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

Name: Du Pont Kabushiki Kaisha
Applicant: Minoru Amo, 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 tolerant to glufosinate and glyphosate herbicides (modified *cry1F*, *pat*, *cry1Ab*, modified *vip3A*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (1507×MON810×MIR162×NK603, OECD UI: DAS-Ø15Ø 7-1×MON-ØØ81Ø-6×SYN-IR162-4×MON-ØØ6Ø3-6) (Including the progeny lines from this maize line which have combination of respective transferred genes of *B.t.* Cry1F maize line 1507, MON810, MIR162, and NK603 (except those already granted an approval regarding Type I 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 Effects on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

Maize resistant to *Lepidoptera* pests and tolerant to glufosinate and glyphosate herbicides (modified *cry1F*, *pat*, *cry1Ab*, modified *vip3A*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis)(1507×MON810×MIR162×NK603, OECD UI: DAS-Ø15Ø7-1×MON-ØØ81Ø-6×SYN-IR162-4×MON-ØØ6Ø3-6) (hereinafter referred to as "this stacked maize line") is the line interbred with the following four modified maize lines using the conventional crossing.

This stacked maize line will be commercialized as a F1 hybrid line (F1) and then due to the genetic segregation the grains harvested from this stacked maize line include the maize with a combination of the genes transferred in the respective parent lines of this stacked maize line.

(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 *Lepidoptera* (*cry1Ab*, *Zea mays* L.) (MON810, OECD UI:MON-ØØ81Ø -6) (hereinafter referred to as "MON-00810-6")

(c) Maize resistant to *Lepidoptera* (modified *vip3A*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR162, OECD UI: SYN-IR162-4) (hereinafter referred to as "SYN-IR162-4")

(d) Maize tolerant to glyphosate herbicide (modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis)(NK603, OECD UI: MON- ØØ6Ø3-6) (hereinafter referred to as "MON-00603-6")

As for the four parent lines of this stacked maize lines, DAS-01507-1 (USDA, 2000a; Outline of the application form, etc., 2004a), was developed jointly by Dow AgroSciences (US) and Pioneer Hi-Bred International, Inc (US), and MON-00810-6 (USDA, 1996; Outline of the application form, etc., 2004b) and MON-00603-6 (USDA, 2000b; Outline of the application form, etc., 2004c) were developed by Monsanto Company (US), and SYN-IR162-4 (USDA, 2007; Outline of the Biological Diversity Risk Assessment Report, 2008) by Syngenta AG (Switzerland).*

* For how to view the Outline of the application form, etc. and the Outline of the Biological Diversity Risk Assessment Report of each parent line:

1. Visit the page of "LMO Search" on the website of the Japan Biosafety Clearing House, https://ch.biodic.go.jp/bch/OpenSearch.do.
2. Input "Maize" in the box of "Name of Organism" and then select "Search/View."

3. Select “Maize” from “Name of Organism” of the appropriate line.
4. Select “Attached documents.”
5. Select “Document 1.”

To individual parent lines, the following genes were transferred.

DAS-01507-1 : The modified *cry1F* and *pat* genes conferring resistance to *Lepidoptera* and tolerance to glufosinate herbicide, respectively

MON-00810-6 : The *cry1Ab* gene conferring resistance to *Lepidoptera*

SYN-IR162-4 : The modified *vip3A* and *pmi* genes conferring resistance to *Lepidoptera* and characteristics of the selective marker, respectively

MON-00603-6 : The modified *cp4 epsps* gene conferring glyphosate herbicide

1) Composition and origins of component elements

The composition of donor nucleic acids and the origins of component elements used for development of the parent lines are shown in Tables 1-4 (p. 3-6).

2) Functions of the component element

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

The functions of individual component elements of the donor nucleic acids are shown in Tables 1-4 (p. 3-6).

Table 1. Component of the donor nucleic acids and origins and functions of the component elements used for the development of TAS-01507-1

Component elements	Size(kbp)	Origins and functions
Modified *cry1F* gene expression cassette		
UBIZM1(2) Promoter	1.98	Ubiquitin constitutive promoter derived from *Zea mays** (including intron and 5' untranslated region).
Modified *cry1F*	1.82	Gene coding for the Cry1F protein derived from *Bacillus thuringiensis* var. *aizawai*. It is optimized to enhance the expression in plants.
ORF25PolyA Terminator	0.72	Terminator to terminate transcription derived from *Agrobacterium tumefaciens* pTi5955.
pat gene expression cassette		
CAMV35S Promoter	0.53	35S constitutive promoter derived from the cauliflower mosaic virus.*
pat	0.55	Gene coding for phosphinothricin acetyltransferase (PAT protein) derived from *Streptomyces viridochromogenes*. It is optimized to enhance the expression in plants.
CAMV35S Terminator	0.21	35S terminator to terminate transcription derived from the cauliflower mosaic virus.

* Constitutive promoter: Promoter to express the target genes in the entire plant body.

Table 2. Component of the donor nucleic acids and origins and functions of the component elements used for the development of MON-00810-6

