , p.15).

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

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

SYN-IR604-5: Plasmid pZM26 constructed based on the plasmid pUC19 derived from *E. coli* (Figure , p. 18).

2) Properties

(i) The number 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
SYN-IR604-5 (pZM26): 13,811 bp

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

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

DAS-01507-1: The *nptII* gene to confer resistance to kanamycin and neomycin
DAS-59122-7: The *tet* gene to confer resistance to tetracycline, and the *spc* gene to confer resistance to spectinomycin
MON-00810-6: Partial coding sequence (*LacZ* gene) for β-D-galactosidase (LacZ protein), and the *nptII* gene to confer resistance to kanamycin and neomycin
MON-00603-6: The *nptII* gene to confer resistance to kanamycin and neomycin
SYN-IR604-5: The *spec* gene to confer resistance to streptomycin, erythromycin, and spectinomycin

(iii) Presence or absence of infectious characteristics of vector and, if present, the information concerning the host range

None of these vectors is known to be infectious.

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid transferred to 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, MON-00603-6, and SYN-IR604-5, and the positions and sites cleaved by restriction enzymes are shown in Figure to Figure (p. 14–18).

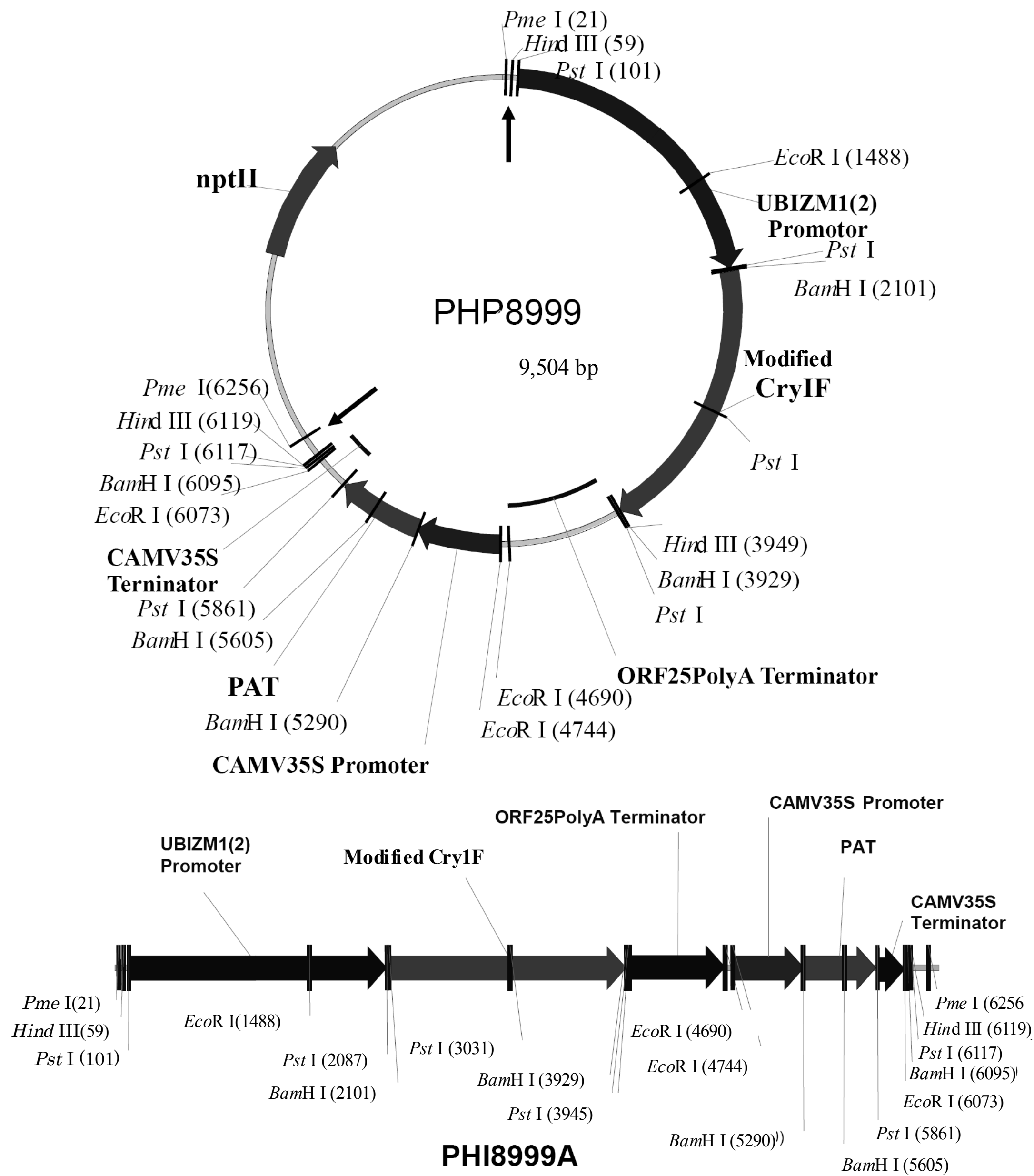

Figure 1 Composition of the plasmid PHP8999* (top) and the transferred DNA region PHI8999A (bottom)

* 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 used for transferring genes into the recipient organism.

