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

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

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides (modified *cry1F,* modified *cry3Aa2, pat*, modified *cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (1507×MIR604×NK603, OECD UI: DAS-Ø15Ø7-1×SYN-IR6Ø4-5×MON-ØØ6Ø3-6) [including the progeny lines isolated from the maize lines, *B.t.* Cry1F maize line 1507, MIR604 and NK603, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type 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 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*, modified *cry3Aa2*, *pat*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (1507×MIR604×NK603, OECD UI: DAS-Ø15Ø7-1×SYN-IR6Ø4-5×MON-ØØ6Ø3-6) (hereinafter referred to as “this stack maize line”) is a cross progeny line developed by crossing the following three (3) 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-01507-1) (hereinafter referred to as "DAS-01507-1")
(b) 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 ")
(c) 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")

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

DAS-01507-1: The modified *cry1F* gene to confer resistance to the insects of order Lepidoptera and the *pat* gene to confer tolerance to glufosinate herbicide
SYN-IR604-5: The modified *cry3Aa2* gene to confer resistance to the insects of order Coleoptera and the *pmi* gene to confer the characteristic of selective marker
MON-00603-6: The *cp4 epsps* gene to confer tolerance to glyphosate herbicide

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of parent lines are shown individually in Table 1 to 3 (p. 4 – 6).

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid are shown individually in Table 1 to 3 (p. 4 – 6).

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

Component elements	Size (kbp)	Origin and function
Modified *cry1F* gene expression cassette		
UBIZM1(2) Promoter	1.98	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 expression cassette		
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 (CaMV)

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

Table 2 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of SYN-IR604-5

Component elements	Size (kbp)	Origin and function
Insect pest-resistant gene cassette		
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 target insect of order Coleoptera, the nucleotide sequence was modified as follows; the 108th to 110th amino acid sequence (valine-serine-serine) of the Cry3Aa2 protein was changed to be four (4) 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 has 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 cassette		
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 adenylyltransferase 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 *Agrobacterium 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 *Agrobacterium tumefaciens* nopaline Ti-plasmid.
RB	0.03	T-DNA right border region derived from *Agrobacterium tumefaciens* nopaline Ti-plasmid.
VirG	0.73	A region involved in transfer of T-DNA, derived from *Agrobacterium 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.

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Table 3 Composition of the donor nucleic acid and the origins and functions of component elements used for the development of MON-00603-6

Component elements	Size (kbp)	Origin and function
Modified *cp4 epsps* gene cassette (1)		
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.
CTP 2	0.2	N-terminal chloroplast transit peptide sequence in the EPSPS protein, derived from the *Arabidopsis thaliana epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
Modified *cp4 epsps*	1.4	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived from *Agrobacterium* strain CP4. 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 *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.
Modified *cp4 epsps* gene cassette (2)		
E35S	0.6	35S promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). This promoter makes the target gene expressed in all the tissues constitutively.
ZmHsp70 Intron	0.8	Intron of heat stress protein (heat shock protein) gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
CTP2	0.23	N-terminal chloroplast transit peptide sequence in the EPSPS protein, derived from the *Arabidopsis thaliana epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
Modified *cp4 epsps*	1.37	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived from *Agrobacterium* strain CP4. 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 *Agrobacterium tumefaciens* T-DNA. It terminates transcription of mRNA and induces polyadenylation.

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

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

[The insecticidal protein]

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

Modified Cry1F protein:
The modified Cry1F protein is the δ-endotoxin which 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 Lepidopteran 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 order Coleoptera, Hymenoptera, Neuroptera and Collembola, and the mammals, birds, fish and non-target organisms (EPA, 2005).