Component elements	Origins and functions
cry1Ab gene cassette	
E35S	It has the 35S promoter of the cauliflower mosaic virus (CaMV) and a double enhancer region. It induces the constitutive expression of the target genes in all tissues.
hsp70 intron	Intron of the heat shock protein gene of maize. The hsp70 intron is used to increase the level of expression of foreign genes in plants.
cry1Ab	Gene coding for the Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain existing in soil.
NOS 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 cassette (It was not inserted into this modified maize, based on the results of analysis of inserted genes.)	
E35S	It has the 35S promoter of the cauliflower mosaic virus (CaMV) and a double enhancer region. It induces the constitutive expression of the target genes in all tissues.
hsp70 intron	Intron of the heat shock protein gene of maize. The hsp70 intron is used to increase the level of expression of foreign genes in plants.
CTP2	N-terminal sequence of chloroplast transit peptide sequence of the *EPSPS* gene of *Arabidopsis*. It transports the target proteins from the cytoplasm to chloroplast.
Modified *cp4 epsps*	Synthesized sequence based on the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium*. It expresses the modified CP4 EPSPS protein with high tolerance to glyphosate.
NOS 3'	3' untranslated region of the nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
gox gene cassette (It was not inserted into this modified maize, based on the results of analysis of inserted genes.)	
E35S	It has the 35S promoter of the cauliflower mosaic virus (CaMV) and a double enhancer region. It induces the constitutive expression of the target genes in all tissues.
hsp70 intron	Intron of the heat shock protein gene of maize. The hsp70 intron is used to increase the level of expression of foreign genes in plants.
CTP1	N-terminal of chloroplast transit peptide sequence of the small subunit 1A of the rubisco gene derived from *A. thaliana*. It transports the target proteins from the cytoplasm to chloroplast.
gox	Synthesized sequence based on the glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. Glyphosate is degraded by the GOX protein.
NOS 3'	3' untranslated region of the nopaline synthase gene of *Agrobacterium tumefaciens*. It contains the transcription terminator and the mRNA polyadenylation signal.
Vector backbone (common to both PV-ZMBK07 and PV-ZMGT10) (It was not inserted into this modified maize, based on the results of analysis of inserted genes.)	
lacZ	Partial coding sequence of β-D-galactosidase or the LacZ protein. The substrate, Xgal, is degraded by β-D-galactosidase to turn blue. It is used as a selective marker during cloning in *Escherichia coli*.
ori-pUC	Segment containing the replication initiation region of the *Escherichia coli* plasmid pUC. It initiates the replication of the plasmid.

nptII	Gene separated from prokaryotic transposon Tn5. It codes for the neomycin phosphotransferase II. The expression of this gene in microorganisms confers to kanamycin tolerance. It functions as a selective marker for transformation.

Table 3. Component of the donor nucleic acids and origins and functions of the component elements used for the development of SYN-IR162-4

Component elements	Size (bp)	Origins and functions
Pest resistant gene cassette		
ZmUbiInt promoter	1,993	Promoter containing the first intron region (1,010bp) derived from the maize polyubiquitin gene. It induces the constitutive expression of the target genes in all tissues of monocot.
Modified *vip3A* gene	2,370	Gene modified from the *vip3A* gene derived from a gram-positive bacillus commonly living in soil, *Bacillus thuringiensis* AB88 strain, to have a codon appropriate for expression in plants. It codes for the modified Vip3A protein having insecticidal activity to *Lepidoptera* insects. In the modified Vip3A protein, an amino acid at position 284 of the amino acid sequence, lysine, is replaced by glutamine. In the modified Vip3A protein expressed in this recombinant, methionine at position 129 is replaced by isoleucine in preparing a transformant.
iPEPC9	108	Intron #9 sequence derived from the maize phosphoenolpyruvate carboxylase gene. It was used to enhance the expression of the target genes.
35S terminator	70	Polyadenylation sequence derived from the 35S RNA of the cauliflower mosaic virus.
Selective marker gene cassette		
ZmUbiInt promoter	1,993	Same as above.
pmi gene	1,176	*manA* gene derived from the *Escherichia coli* K-12 strain producing phosphomannose isomerase (hereinafter referred to as "PMI protein"). It was used as a selective marker for the transgenic transformant.
NOS terminator	253	Terminator sequence of the nopaline synthase gene of *Agrobacterium tumefaciens*. It terminates the mRNA transcription by polyadenylation.
Other regions (hereinafter referred to as "vector backbone region")		
LB	25	T-DNA left border region derived from the nopaline Ti-plasmid derived from *A. tumefaciens*.
spec	789	Streptomycin adenylyltransferase gene (*aadA*) of the *E. coli* transposon Tn7. It was used as a selective marker of the vector in order to confer resistance to streptomycin and spectinomycin.
cos	432	Cohesive end region of the linear DNA of the lambda phage necessary to transfer the plasmid to *E. coli* and for self-replication of the plasmid in *E. coli*.
ColE1 ori	807	Replication origin of the plasmid derived from *E.coli*.
RB	25	T-DNA right border region derived from the nopaline Ti-plasmid derived from *A. tumefaciens*.

Table 4. Component of the donor nucleic acids and origins and functions of the component elements used for the development of MON-00603-6

Component elements	Size (bp)	Origins and functions
Modified *cp4 epsps* gene cassette (1)		
P-ract1	0.9	Promoter region of the actin 1 gene derived from rice. It induces the expression of the target genes.
ract1 intron	0.5	Intron of the rice actin gene. It induces the expression of the target genes by improving splicing efficiency.
CTP2	0.2	Sequence coding for the N-terminal chloroplast transit peptide of the protein EPSPS in the *epsps* gene of *Arabidopsis*. It transports the target proteins from the cytoplasm to chloroplast.
Modified *cp4 epsps*	1.4	5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* CP4 strain.
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 cassette (2)		
E35S	0.6	It has the 35S promoter of the cauliflower mosaic virus (CaMV) and a double enhancer region. It induces the constitutive expression of the target genes in all tissues.
ZmHsp70 Intron	0.8	Intron of the heat shock protein gene of maize. The ZmHsp 70 intron is used to increase the level of expression of foreign genes in plants.
CTP2	0.23	Sequence coding for the N-terminal chloroplast transit peptide of the protein EPSPS in the *epsps* gene of *Arabidopsis*. It transports the target proteins from the cytoplasm to chloroplast.
Modified *cp4 epsps*	1.37	5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* CP4 strain.
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 that is known to possess any allergenicity

a. Functions of proteins produced by target gene expression

[Pest resistance proteins]

The modified Cry1F, Cry1Ab, and modified Vip3A proteins produced in the DAS-01507-1, MON-00810-6, and SYN-IR162-4, respectively, are all an insecticidal protein derived from *Bacillus thuringiensis* (hereinafter referred to as "Bt protein") and have high insecticidal activity against *Lepidoptera* insects. The Bt protein generally exhibits the insecticidal activity by forming pores in the midgut cells of a pest to destroy them (Schnepf *et al.*, 1998; Lee *et al.*, 2003). Because each of the modified Cry1F, Cry1Ab, and modified Vip3A proteins function by binding to each specific receptor, those proteins are active against specific spectrum of insects (Shirai, 2003; Lee *et al.*, 2003; Jurat-Fuentes *et al.*, 2006).