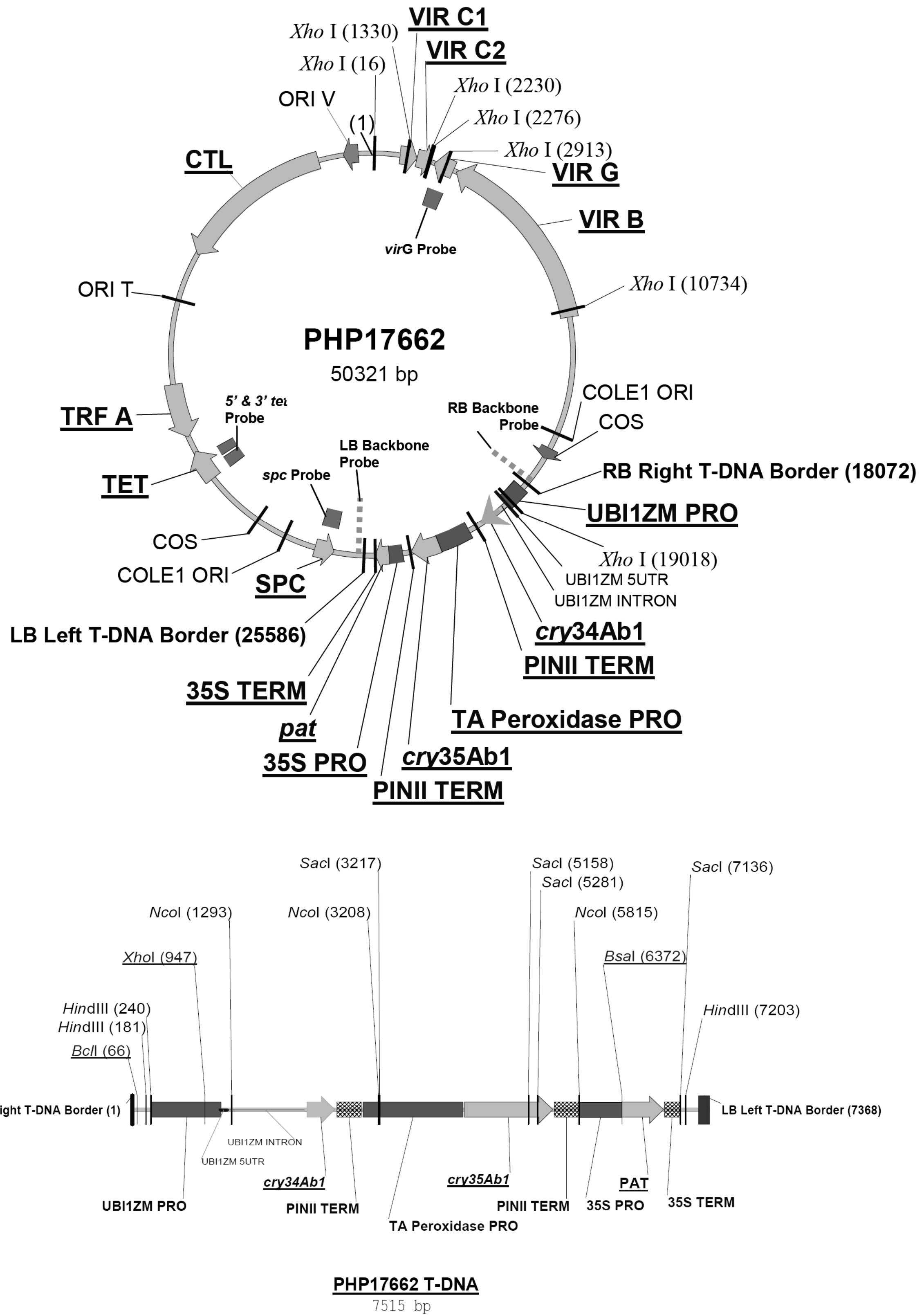

Figure 2 Composition of the plasmid PHP17662* and T-DNA region

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

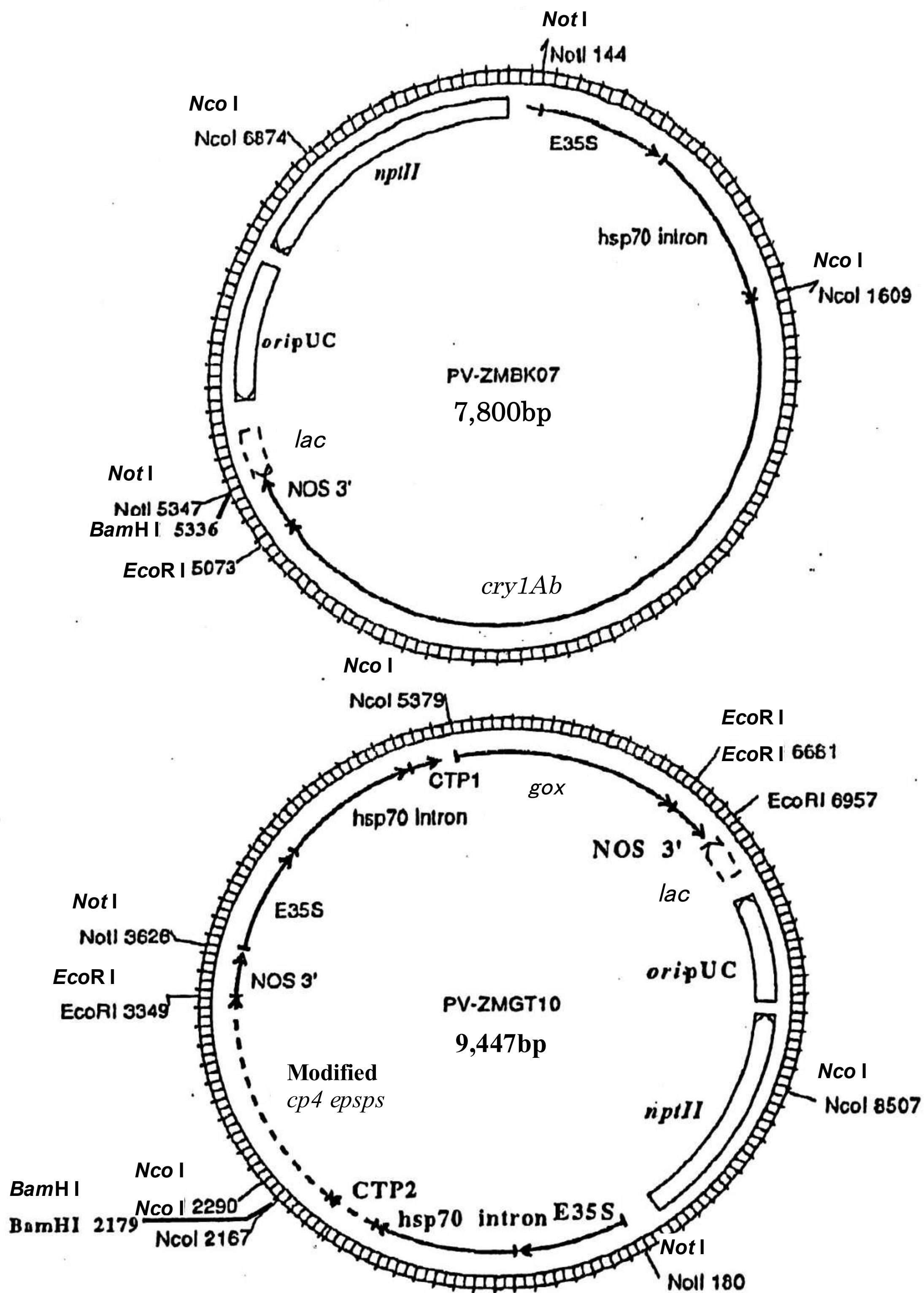

Figure 3 Composition of the plasmids PV-ZMBK07 and PV-ZMGT10*

* The vector used for the development of MON-00810-6

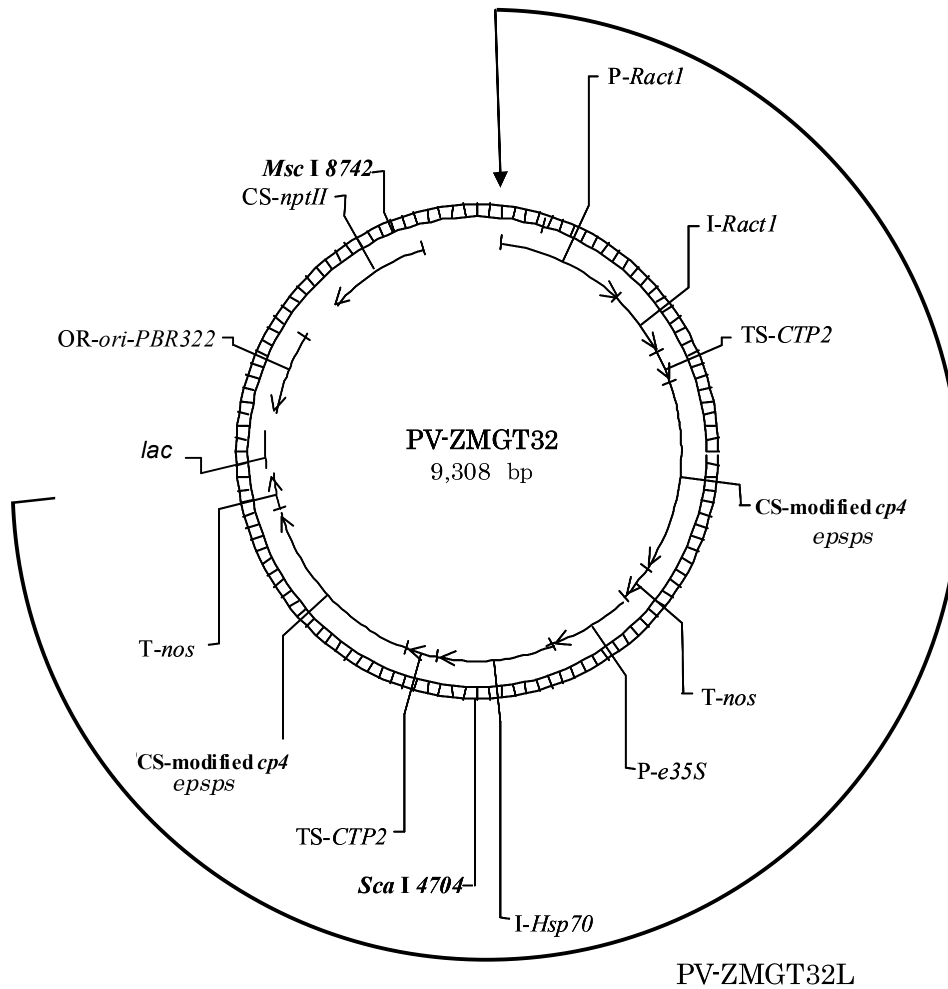

Figure 4 Composition of the plasmid PV-ZMGT32*

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

The plasmid PV-ZMGT32 was treated by the restriction enzyme *Mlu*I to prepare the linear DNA fragment PV-ZMGT32L, which was used for transferring genes into the recipient organism.

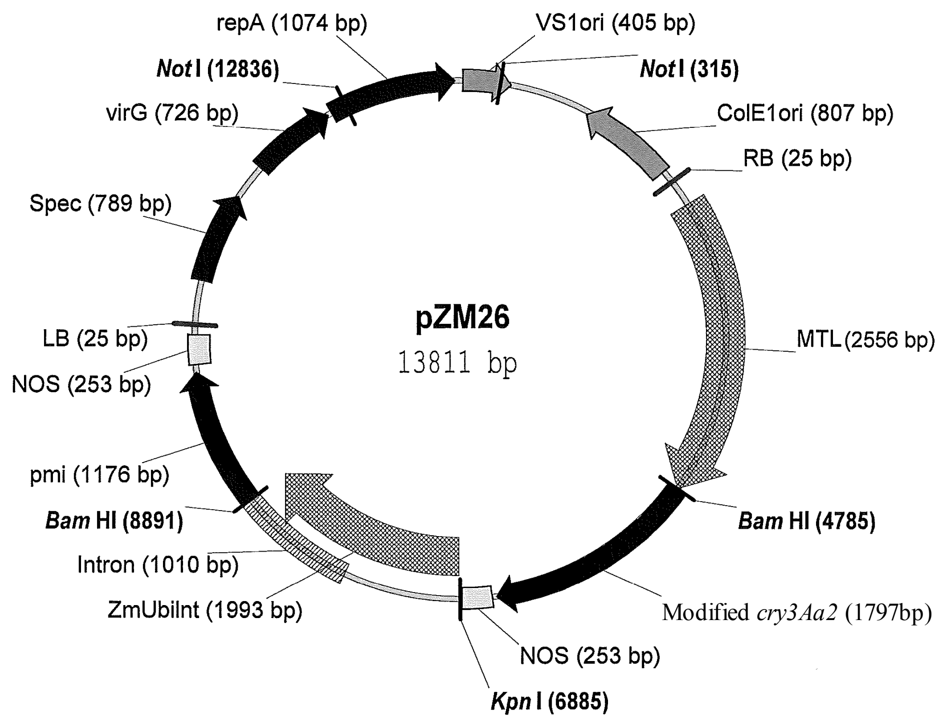

Figure 5 Composition of the plasmid pZM26

* The vector used for the development of SYN-IR604-5

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 and SYN-IR604-5.

3) Process of rearing of living modified organisms

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

Selection of nucleic acid-transferred cells was carried out by culturing on the growth medium containing the substances listed below.
DAS-01507-1 and DAS-59122-7: Glufosinate
MON-00810-6 and MON-00603-6: Glyphosate
SYN-IR604-5: Mannose

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

For DAS-59122-7 developed using the *Agrobacterium* method, the antibiotic Carbenicillin was added to the glufosinate medium to remove any residual *Agrobacterium*.
For SYN-IR604-5, the antibiotic Cefotaxime was added to the mannose medium to remove any residual *Agrobacterium*. PCR was then carried out for regenerated plants to select individual plants not containing the antibiotic-resistant marker gene. Consequently, it is considered that there is no remaining *Agrobacterium*.

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