Modified Cry3Aa2 protein:
The modified Cry3Aa2 protein is the δ-endotoxin which was derived from *Bacillus thuringiensis* subsp. *tenebrionis.*

The modified Cry3Aa2 protein exhibits insecticidal activity against the following four (4) Coleopteran insects [Western corn rootworm (*Diabrotica virgifera virgifera*), Northern corn rootworm (*Diabrotica longicornis barberi*), 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 the mammals, birds, fish and non-target organisms (Outline of the Biological Diversity Risk Assessment Report, 2009; USDA, 2006).

[Herbicide tolerant protein]

PAT protein:
Glufosinate herbicide inhibits glutamine synthase in plants by its active ingredient *L*-glufosinate, causing plants to die due to accumulation of ammonia in the cells, one of the enzyme's substrate. However, the PAT protein acetylates and inactivates *L*-glufosinate, so that the tolerance to glufosinate was conferred to the plant (OECD, 2002).

Modified CP4 EPSPS protein:
The glyphosate herbicide inhibits the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic

amino acid biosynthesis, and interrupts the aromatic amino acid biosynthesis, thereby causing 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.

[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 available database in 2010 (AD_2010, TOX_2010 and PRT_2010 for the modified CP4 EPSPS protein, and FARRP10.0 for the other proteins). The result indicated that the modified Cry1F protein, the modified Cry3Aa2 protein, the PAT protein, the modified CP4 EPSPS protein and the PMI protein do not share structural homology with any of the known allergens.

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

<u>Bt protein</u>
There is no report that the modified Cry1F protein and the modified Cry3Aa2 protein possess enzymatic activity.

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

<u>Modified CP4 EPSPS protein</u>
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 acids. As such, the enhanced EPSPS activity due to the modified CP4 EPSPS protein would not increase the concentration of aromatic amino acids, the end products of this pathway (Annex 3; Ridley *et al*., 2002; Padgette *et al*., 1996). Actually, it has been confirmed that the content of aromatic amino acid in the seed exhibits no difference between the glyphosate-tolerant recombinant crops (soybean, oilseed rape, cotton and maize) and non-recombinant crops.

In addition, the EPSPS protein is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Gruys *et al*., 1992). The only substance that is known to react with the EPSPS protein other than these is shikimate, but the reactivity with shikimate is only one-two millionth of that of S3P; therefore it is considered unlikely to react as the substrate of EPSPS in any living organisms (Annex 3).

PMI protein

The PMI protein 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 there is no other natural substrate known for the PMI protein (Freeze *et al*., 2002).

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

(2) Information concerning vectors

1) Name and origin

The plasmid vectors used for the development of the parent lines are as follows.
DAS-01507-1: Plasmid PHP8999 constructed based on the plasmid pUC19 derived from *Escherichia coli* (Figure 1, p.10).
SYN-IR604-5: Plasmid pZM26 constructed based on the plasmid pUC19 derived from *E. coli* (Figure 2, p.11).
MON-00603-6: Plasmid PV-ZMGT32 constructed based on the plasmid pUC119 derived from *E. coli* (Figure 3, p.12).

2) Properties

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

The total number of base pairs in the plasmid vectors used for the development of parent lines is as follows.
DAS-01507-1 (PHP8999): 9,504 bp
SYN-IR604-5 (pZM26): 13,811 bp
MON-00603-6 (PV-ZMGT32): 9,308 bp

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

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

DAS-01507-1: The *nptII* gene to confer the resistance to kanamycin and neomycin
SYN-IR604-5: The *spec* gene to confer the resistance to streptomycin, erythromycin and spectinomycin
MON-00603-6: The *nptII* gene to confer the resistance to kanamycin and neomycin

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

Neither these vectors is known to be infectious.

(3) Method of preparing living modified organisms

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

Composition of the donor nucleic acid used for the development of the parent lines DAS-01507-1, SYN-IR604-5 and MON-00603-6, and along with restriction sites with relative positions are shown in Figure 1, 2, and 3 (p.10 – p.12).