Modified Cry1F protein:

The modified Cry1F protein is a δ-endotoxin derived from *B. thuringiensis* var. *aizawai*. It exhibits high insecticidal activity against several *Lepidoptera* pests, European com borer (*Ostrinia nubilalis*), Fall armyworm (*Spodoptera frugiperda*), and Beet armyworm (*Spodoptera exigua*), but the toxicity of the protein has not been observed against insects other than *Lepidoptera* insects, including *Coleoptera*, *Hymenoptera*, *Neuroptera* and *Collembola*, and non-target organisms, including mammals, birds, and fish (EPA, 2010).

Cry1Ab protein:

The Cry1Ab protein is a δ-endotoxin derived from *B. thuringiensis* subsp. *kurstaki*. It exhibits insecticidal activity against several *Lepidoptera* pests, European corn borer (*Ostrinia nubilalis*), Southwestern corn borer (*Diatraea grandiosella*), Southern cornstalk borer (*Diatraea crambidoides*), Corn earworm (*Helicoverpa zea*), Fall armyworm (*Spodoptera frugiperda*), and Stalk borer (*Papaipema nebris*), but the toxicity of the protein has not been observed against insects other than *Lepidoptera* insects, including *Coleoptera*, *Hymenoptera*, *Neuroptera* and *Collembola*, and non-target organisms, including mammals, birds, and fish (EPA, 2010).

Modified Vip3A protein:

The modified Vip3A protein is an extracellular secretory protein derived from *B. thuringiensis* AB88 strain. It exhibits high insecticidal activity against several *Lepidoptera* pests, Black cutworm (*Agrotis ipsilon*), Fall armyworm (*Spodoptera frugiperda*), and Corn earworm (*Helicoverpa zea*), but the toxicity of the protein has not been observed against insects other than *Lepidoptera* insects, including *Coleoptera*, *Hymenoptera*, *Hemiptera*, *Neuroptera* and *Collembola*, and non-target organisms, including mammals, birds, and fish (USDA, 2007).

[Herbicide tolerant proteins]

PAT protein:

L-glufosinate, an active ingredient of the glufosinate herbicide inhibits the activity of glutamine synthetase and the resulting accumulation of ammonia in the plant body lead the plant to die. The PAT protein produced in DAS-01507-1 acetylates and detoxifies L-glufosinate to confer tolerance to glufosinate herbicide on plants (OECD, 2002).

Modified CP4 EPSPS protein:

The glyphosate herbicide inhibits the activity of 5-enolpyruvylshikimate 3-phosphate synthetase (enzyme number: E.C.2.5.1.19, hereinafter referred to as "EPSPS protein") in the shikimic acid pathway, the aromatic amino acid synthetic pathway in plants. Therefore, the aromatic amino acid cannot be synthesized resulting in plant death. Because the modified CP4 EPSPS protein produced in MON-00603-6 has the enzymatic activity even under the presence of glyphosate and the shikimic acid pathway is not inhibited, this protein confers tolerance to glyphosate herbicide on plants.

[Selective marker]

PMI protein:

Maize cannot use mannose as a carbon source. The PMI protein produced in SYN-IR162-4 reversibly interconverts mannose-6-phosphate and fructose-6-phosphate, and then mannose can be used as a carbon source. Therefore, this protein was used as a selective marker. The PMI protein is not present in maize but has been confirmed to be widely present in human digestive organs and plants such as soybeans.

b. Homology to known allergen proteins

Using several databases, the amino acid sequences homology between the modified Cry1F, PAT, Cry1Ab, modified Vip3A, PMI, and modified CP4 EPSPS proteins and the known allergens were searched[1)]. As a result, it has been confirmed that there was no homology between these proteins and the known allergens.

[1)] Modified Cry1F and PAT proteins: Database FARRP 12.0, searched in May, 2012.
Cry1Ab protein: Database NCBI Release 181.0, searched in July, 2011.
Modified CP4 EPSPS protein: Database AD_2011, TOX_2011, and PRT_2011, searched in February, 2011.
Modified Vip3A and PMI proteins: Database FARRP 12.0, searched in April, 2012.

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

Bt proteins:

The modified Cry1F, Cry1Ab, and modified Vip3A proteins are all the Bt proteins derived from *B. thuringiensis*. A number of studies on the functions of the Bt proteins have been performed. It has been concluded that the Bt proteins have the insecticidal activity because they destroy the midgut of the target insect by forming pores in it (OECD, 2007; Lee *et al.*, 2003). However, there have been no reports that the Bt proteins have enzymatic activities.

PAT protein:

The PAT protein acetylates the free amino group of L-glufosinate, an active ingredient of the glufosinate herbicide. However, this protein does not use D-glufosinate, an enantiomer of L-glufosinate, L-glutamic acid especially whose structure is similar to L-glufosinate, and other L-amino acids as a substrate. The acetylated reaction of L-glufosinate by this protein is not inhibited even in the excessive presence of various amino acids. Therefore, it has been concluded that the PAT protein has extremely high substrate specificity to L-glufosinate (OECD, 1999).

Modified CP4 EPSPS protein:

The modified CP4 EPSPS protein has the same function as the EPSPS protein. The EPSPS protein is not a rate-limiting enzyme in the shikimic acid pathway for synthesis of aromatic amino acids. Therefore, it has been considered that the levels of the aromatic amino acids, the end products of this pathway, do not increase even with the increased activities of the EPSPS proteins by producing the modified CP4 EPSPS protein. In fact, it has been confirmed that there is no difference between the amount of the aromatic amino acids of genetically modified crops tolerant to glyphosate herbicide (rapeseed, cotton, soybean, and maize) and that of non-genetically-modified crops (CFIA, 1995; Nida *et al.*, 1996; Padgette *et al.*, 1996; Ridley *et al.*, 2002). In addition, the EPSPS protein specifically reacts with the substrates, phosphoenolpyruvate (hereinafter referred to as "PEP") and shikimate-3-phosphate (hereinafter referred to as "S3P"). It is known that it also reacts with shikimic acid, an analog of S3P. However, the comparison by the specificity constant (k_{cat}/K_m), which represents the degree of occurrence of reaction, showed that the reaction specificity between the EPSPS protein and shikimic acid is one two-millionth of that between the EPSPS protein and S3P, and shikimic acid is unlikely to react as a substrate of the EPSPS protein (Gruys *et al.*, 1992).