This stack maize line was developed by cross breeding between DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5. The process is shown in Figure 6 (p. 19; Confidential: Not disclosed to unauthorized person). In addition, the status of approval of these parent lines in Japan is listed in Table 6 (p. 20).

(Not made available or disclosed to unauthorized person)

Figure 6 Example of the process of rearing of this stack maize line

Table 6 Status of approval of the parent lines and this stack maize line in Japan

Line	Safety as food			Safety as feed			Environmental safety		
DAS-01507-1	2002	July	8	2003	March	27	2005	March	2
DAS-59122-7	2005	October	25	2006	March	31	2006	April	10
MON-00810-6	2001	March	30	2003	March	27	2004	June	1
MON-00603-6	2001	March	30	2003	March	27	2004	November	22
SYN-IR604-5	2007	August	17	2007	August	22	2007	August	23
This stack maize line	2012 Pending application			2012 Pending application			2011 Submitted		

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

(i) 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, MON-00603-6, and SYN-IR604-5 are transferred in accordance with Mendel's law and that the replication product of the transferred nucleic acid exists on the maize genome.

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

DAS-01507-1:

The result of Southern blot analysis confirmed that one copy each of the modified *cry1F* gene expression cassette and the *pat* gene expression cassette are transferred into the maize genome, and that the transferred genes are inherited stably by offspring.

The result of nucleotide sequence analysis of transferred DNA confirmed that the transferred DNA contained a part of the modified *cry1F* gene sequence in the 5'-end region, parts of the *pat* gene sequence in the 5'-end and 3'-end regions, and a part of the *ORF25PolyA Terminator* sequence in the 3'-end region. However, Northern blot analysis confirmed that these gene fragments were not transcribed into mRNA, therefore not functioning.