PMI protein:

The PMI protein is an enzyme protein, which catalyzes the reversible interconversion between mannose-6-phosphate and fructose-6-phosphate. The reaction of the PMI protein is specific to mannose-6-phosphate and fructose-6-phosphate. No other natural substrates have been reported (Freeze, 2002).

Based on the above, these proteins are unlikely to change the metabolic system of recipient organisms.

(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 from the *E. coli* plasmid pUC19 (Figure 1, p. 12)

MON-00810-6: Plasmids PV-ZMBK07 and PV-ZMGT10 constructed from the *E. coli* plasmid pUC119 (Figure 2, p. 13)

SYN-IR162-4: Plasmid pNOV1300 constructed from the *E. coli* plasmid pSB12 (Figure 3, p. 14)

MON-00603-6: Plasmid PV-ZMGT32 constructed from the *E. coli* plasmid pUC119 (Figure 4, p. 15)

2) Properties

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

The numbers of base pairs in the plasmids used for the development of parent lines are as follows.

DAS-01507-1: 9,504 bp (PHP8999)

MON-00810-6: 7,800 bp (PV-ZMBK07) and 9,447 bp (PV-ZMGT10)

SYN-IR162-4: 14,405 bp (pNOV1300)

MON-00603-6: 9,308 bp (PV-ZMGT32)

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

The following genes were used as a selective marker of the vectors. None of these marker genes have been confirmed to be transferred into parent lines.

DAS-01507-1: Gene resistant to kanamycin/neomycin (*nptII* gene)

MON-00810-6: Partial coding sequence of β-D-galactosidase (LacZ protein) (*lacZ* gene) and gene resistant to kanamycin/neomycin (*nptII* gene)

SYN-IR162-4: Gene resistant to streptomycin/spectinomycin (*spec* gene)

MON-00603-6: Partial coding sequence of β-D-galactosidase (LacZ protein) (*lacZ* gene) and gene resistant to kanamycin/neomycin (*nptII* gene)

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

In the vector pNOV1300 used for developing SYN-IR162-4, exists a cohesive end region derived from the lambda phage, *cos*, which allows the plasmid to transfer to *E. coli*. However, no recipient organisms of the lambda phage other than *E. coli* have been known. The other vectors are not infectious.

(3) Method of preparing living modified organisms

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

Figures 1 to 4 (p. 12-15) show the structure of the donor nucleic acids and the restriction enzyme cleavage sites used for the development of new parent lines, DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6, respectively.

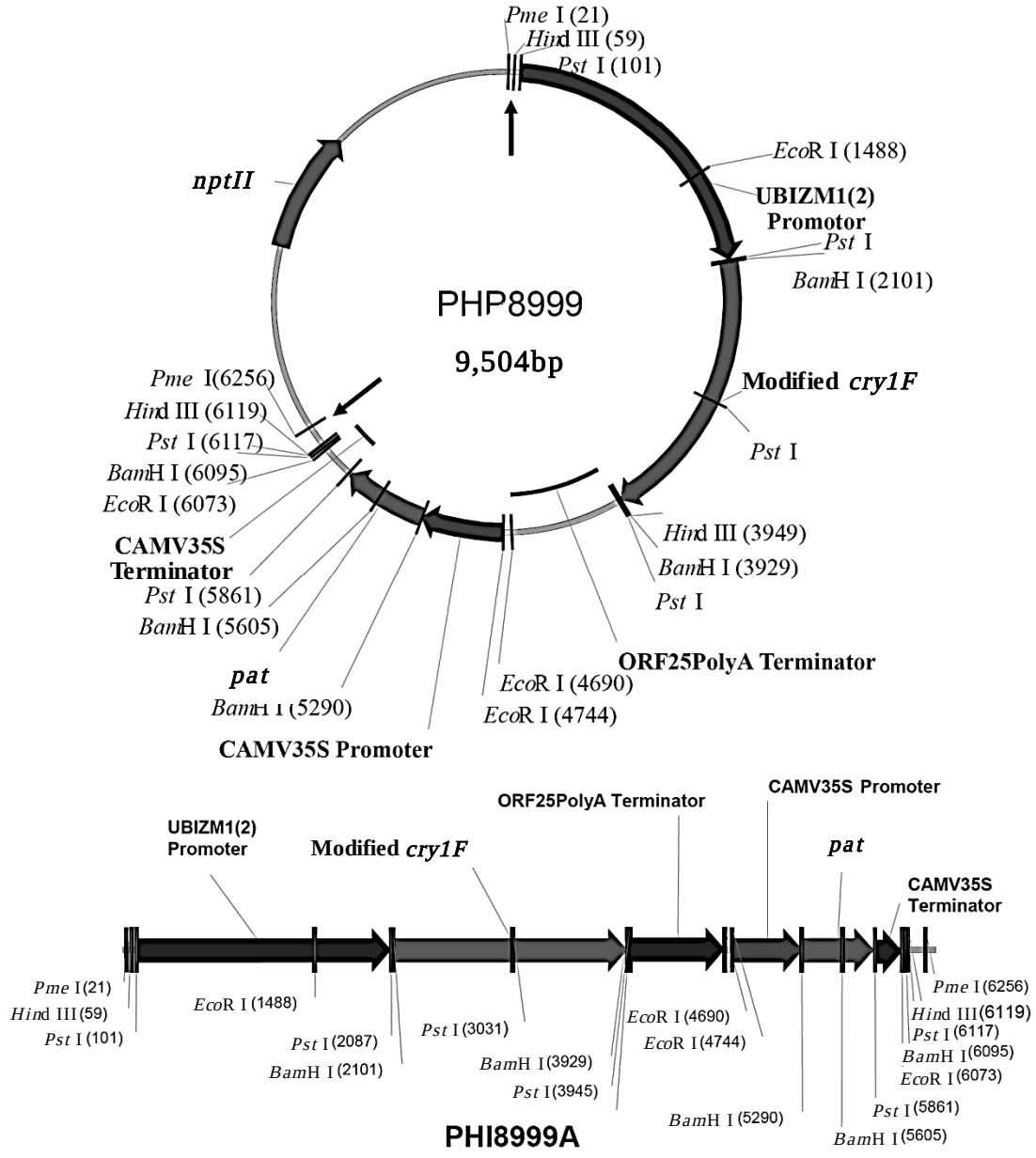

Figure 1 Structure of the plasmid PHP8999* (upper figure) and inserted DNA region PHI8999A (lower figure)

* Vector used for developing DAS-01507-1

The plasmid PHP8999 was treated with the restriction enzyme *Pme* I (cleave at the two arrows in the upper figure) to prepare a linear DNA fragment, PHI8999A (the lower figure). It was used for transfection into the recipient organism.