DAS-59122-7:

The result of Southern blot analysis confirmed that one copy each of the *cry34Ab1* gene expression cassette, the *cry35Ab1* gene expression cassette, and the *pat* gene expression cassette are transferred into the maize genome, and that the transferred genes are inherited stably by offspring.

MON-00810-6:

The result of Southern blot analysis confirmed that one copy of a DNA fragment essential for the expression of the *cry1Ab* gene derived from PV-ZMBK07 is transferred into the maize genome and inherited stably by offspring.

Southern blot analysis indicated that only the 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. A possible reason why the modified *cp4 epsps* gene was not detected by the Southern blot analysis, despite the fact that the nucleic acid-transferred cells were selected on the glyphosate-containing medium (I.2.(3).3).(i), p. 19), was because the transferred genes might have segregated in the following generation of the regenerated individuals.

MON-00603-6:

The result of Southern blot analysis confirmed that one copy of PV-ZMGT32L (composed of two modified *cp4 epsps* gene expression cassettes) is transferred into the maize genome and that the transferred genes are inherited stably by offspring.

It was found that a 217bp fragment of *P-ract1* was inserted near the 3'-end of the transferred gene in the reverse direction; however, this fragment was confirmed not to be involved in the production of any new protein by a Western blot analysis. In addition, a base in the modified *cp4 epsps* gene induced by *E35S* was changed during the development of MON-00603-6, and as a result, an amino acid forming the modified CP4 EPSPS protein was changed. However, the structure and function of the protein is considered to remain unchanged for the following three reasons: this amino acid is not included in the seven amino acids essential for activating the EPSPS protein family; this change does not affect the active site of the protein and its three-dimensional structure; and the traits of the modified protein, such as enzyme activity and immune response, are comparable to those of the original protein.

SYN-IR604-5:

The result of Southern blot analysis confirmed that one copy each of the modified *cry3Aa2* gene and the *pmi* gene are transferred into the maize genome, and that the transferred genes are inherited stably by offspring.

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

—

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

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, a 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, a bioassay for resistance to pest insects of the order Coleoptera, and glufosinate herbicide spraying test

MON-00810-6: A bioassay for resistance to pest insects of the order Lepidoptera

MON-00603-6: A glyphosate herbicide spraying test

SYN-IR604-5: Confirming the expression of the modified Cry3aA2 protein and the PMI protein by ELISA method, and a bioassay for resistance to pest insects of the order Coleoptera

(v) 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 that allows gene transmission, and thus, there is no possibility that the nucleic acid transferred to the maize lines could be transmitted to any other wild animals or 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, MON-00603-6, and SYN-IR604-5) based on the real-time quantitative PCR analysis is available at the website of the European Commission (Joint Research Centre, 2005–2007, 2010).

Sensitivity:

The quantification limits were: ≤0.08% for DAS-01507-1; 0.1% for DAS-59122-7, MON-00810-6, and MON-00603-6; and <0.09% for SYN-IR604-5.

Reliability:

As for the methods for detection of DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5, the reliability has been verified as a result of tests performed at 14 (repeated twice), 14 (repeated 4 times), 14 (repeated 3 times), 12 (repeated twice), and 14 (repeated 4 times) member test laboratories of the European Network of GMO Laboratories, respectively.

In order to detect and identify this stack maize line, the above-mentioned methods must be applied to each grain of maize seeds or plant body.

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

(i) 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 as described below: resistance to Lepidoptera

due to the modified *cry1F* gene and tolerance to glufosinate herbicide due to the *pat* gene, both of which are derived from DAS-01507-1; resistance to Coleoptera due to the *cry34Ab1*/*cry35Ab1* gene and tolerance to glufosinate herbicide due to the *pat* gene, both of which are derived from DAS-59122-7; resistance to Lepidoptera due to *cry1Ab* gene that is derived from MON-00810-6; tolerance to glyphosate herbicide due to the modified *cp4 epsps* that is derived from MON-00603-6; and resistance to Coleoptera due to the modified *cry3Aa2* gene that is derived from SYN-IR604-5.

The possibility of functional interaction between proteins produced by these genes is discussed below from three standpoints: between the pest insect-resistant proteins, between the herbicide-tolerant proteins, and between these two types of proteins.

<u>Functional interaction between the pest insect-resistant proteins</u>

The modified Cry1F protein and the Cry1Ab protein exhibit insecticidal activity against pest insects of the order Lepidoptera. The Cry34Ab1/Cry35Ab1 proteins and the modified Cry3Aa2 protein exhibit insecticidal activity against pest insects of the order Coleoptera (I. 2. (1). 2). (ii), p. 9). The specificity of the insecticidal activity given to these pest insect-resistant proteins depends on their structure; therefore, their insecticidal effect on respective target insects remains unaffected unless there is a change to the regions associated with their target specificity.

In addition, there is no report that any pest insect-resistant protein has exhibited a synergy effect in the previously approved stack lines. 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.

<u>Functional interaction between the herbicide-tolerant proteins</u>

The PAT protein and the modified CP4 EPSPS protein both have enzymatic activities. As mentioned earlier, the two proteins differ from each other in terms of their substrates: the PAT protein uses *L*-glufosinate as a substrate while the modified CP4 EPSPS protein uses PEP and S3P as substrates. They are known to react specifically with their respective substrates, and the metabolic pathways in which they are involved are independent from each other (I. 2. (1). b. (iii), p. 11). Therefore, it is unlikely that any unexpected metabolites would be produced.

<u>Functional interaction between the pest insect-resistant proteins and the herbicide-tolerant proteins</u>

The pest insect-resistant proteins and the herbicide-tolerant proteins are unlikely to interact with each other, as their functions differ. In addition, there is no report of interaction between the pest insect-resistant protein and the herbicide-tolerant protein in the previously approved stack lines.

In order to examine whether the expressed proteins, which are derived from the parent lines, interact with each other in this stack maize line, the stack maize line, the parent lines, and the non-recombinant control maize were analyzed for resistance to the European corn borer, a pest insect of the order Lepidoptera, and to Western corn rootworm, a pest insect of the order Coleoptera, as well as the tolerance to glufosinate and glyphosate herbicides.

The PMI protein, which is produced by the *pmi* gene inserted into SYN-IR604-5 as a selective marker, catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. The PMI protein reacts specifically with mannose-6-phosphate and fructose-6-phosphate, and it is very unlikely to affect other metabolic pathways. Furthermore, its function differs from those of the pest insect-resistant proteins and the herbicide-tolerant proteins. Therefore, it is unlikely that these proteins would exhibit functional interaction.

Resistance to European corn borer (*Ostrinia nubilalis*)

In order to observe the levels of feeding damage, larvae of European corn borer (*Ostrinia nubilalis*) were raised on the leaves of this stack maize line, the parent lines DAS-01507-1 and MON-00810-6, and the non-recombinant control maize (Annex 6; Confidential: Not disclosed to unauthorized person). No statistically significant difference between this stack maize line and the parent lines was found regarding the levels of feeding damage to leaves by European corn borer larvae (Table 7, p. 25).