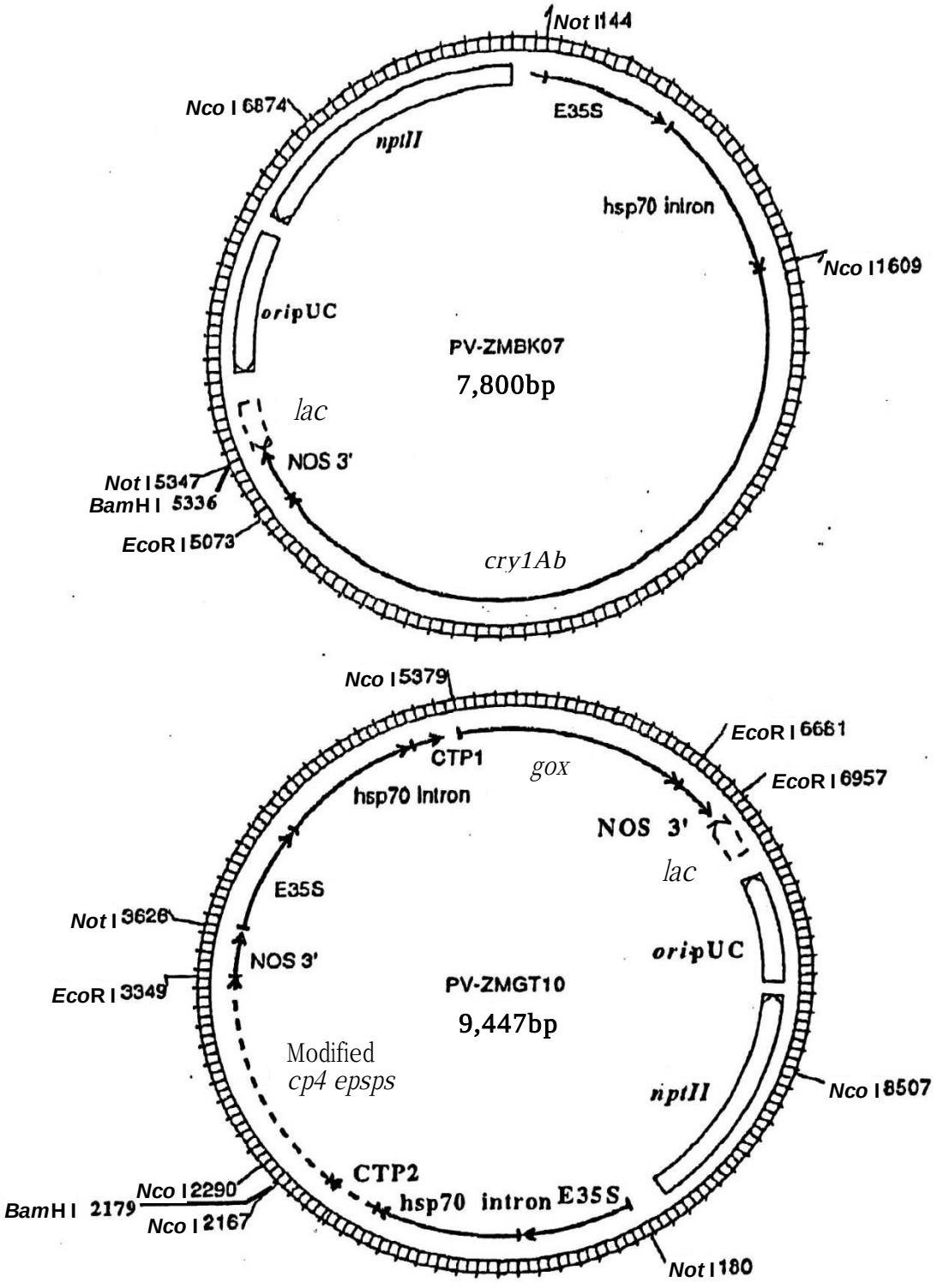

Figure 2 Structures of plasmids PV-ZMBK07 and PV-ZMGT10*

* Vector used for developing MON-00810-6

The mix of cyclic plasmids, PV-ZMBK07 and PV-ZMGT10, was used for transfection into the recipient organism. It was confirmed that only the *cry1Ab* expression cassette of the PV-ZMBK07 (Table 2, p. 9) was actually inserted (I, 1, (4), (b); p. 20).