Table 7 Levels of feeding damage to leaves by the larvae of European corn borer (*Ostrinia nubilalis*)

Samples tested *	Levels of feeding damage
This stack maize line	0.0065 b ± 0.0402
Control: Parent line DAS-01507-1	0.0283 b ± 0.0370
Control: Parent line MON-00810-6	0.0280 b ± 0.0398
(Reference) Non-recombinant control maize	0.7114 a ± 0.1717

n = 20, mean value ± standard deviation

* All genetic background = PHEF09×SYNZLL6011

Test condition: Each line was cultivated in growth chambers in the U.S. in 2010. At the 6-leaf stage (V6 stage), leaves were collected, and the larvae of the European corn borer (*Ostrinia nubilalis*) immediately after hatching were raised on the leaves for 48 hours. Tests were repeated 5 times with 2 samples each from 2 plants per repeat.

Evaluation method: Level of feeding damage was determined through digital images of the leaves fed to the larvae. The damage-free part of a leaf was represented as the number of pixels on its digital image, and the number was divided by the mean number of pixels over all control leaves (not given to larvae), and the calculated value was subtracted from unity (1) to determine the level of feeding damage of individual leaves.

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

Resistance to Western corn rootworm

The levels of feeding damage to roots by Western corn rootworm was examined on this stack maize line, the parent lines DAS-59122-7 and SYN-IR604-5, and the non-recombinant control maize, (Annex 6; Confidential: Not disclosed to unauthorized person). No statistically significant difference was observed between this stack maize line and the parent lines regarding the levels of feeding damage to roots by Western corn rootworm (Table 8, p. 26).

Table 8 Levels of feeding damage to root by Western corn rootworm

Samples tested *	Nodal Injury score (NIS)
This stack maize line	0.242 b ± 0.218
Control: Parent line DAS-59122-7	0.230 b ± 0.174
Control: Parent line SYN-IR604-5	0.368 b ± 0.315
(Reference) Non-recombinant control maize	1.638 a ± 0.483

n = 20, mean value ± standard deviation

* All genetic background = PHEF09×SYNZLL6011

Test condition: Each line was cultivated in growth chambers in the U.S. in 2010. At the 3-leaf stage (V3 stage), the roots were inoculated with the eggs of Western corn rootworm. After hatching, the severity of feeding damage at the roots was examined. Tests were repeated 10 times with 2 plant samples per repeat.

Evaluation method: The total number of roots and damaged roots (up to 3.8 cm from the stem) was counted for each node, and degree of root damage was calculated (the number of damaged roots/ the total number of roots).

Score 0.00: No damage

Score 1.00: All the roots were damaged at 1 node.

Score 2.00: All the roots were damaged at 2 nodes.

Score 3.00: All the roots were damaged at 3 or more nodes.

If multiple nodes were damaged, the score of each node is added (maximum = 3.00).

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

Tolerance to glufosinate herbicide

Glufosinate herbicide was sprayed on this stack maize line, the parent lines DAS-01507-1 and DAS-59122-7, and the non-recombinant control maize, and their severity of herbicide injury was examined (Annex 7; Confidential: Not disclosed to unauthorized person). No statistically significant difference in the severity of herbicide injury between this stack maize line and the parent line DAS-01507-1 was found (Table 9, p. 27). When the herbicide was sprayed at a 32-times higher dosage, a statistically significant difference was observed between this stack maize line and the parent line DAS-59122-7. This could be attributed to the additive enhancement of herbicide tolerance in this stack maize line, due to the redundant production of PAT proteins derived from both of the parent lines DAS-01507-1 and DAS-59122-7. When the herbicide was sprayed at a normal dosage or 16-times higher dosage, no statistically significant difference was observed between this stack maize line and the parent line.

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

Samples tested[1)]	Levels of herbicide injury (%)			
	Not sprayed	Normal dosage[2)]	16-times higher dosage	32-times higher dosage
This stack maize line	0 ± 0	0 b[3)] ± 0	0 b ± 0	3.33 c ± 4.88
Control: Parent line DAS-01507-1	0 ± 0	0 b ± 0	1.33 b ± 5.16	10.7 bc ±25.5
Control: Parent line DAS-59122-7	0 ± 0	6.67 b ±12.3	8.00 b ±13.2	40.7 b ±30.3
(Reference) Non-recombinant control maize	0 ± 0	27.3 a ±20.5	100 a ± 0	100 a ± 0

n = 15, mean value ± standard deviation

Test condition: Each line was cultivated in greenhouses in the U.S. in 2010–2011. At the 2-leaf stage (V2 stage), the glufosinate herbicide was sprayed. Tests were repeated 3 times with 5 plants per repeat.

Evaluation method: On the 12th day after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis, or bleaching) was visually evaluated based on the 11-step scale from 0% (intact) to 100% (complete death) at 10% intervals.

1) All genetic background = PHEF09 × SYNZLL6011

2) The concentration of glufosinate sprayed (actual value): normal dosage of 0.498 kg active ingredient (a.i.)/ha, 16-times higher dosage of 7.96 kg a.i./ha, and 32-times higher dosage of 15.9 kg a.i./ha. The spraying at the concentrations of 16- and 32-times higher dosages was intended for the evaluation of herbicide tolerance levels, and spraying of herbicide at such concentrations is not intended in the commercial cultivation.

3) Different letters in a given column indicate that a statistically significant difference ($P < 0.05$) was observed between the relevant mean values (multiple tests consisting of ANOVA and the Sidak method for each herbicide concentration [Westfall *et al.,* 2006]).

Tolerance to glyphosate herbicide

Glyphosate herbicide was sprayed on this stack maize line, the parent line MON-00603-6, the parent line MON-00810-6 (reference), the parent line SYN-IR604-5 (reference), and non-recombinant control maize (reference), and their severity of herbicide injury was examined (Annex 7; Confidential: Not disclosed to unauthorized person). No statistically significant difference in the severity of herbicide injury between this stack maize line and individual control parent lines was found (Table 10, p. 28).

Table 10 Levels of herbicide injury to this stack maize line and parent lines by spraying glyphosate herbicide

Samples tested[1)]	Levels of herbicide injury (%)			
	Not sprayed	Normal dosage[2)]	16-times higher dosage	32-times higher dosage
This stack maize line	0 ± 0	0 c[3)] ± 0	0 b ± 0	0 b ± 0
Control: Parent line MON-00603-6	0 ± 0	0 c ± 0	0 b ± 0	0 b ± 0
(Reference) Parent line MON-00810-6	0 ± 0	83.3 a ± 6.17	100 a ± 0	100 a ± 0
(Reference) Parent line SYN-IR604-5	0 ± 0	76.7 b ± 4.88	100 a ± 0	100 a ± 0
(Reference) Non-recombinant control maize	0 ± 0	80.0 ab ± 0	100 a ± 0	100 a ± 0

n = 15, mean value ± standard deviation

Test condition: Each line was cultivated in greenhouses in the U.S. in 2010–2011. At the 2-leaf stage (V2 stage), the glyphosate herbicide was sprayed. Tests were repeated 3 times with 5 plants per repeat.

Evaluation method: On the 12th day after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis, or bleaching) was visually evaluated based on the 11-step scale from 0% (intact) to 100% (complete death) at 10% intervals.

1) All genetic background = PHEF09 × SYNZLL6011

2) The concentration of glyphosate sprayed (actual value): Normal dosage of 1.33 kg acid equivalent (glyphosate free acid equivalent: a.e.)/ha, 16-times higher dosage of 20.3 kg a.e./ha, and 32-times higher dosage of 41.6 kg a.e./ha. The spraying at the concentrations of 16- and 32-times higher dosages was intended for the evaluation of herbicide tolerance levels, and spraying of herbicide at such concentrations is not intended in commercial cultivation.

3) Different letters in a given column indicate that a statistically significant difference ($P < 0.05$) was observed between the relevant mean values (multiple tests consisting of ANOVA and the Sidak method for each herbicide concentration [Westfall *et al.*, 2006]).

Both the considerations on the possibility of functional interaction between the proteins expressed in this stack maize line, as well as the examinations of the characteristics of each protein, lead to a conclusion that any interaction between these proteins is unlikely to take place in this stack maize line. In addition, this conclusion was supported by the results of bioassays which confirmed that the traits originally conferred

to the parent lines had been unchanged in this stack maize line. Therefore, it was concluded that the individual proteins expressed in the relevant parent lines do not interact with each other and that the traits acquired through the transferred genes remain unchanged in this stack maize line.

Consequently, with regard to the differences in physiological or ecological 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 individual examinations of the parent lines at the times of their approval (Annexes 1 to 5).

(ii) 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

The parent lines (DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5) and their respective non-recombinant controls were examined on their morphological and growth characteristics listed in Table 11 (p. 31). No statistically significant difference was observed between the parent lines and their controls except the germination rate and ear diameter of DAS-01507-1, the culm lengths of DAS-59122-7 and MON-00810-6, and the 100-kernel weight of MON-00603-6. However, the differences were found in only one of the two varieties with different genetic backgrounds in all cases.

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

It has been confirmed that the low-temperature treatment at the early stage of growth caused DAS-01507-1, MON-00603-6, DAS-59122-7, MON-00810-6, and SYN-IR604-5 to wither, wilt, or die as their non-recombinant controls did.

c. Wintering ability and summer survival of the mature plant

Maize is a summer type annual plant. After ripening, it normally dies out in winter and is not known to survive the winter. It neither re-grows after harvest and propagates vegetatively nor produces seeds. In fact, DAS-01507-1 and DAS-59122-7 were confirmed to wither when they were cultivated and harvested in test fields in the U.S. Furthermore, in the isolated field tests of MON-00810-6, MON-00603-6, and SYN-IR604-5, the start of withering and death after ripening was observed by the end of the tests (Outline of the Biological Diversity Risk Assessment Report, 2009).

d. Fertility and size of the pollen

As a result of pollen staining and their observation under a microscope, no difference was observed in fertility, size, or shape of the pollens between DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5 and their respective non-recombinant controls.

Table 11 Investigational results of morphological and growth characteristics

Item for investigation	DAS-01507-1	DAS-59122-7	MON-00810-6	MON-00603-6	SYN-IR604-5
Germination rate	○*	○	○	○	○
Uniformity of germination	○	○	○	○	○
Time to tasseling	○	○	○	○	○
Time to silking	○	○	○	○	○
Start of flowering	○	○	○	—	—
End of flowering	○	○	○	—	—
Flowering period	○	○	○	—	—
Time to maturity	○	○	○	○	○
Plant form or plant type	○	○	○	○	○
Tiller number	○	○	○	○	○
(Total) number of ears	○	—	○	○	○
Number of productive ears	○	○	○	—	○
Grain color and grain shape	○	○	○	○	○
Culm length	○	○*	○*	○	○
Ear height	○	○	○	○	○
Ear length	○	○	○	○	○
Ear diameter	○*	○	○	○	○
Row number per ear	○	○	○	○	○
Grain number per row	○	○	○	○	○
100-kernel weight	○	○	○	○*	○
Weight of above-ground parts	○	○	○	○	○
Flower shape	—	○	—	—	—

○ : Examined
— : Not examined
* : For one of the two hybrid 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: As a part of the studies discussed in (ii)-a (p.29), ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight were

examined. Statistically significant differences from non-recombinant maize were observed in the ear diameter of DAS-01507-1 and the 100-kernel weight of MON-00603-6; however, the differences were observed in only one out of two varieties tested.

Seed shedding: The possibility of spontaneous shedding and dispersing of maize seeds is low, since the ears are covered with husks (OECD, 2003). In DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5, seed shedding has not been observed under natural conditions.

Dormancy and germination rate: Maize exhibits almost no seed dormancy (CFIA, 1994). In the germination rate tests, DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5 showed high germination rates, and there was no difference from their respective non-recombinant controls.

f Crossability

Crossability test was not performed, since no wild relatives (Teosinte) capable of naturally crossing with maize, the recipient organism, grows wild in Japan.

g Productivity of harmful substances

In order to examine whether the parent lines DAS-01507-1, DAS-59122-7, MON-00810-6, MON-00603-6, and SYN-IR604-5 produce any harmful substances, plow-in tests, succeeding crop tests and soil microflora tests were carried out. The results of the plow-in test and the succeeding crop test conducted on DAS-01507-1 showed a significant difference in the fresh weight of lettuce grown as the succeeding crop. However, a significant difference was observed for only one out of two varieties examined. In addition, no significant difference was observed in the germination rate of the lettuce.

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 Organisms based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms. Results of the review are listed below.

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

This stack maize line was developed by crossing the following five recombinant maize lines, using the traditional crossbreeding method:

(i) maize resistant to Lepidoptera and tolerant to glufosinate herbicide (*B.t.* Cry1F maize line 1507) due to the modified *cry1F* gene that encodes the modified Cry1F protein and the *pat* gene that encodes the PAT protein (phosphinothricin acetyltransferase);

(ii) maize resistant to Coleoptera and tolerant to glufosinate herbicide (*B.t.* Cry34/35Ab1 Event DAS-59122-7) due to the *cry34Ab1*/*cry35Ab1* gene that encodes the Cry34Ab1/Cry35Ab1 protein and the *pat* gene that encodes the PAT protein (phosphinothricin acetyltransferase);

(iii) maize resistant to Lepidoptera (MON810) due to the *cry1Ab* gene that encodes the Cry1Ab protein;

(iv) maize tolerant to glyphosate herbicide (NK603) due to the modified *cp4 epsps* gene that encodes the modified CP4 EPSPS protein (5-enol-pyruvyl-shikimate-3-phosphate synthase); and

(v) maize resistant to Coleoptera (MIR604) due to the modified *cry3Aa2* gene that encodes the modified Cry3Aa2 protein and the *pmi* gene that encodes the PMI protein (phosphomannose isomerase). These five 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 maize line.