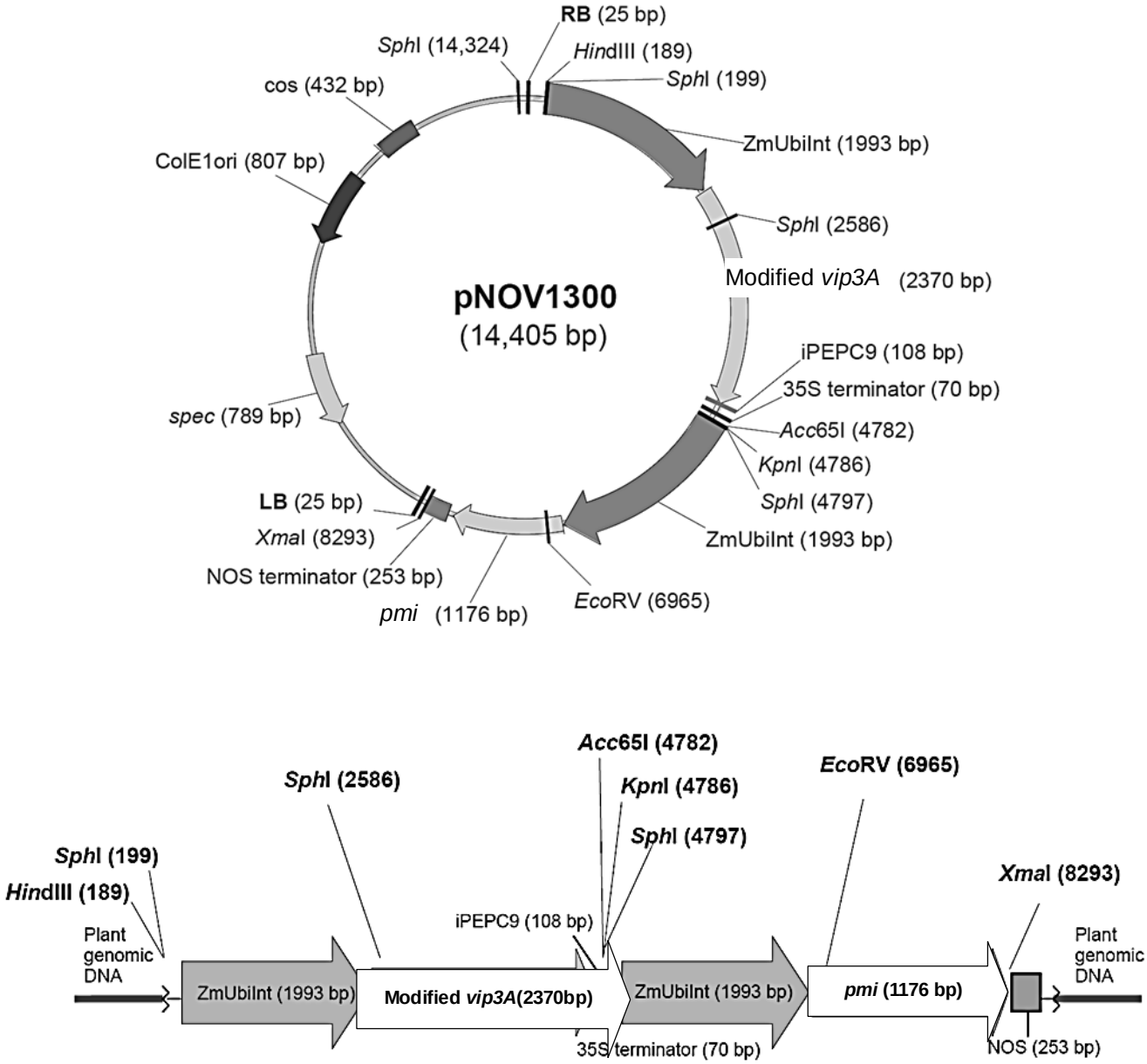

Figure 3 Structures of the plasmid pNOV1300* (upper figure) and the inserted T-DNA region (lower figure)

* Vector used for developing SYN-IR162-4

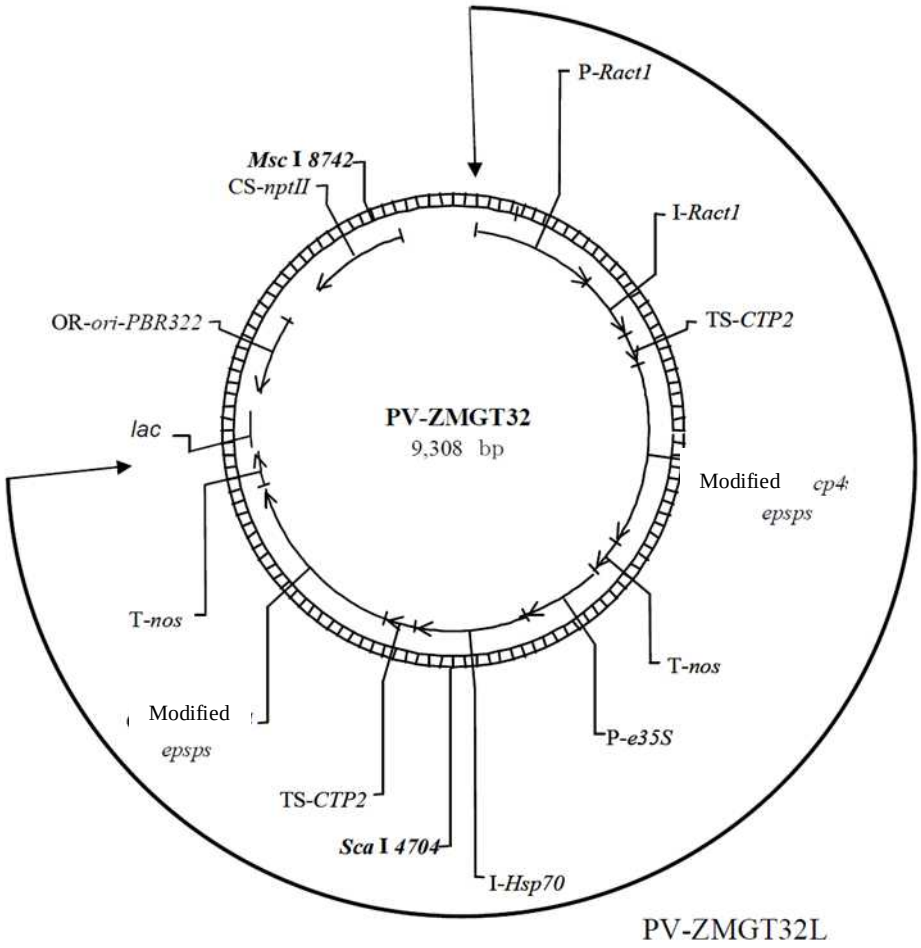

Figure 4 Structure of the plasmid PV-ZMGT32*

* Vector used for developing MON-00603-6

The plasmid PV-ZMGT32 was treated with the restriction enzyme *Mlu*I to prepare a linear DNA fragment, PV-ZMGT32L. It was used for transfection into the recipient organism.

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

The transfer of nucleic acids to the recipient organisms was performed using the particle gun method for DAS-01507-1, MON-00810-6 and MON-00603-6, and the agrobacterium method for SYN-IR162-4.

3) Breeding process of living modified organisms

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

The cells containing the transferred nucleic acids were selected by culture in the media added with the following.