The specificity of Bt proteins is thought to be governed by their structure. They bind to different receptors of the midgut cells of pest insects. There is no report among the stack lines granted approvals to date that Bt proteins from different parent lines exhibited a synergic effect. These approved stack lines show only the inherited insecticidal traits. Thus, it was deemed that the individual Bt proteins in this stack maize line (the modified Cry1F protein, the Cry34Ab1/Cry35Ab1 protein, the Cry1Ab protein, and the modified Cry3Aa2 protein) were unlikely to interact with each other and to affect the specificity of another. Furthermore, the PAT protein, the modified CP4 EPSPS protein, and the PMI protein differ from each other in their substrate and mechanism of action, and the metabolic pathways in which they are involved are independent from each other. There is no report of any Bt protein

exhibiting an enzymatic activity. Therefore, these proteins were considered unlikely to interact with each other when expressed in this stack maize line, affecting the metabolic system of their recipient organisms and producing any unexpected metabolite.

In addition, based on the bioassays, the resistance to Lepidoptera and Coleoptera and the tolerance to glyphosate herbicide in this stack maize line were found at similar levels as exhibited by the individual parent lines. Regarding the tolerance to glufosinate herbicide, this stack maize line exhibited an additively enhanced effect possibly due to the expression of the relevant protein.

Consequently, it is considered unlikely that the proteins expressed in this stack line derived from individual 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, 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-breeding in the natural environment in Japan.

As a result of investigation for various characteristics referring to competitiveness of *B.t.* Cry1F maize line 1507, *B.t.* Cry34/35Ab1 Event DAS-59122-7, MON810, NK603, and MIR604, the parent lines of this stack maize line, statistically significant differences were observed between this stack maize line and its non-recombinant maize in some of the items examined. However, the differences were judged not to be so large as enhancing the competitiveness of this stack maize line.

The resistance trait to pest insects of the order Lepidoptera is given to this stack maize line by the modified Cry1F protein and the Cry1Ab protein, and that of the order Coleoptera is given by the Cry34Ab1/Cry35Ab1 and the modified Cry3Aa2 protein. However, the insect damage by Lepidopteran and Coleopteran insect pests is not the major factor to inhibit the growth of maize under a 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 its competitiveness.

In addition, this stack maize line is given traits to be tolerant to glufosinate and glyphosate herbicides due to the PAT protein and the modified CP4 EPSPS protein. 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.

In addition, this stack maize line is also given traits to use mannose as a carbon source. However, it is unlikely that this stack maize line uses mannose as a major carbon source in the natural environment in Japan, and thus, it is considered unlikely that these traits would enhance 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: regarding this stack maize line, there are no specific wild animals and wild plants that are possibly affected by this stack maize line, and it would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

(2) Productivity of harmful substances

Maize, the taxonomical species to which the recipient organism belongs, has been long used in Japan, though it is not generally known to produce 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/Cry35Ab1 protein, the Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the modified CP4 EPSPS protein, and the PMI protein) do not have any homology with any of the known allergens. Moreover, the PAT protein produces *N*-acetyl-1-glufosinate when glufosinate herbicide is sprayed to the plant, although it has been confirmed that the toxicity of *N*-acetyl-1-glufosinate to animals is lower than that of glufosinate.

In addition, for examining the ability of the parent lines of this stack maize line to produce any harmful substances (the substances secreted from the roots that can affect other plants and microorganisms in soil, the substances existing in the plant body that can affect other plants after dying), plow-in tests, succeeding crop tests, and the soil microflora tests were conducted. In all tests, there was no difference suggesting that the productivity of harmful substances of the parent lines has increased. Consequently, it is considered unlikely that this stack maize line possesses productivity of unintended harmful substances.

The modified Cry1F protein and the Cry1Ab protein expressed in this stack maize line exhibit insecticidal activity against insects of the order Lepidoptera, and the Cry34Ab1/Cry35Ab1 protein, and the modified Cry3Aa2 protein exhibit insecticidal activity against insects of the order Coleoptera. Therefore, the Lepidopteran and Coleopteran insects were specified as wild animals and wild plants that are possibly affected by this recombinant maize. Then, there is a concern about possible effects on the non-target species of Lepidopteran and Coleopteran insects that could directly eat this stack maize line or eat pollens dispersed from this stack maize line and attached to their food plants. However, it is considered unlikely that the Lepidopteran and Coleopteran insects inhabit locally near the fields for cultivation of this stack maize line and thus, it is considered very unlikely 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 would pose no risk of Adverse Effect on Biological Diversity that is attributable to productivity 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 stack maize line, 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 the 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, in accordance with the Type 1 Use Regulation, cause Adverse Effects on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is valid.

References

CFIA (Canadian Food Inspection Agency). 1994. The biology of *Zea mays* (L.). (http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.pdf). Accessed on March 30th, 2011.

EPA (U.S. Environmental Protection Agency), 2005a. Biopesticide Registration Action Document. Bacillus thuringiensis Cry1F Corn. (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006481.pdf). Accessed on March 30th, 2011.

EPA (U.S. Environmental Protection Agency), 2005b. Biopesticide Registration Action Document. Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production in Event DAS-59122-7 Corn. (http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006490.pdf). Accessed on Aug 22nd, 2011.

FAOSTAT, 2011. (http://faostat.fao.org/site/567/default.aspx#ancor). Accessed on January 5th, 2011.

FARRP (Food Allergy Resource and Resource Program), 2010. Protein AllergenOnline database, version 10.0. (http://www.allergenonline.org/). Accessed in March 2010.

Freeze, H. H. 2002. Phosphomannose isomerase. Handbook of glycosyltransferases and related genes. Edition 1. Taniguchi, N., Honke, K. and Fukuda, M., Eds. Springer-Verlag, Tokyo and New York, pp. 595-599.

Gruys, K.J., Walker, M.C., and Sikorski, J.A. 1992. Substrate synergism and the steady-state kinetic reaction mechanism for EPSP synthase from *Escherichia coli*. Biochem. 31: 5534-5544

Hansen-Jesse, L.C., and J.J. Obrycki. 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*, 125: 241-248.

Hua, G., Masson, L., Jurat-Fuentes, J. L., Schwab, G., and Adang, M. J. 2001. Binding analyses of *Bacillus thuringiensis* Cry δ-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis*. *Appl. Environ. Microbiol.* 67: 872-879.

Joint Research Centre. 2005-2007, 2010.
(http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-WEB-Protocol-Validation.pdf,
http://gmo-crl.jrc.ec.europa.eu/summaries/59122-Protocol%20Validation.pdf,
http://gmo-crl.jrc.ec.europa.eu/summaries/Mon810_validation_report.pdf,
http://gmo-crl.jrc.ec.europa.eu/summaries/NK603-WEB-Protocol%20Validation.pdf,
http://gmo-crl.jrc.ec.europa.eu/summaries/MIR604_validated_Method_correctedversion1.pdf). Accessed on March 30th, 2011.

Kikuchi, K. 1987. Toumorokoshi no seisan to riyou (Production and Use of Maize). Korin Publishing Co. Ltd.

NCBI. 2010～2011. The BLASTP 2.2.13 algorithm against Release 177.0 and 181.0 (4/15/10) of the NCBI Entrez Protein dataset. (http://www.ncbi.nlm.nih.gov/) Accessed in June 2010 and February 2011.

Nougyou Gijutsu Taikei Sakumotsu-hen (Encyclopedia of Agricultural Technique, Crops), Vol. 7, 2004. Rural Culture Association Japan.

Ministry of Agriculture, Forestry and Fisheries. 2011. Nourin Suisan Sesaku ni tsuite (Toukei) Sakutsuke Menseki, Seisanryou, Kachiku no Tousuu nado (About Policies on Agriculture, Forestry and Fisheries (Statistics) Planted Area, Production and Number of Livestock Animals) (http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_kome/pdf/syukaku_siryou_10.pdf). Accessed on May 19th, 2011.

OECD. 1999. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 11: Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. ENV/JM/MONO(99)13. (http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(99)13&doclanguage=en). Accessed on March 30th, 2011.

OECD. 2002. Series on Harmonization of Regulatory Oversight in Biotechnology, No. 25. MODULE II: PHOSPHINOTHRICIN. ENV/JM/MONO(2002)14. (http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2002)14&doclanguage=en). Accessed on March 30th, 2011.

OECD. 2003. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 27: Consensus Document of the Biology of *Zea mays* subsp. *mays* (Maize). (http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2003)11&doclanguage=en). Accessed on March 30th, 2011.

Padgette, S.R., Taylor, N.B., Nida, D.L., Bailey, M.R., MacDonald, J., Holden, L.R., and Fuchs, R.L. 1996. The Composition of Glyphosate-Tolerant Soybean Seeds is Equivalent to That of Conventional Soybeans. J. of Nutrition. 126(3): 702-716.

Pleasants, J.M., Hellmich, R.L., Dively, G.P., Sears, M.K., D. Stanley-Horn, D.E., Mattila, H.R., Foster, J.E., Clark, P., and Jones, G.D.. 2001. Corn pollen deposition on milkweeds in and near cornfields**.** *PNAS*, 98: 11919-24.

Ridley, W.P., Sidhu, R.S., Pyla, P.D., Nemeth, M.A., Breeze, M.L., and Astwood, J.D. 2002. Comparison of the Nutritional Profile of Glyphosate-Tolerant Corn Event NK603 with That of Conventional Corn (*Zea mays* L.). J. Agric. Food. Chem., 50(25)7235-7243

Biological Diversity Risk Assessment Report. 2011. Outline of the Biological Diversity Risk Assessment Report on Glyphosate-induced male sterile, glyphosate resistant maize (modified *cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (MON87427, OECD UI: MON-87427-7) (http://www.bch.biodic.go.jp/download/lmo/public_comment/H23_11_17_MON87427ap3.pdf).

Outline of the Biological Diversity Risk Assessment Report. 2009. Maize producing thermostable α-amylase, resistant to Lepidopteran and Coleopteran insects and resistant to glufosinate and glyphosate herbicides (modified *amy797E*, modified *cry1Ab*, modified *cry3Aa2*, *pat*, *mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (3272 × Bt11 × MIR604 × GA21, OECD UI:SYN-E3272-5 × SYN-BTØ11-1 × SYN-IR6Ø4-5 × MON-ØØØ21-9) [including the progeny lines isolated from the maize lines, 3272, Bt11, MIR604, and GA21, 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]) Summary of applications etc. (https://ch.biodic.go.jp/bch/OpenSearch.do).

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62 (3) : 775-806.

Shirai, Y. 2003. Gaichuu teikousei idenshi kumikae sakumotsu ga hihyouteki konchuu ni oyobosu eikyou: genzai madeno kenkyuu jirei (Influence of Transgenic Insecticidal Crops on Non-target Insects: Overview from the data to data). *Jpn. J. Appl. Entomol. Zool.* 47: 1-11.

Shirai, Y. and Takahashi, M. 2005. Effects of transgenic Bt corn pollen on a non-target lycaenid butterfly, *Pseudozizeeria maha. Appl. Entomol. Zool.* 40(1) : 151-159.

Tozawa H. 2005. Toumorokoshi-Rekishi, Bunka, Tokusei, Saibai, kakou, Riyou- (Maize – History, Culture, Property, Cultivation, Processing and Use). Rural Culture Association Japan.

USDA. 1995. Petition for Determination of Nonregulated Status for Insect Protected Corn (*Zea mays* L.) with the *cryIA(b)* Gene from *Bacillus thuringiensis* subsp. *kurstaki*. (http://www.aphis.usda.gov/brs/aphisdocs/95_09301p.pdf). Accessed on August 23rd, 2011.

USDA. 1996. Petitions for Determination of Nonregulated Status Granted: Additional YieldGuard™ Corn (*Zea mays* L.) Lines with the *cryIA(b)* Gene from *Bacillus thuringiensis* subsp. *kurstaki*. (http://www.aphis.usda.gov/brs/aphisdocs/96_01701p.pdf). Accessed on August 23rd, 2011.

USDA. 2000. Request for Extension of Determination of Nonregulated Status to the Additional Regulated Article: Roundup Ready Corn Line NK603. (http://www.aphis.usda.gov/brs/aphisdocs/00_01101p.pdf). Accessed on March 30th, 2011.

USDA. 2006. Petition for the Determination of Non-Regulated Status. Corn Rootworm Protected Transformation Event MIR604. (http://www.aphis.usda.gov/brs/aphisdocs/04_36201p.pdf). Accessed on March 30th, 2011.

Westfall, P.H., Tobias, R.D., Rom, D., Wolfinger, R.D. and Hochberg, Y. 2006. Concepts and basic methods for multiple comparisons and tests. *Multiple Comparisons and Multiple Tests Using the SAS System*. Cary, NC, USA: SAS Institute Inc., 13-40.

Trade Statics of Japan. 2011. (http://www.customs.go.jp/toukei/info/index.htm). Accessed on January 14th, 2011.

Annexes

Annex 1 Summary of applications for 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)
(https://ch.biodic.go.jp/bch/OpenSearch.do)

Annex 2 Summary of applications for 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)
(https://ch.biodic.go.jp/bch/OpenSearch.do)

Annex 3 Summary of applications for maize resistant to Lepidoptera (*cry1Ab*, *Zea mays* L.) (MON810, OECD UI:MON-ØØ81Ø-6)
(https://ch.biodic.go.jp/bch/OpenSearch.do)

Annex 4 Summary of applications for maize tolerant to glyphosate herbicide (*cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (NK603, OECD UI: MON-ØØ6Ø3-6)
(https://ch.biodic.go.jp/bch/OpenSearch.do)

Annex 5 Summary of applications for maize resistant to Coleoptera (modified *cry3Aa2*, *Zea mays* subsp. *mays* (L) Iltis) (MIR604, OECD UI:SYN-IR6Ø4-5)
(https://ch.biodic.go.jp/bch/OpenSearch.do)

Annex 6 (Confidential: Not disclosed to unauthorized person)

Annex 7 (Confidential: Not disclosed to unauthorized person)