DAS-01507-1: Glufosinate herbicide
MON-00810-6: Glyphosate herbicide
SYN-IR162-4: Mannose
MON-00603-6: Glyphosate herbicide

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

For SYN-IR162-4 developed using the agrobacterium method, *Agrobacterium* was removed by adding cefotaxime to the mannose medium. The PCR was performed in the redifferentiated plants for confirmation. As a result, the antibiotic resistance marker gene (*spec* gene), which is contained in the vector backbone region of the plasmid, was not detected. It is therefore concluded that *Agrobacterium* is absent.

(c)Breeding process containing the following lines: cells to which the nucleic acid was transferred; the line in which the state of existence of replication products of transferred nucleic acid was confirmed; the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effects on Biological Diversity

This stacked maize line was developed by crossing DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6. The development process is shown in Figure 5 (p. 17). The status of approval of these parent lines in Japan is described in Table 5 (p. 17).

(Confidential information: not disclosed to any unauthorized person)

Figure 5 Example of breeding this stacked maize line

Table 5 The status of approval of parent lines and this stacked maize line in Japan

Line	Food [1)]	Feed [2)]	Environment [3)]
DAS-01507-1	July 8, 2002	March 27, 2003	March 2, 2005
MON-00810-6	March 30, 2001	March 27, 2003	June 1, 2004
SYN-IR162-4	January 21, 2010	June 1, 2010	June 11, 2010
MON-00603-6	March 30, 2001	March 27, 2003	November 22, 2004
This stacked maize line	Applied, 2012	Scheduled for notification, 2012	Applied, 2012

1) Food Sanitation Act (Act No. 233, 1947)

2) Act on Safety Assurance and Quality Improvement of Feeds (Act No. 35, 1953)

3) Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Act No. 97, 2003)

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

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

It has been confirmed that the traits of DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6 were inherited according to Mendel's law and the replication products of transferred nucleic acids are present in the chromosomal genome of maize.

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

Southern blot analysis was performed for the copy number of the transferred gene and stability of their transmission in each parent line.

DAS-01507-1:

It has been confirmed that one copy each of the modified *cry1F* gene expression cassette and the *pat* gene expression cassette is inserted into the maize genome and they are inherited stably in progenies.

It has been confirmed by base sequence analysis of the transferred DNA that a part of the modified *cry1F* gene sequence, of the *pat* gene sequence and of the ORF25PolyA Terminator sequence are contained in the 5'-terminal region, the 5'- and 3'-terminal regions , and the 3'-terminal region, respectively. However, it has been confirmed by Northern blot analysis that their gene fragments were not transcribed into mRNA or functioned.

MON-00810-6：

It has been confirmed that the DNA fragment derived from PV-ZMBK07 necessary for expression of one copy of the *cry1Ab* gene was inserted into the maize genome and inherited stably in progenies.

In addition, it has been confirmed by Southern blot analysis that only the region necessary for generation of the Cry1Ab protein derived from PV-ZMBK07 was inserted into the maize genome and that *nptII* gene cassettes derived from PV-ZMBK07, the modified *cp4 epsps* and *gox* gene cassettes derived from PV-ZMGT10 were absent.

SYN-IR162-4:

It has been confirmed that one copy each of the modified *vip3* and *pmi* genes was inserted into the maize genome and inherited stably in progenies.

MON-00603-6:

It has been confirmed that one copy of the PV-ZMGT32L (including two modified *cp4 epsps* gene expression cassettes) was inserted into the maize genome and

inherited stably in progenies.

In addition, a 217 bp fragment of the *P-Ract1* was transferred in the reverse direction into close to the 3'-terminal of the inserted gene. However, it has been confirmed by Western blot analysis that this fragment is not involved in the generation of new proteins. Moreover the base of the modified *cp4 epsps* gene induced by the P-*e35S* has changed in the development of MON-00603-6 and one of the amino acids constituting the modified CP4 EPSPS protein has changed. However, the amino acid is not included in the amino acids necessary for activity of the EPSPS protein family, this change does not affect the active site and three-dimensional structure of the protein, and its enzyme activity and immunoreactivity are comparable to those of the original protein. Therefore, it was concluded that the structure and function of the protein had not changed.

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

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

The stability of the expression of the parent lines of this stacked maize line was confirmed by the following methods.

DAS-01507-1: Bioassay for resistance to *Lepidoptera* pests, application study on glufosinate herbicide, and confirmation of generation of proteins by ELISA

MON-00810-6: Bioassay for resistance to *Lepidoptera* pests

SYN-IR162-4: Bioassay for resistance to *Lepidoptera* pests and confirmation of generation of proteins by ELISA

MON-00603-6: Application study on glyphosate herbicide

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

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

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

Detection method:

The line-specific detection method using real-time quantitative PCR for respective parent lines (DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6), published on the European Commission website (Joint Research Centre, 2012).

Sensitivity:

It has been confirmed that the limits of determination of the line-specific detection method of DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6 are 0.08, 0.1, 0.08, and 0.1%, respectively.

Reliability:

Reliability of the line-specific detection method of DAS-01507-1, MON-00810-6, SYN-IR162-4, and MON-00603-6 was confirmed in the collaborative studies performed by 14, 14, 12, and 12 testing laboratories, respectively, which are a member of the European Network of GMO Laboratories.

For detection and identification of this stacked maize line, a seed or a plant is analyzed by the above-mentioned methods.

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

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

This stacked maize line has been conferred of the following traits derived from individual parent lines.

DAS-01507-1: Resistance to *Lepidoptera* pests by the modified *cry1F* gene and tolerance to glufosinate herbicide by the *pat* gene
MON-00810-6: Resistance to *Lepidoptera* pests by the *cry1Ab* gene
SYN-IR162-4: Resistance to *Lepidoptera* pests by the modified *vip3A* gene
MON-00603-6: Tolerance to glyphosate herbicide by the modified *cp4 epsps* gene

In addition, in SYN-IR162-4, the *pmi* gene was transferred as a selective marker.

The possibility of the functional interaction of these proteins generated by these genes was examined in terms of proteins with resistance to pests and herbicides.

<u>Functional interaction among proteins with resistance to pests</u>

The modified Cry1F, Cry1Ab, and modified Vip3A proteins exhibit insecticidal activity against *Lepidoptera* pests (I, 1, (1), 2), (b); p.9). The specificity of insecticidal activity

exhibited by the proteins with resistance to pests is associated with the protein structures. Then, it is concluded that the insecticidal activity against target pests is not affected unless the region related to the specificity is changed.

Therefore, it is concluded that the proteins with resistance to pests do not interact in this stacked maize line, and it is difficult to think that insecticidal activity or insecticidal spectrum of each protein with resistance to pests synergistically increases or expands.

Functional interaction among proteins with tolerance to herbicides

Both the PAT and modified CP4 EPSPS proteins exhibit enzyme activity. The substrates of these proteins are different, L-glufosinate for the PAT protein, and PEP and S3P for the modified CP4 EPSPS protein. Both proteins are known to have substrate specificity. Also, each metabolic pathway is independent of each other (I, 1, (1), 2), (c); p. 11). Therefore, it is difficult to think that these proteins interact to produce an unexpected metabolite.

Functional interaction between proteins with resistance to pests and proteins with tolerance to herbicides

Since the proteins with resistance to pests and proteins with tolerance to herbicides have independent mechanism of action, it is difficult to think that both types of proteins affect each other.

Functional interaction with other proteins

The *pmi* gene is also transferred in SYN-IR162-4 as a selective marker, and the PMI protein generated by this gene reversibly interconverts mannose-6-phosphate and fructose-6-phosphate. The activity of this protein is specific to mannose-6-phosphate and fructose-6-phosphate and is unlikely to affect other metabolic pathways. Therefore, the above proteins with resistance to pests and proteins with tolerance to herbicides are highly unlikely to interact with PMI protein.

Based on the above, it is difficult to think that the proteins derived from each parent line interact with one another. It has been concluded that the new traits conferred by the transferred genes on respective parent lines do not change in this stacked maize line.

Therefore, differences in physiological and ecological properties between this stacked maize line and the species of the taxonomy to which the recipient organism belongs were evaluated based on the results of the individual examination of the parent lines.

(b) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

The Biological Diversity Risk Assessment Report of respective parent lines was completed. Based on the evaluation of the following physiological or ecological properties, from a to g, it is concluded that all parent lines are within the range of the taxonomic species (maize) to which the recipient organism belongs.*

a. Morphological and growth characteristics
b. Cold-resistance and heat-resistance at the early stage of growth
c. Wintering ability and summer survival of the mature plant
d. Fertility and size of the pollen
e. Production, shedding habit, dormancy, and germination rate of the seed
f. Crossability
g. Productivity of harmful substances

* For the Biological Diversity Risk Assessment Report of respective parent lines, see the footnote on page 1 in this report.

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

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

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

Maize resistant to *Lepidoptera* pests and tolerant to glufosinate and glyphosate herbicides (Including the progeny lines from this maize line which have combination of respective transferred genes of *B.t*. Cry1F maize line 1507, MON810, MIR162, and NK603 (except those already granted an approval regarding Type I Use Regulation)) (hereinafter referred to as "this stacked line") was developed with the following lines by crossing.

(a) Maize resistant to *Lepidoptera* pests and tolerant to glufosinate herbicide, to which the modified *cry1F* gene coding for the modified Cry1F protein and the *pat* gene coding for the PAT protein (phosphinothricin acetyltransferase) are transferred (*B.t*. Cry1F maize line 1507)

(b) Maize resistant to *Lepidoptera* pests, to which the *cry1Ab* gene coding for the Cry1Ab protein is transferred (MON810)

(c) Maize resistant to *Lepidoptera* pests, to which the modified *vip3A* coding for the modified Vip3A protein (Bt protein) and the *pmi* gene coding for the PMI protein (mannosephosphate isomerase) are transferred (MIR162)

(d) Maize tolerant to glyphosate herbicide, to which the modified *cp4 epsps* gene coding for the modified CP4 EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase) is transferred (NK603)

It was not thought that the respective Bt proteins (modified Cry1F, Cry1Ab, and modified Vip3A proteins) derived from the genes transferred to this stacked line interact with one another to change the specificity of the insecticidal effect in these proteins, because the regions involved in the specificity were not changed. For two proteins with tolerance to herbicides, the PAT and modified CP4 EPSPS proteins have different substrates and actions respectively and their metabolic pathways are independent of each other. In addition there has been no report that Bt proteins have enzyme activities. Therefore it is unlikely that the proteins with tolerance to herbicides and the Bt proteins interact with one another. The PMI protein with high substrate specificity is also unlikely to interact the proteins with tolerance to herbicides and the Bt proteins. Therefore, it was concluded that these proteins did not

interact to change the metabolic system of the recipient organism to produce an unexpected metabolite in this stacked line.

Based on the above, it was unlikely that these proteins derived from respective parent lines functionally interact with one another in the plant body of this stacked maize line, and therefore it was concluded that there were no trait changes to be evaluated, except having the traits which the parent line had.
The examination of the respective evaluation items listed below of the respective parent line has already been completed*. Based on the results of the examination, the conclusion described in the Biological Diversity Risk Assessment Report that the use of the respective parent lines in accordance with the Type I Use Regulation causes no Adverse Effect on Biological Diversity in Japan has been judged to be reasonable.

a. Competitiveness
b. Productivity of harmful substances
c. Crossability

* The results of the evaluation of the respective parent lines are available as described below.

- *B.t*. Cry1F maize line 1507
  https://ch.biodic.go.jp/bch/OpenDocDownload.do?info_id=138&ref_no=2
- MON810
  https://ch.biodic.go.jp/bch/OpenDocDownload.do?info_id=6&ref_no=2
- MIR162
  https://ch.biodic.go.jp/bch/OpenDocDownload.do?info_id=1493&ref_no=2
- NK603
  https://ch.biodic.go.jp/bch/OpenDocDownload.do?info_id=88&ref_no=2

(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 stacked maize line, in accordance with the Type 1 Use Regulation, causes Adverse Effects on Biological Diversity in Japan. It was judged that the conclusion made above by the applicant is reasonable.

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List of documents

Document 1: Opinion of the person with relevant knowledge and experience. 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)
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