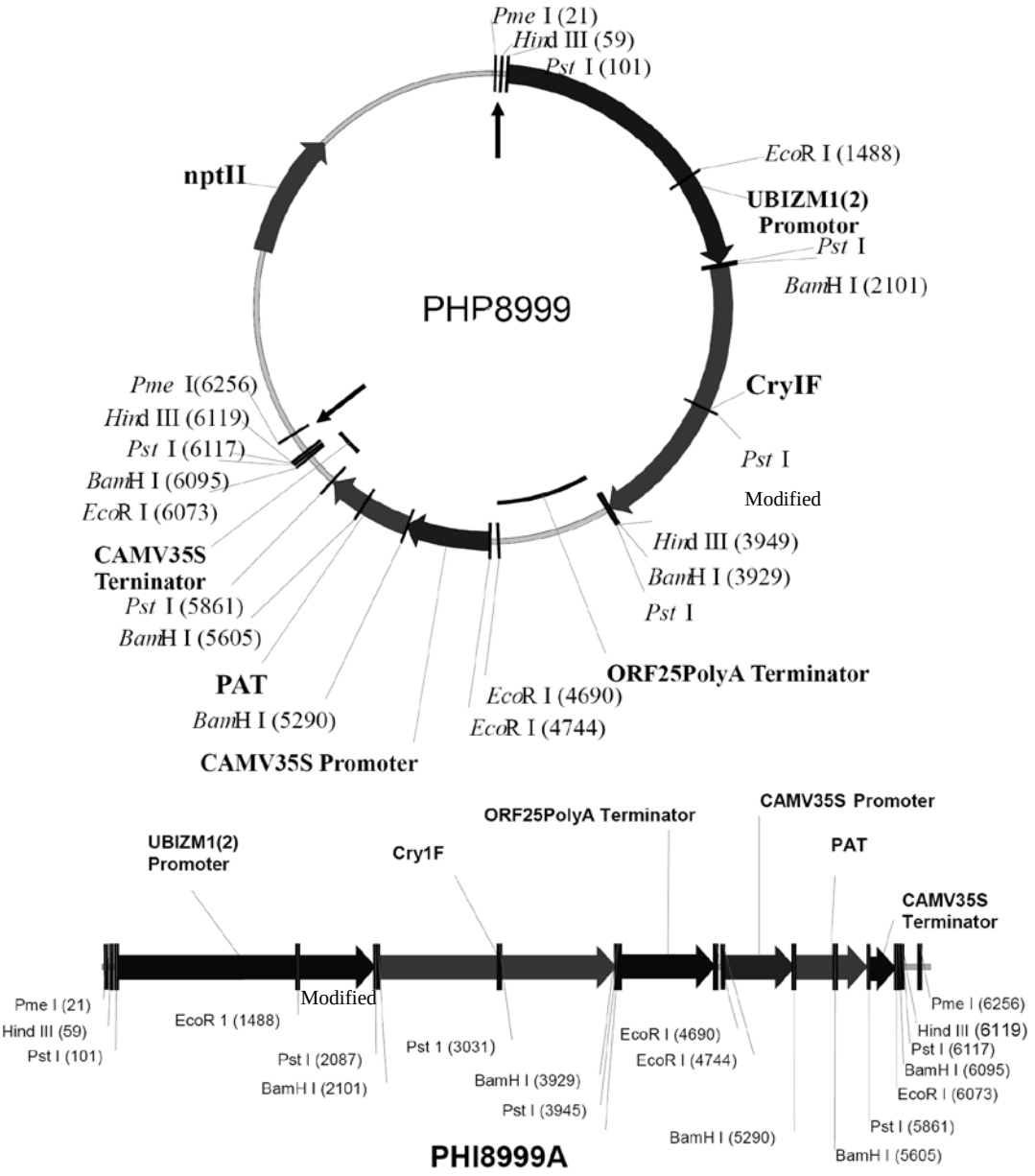

Figure 1 Composition of the plasmid PHP8999* (top) and the transferred DNA region PHI8999A (bottom)

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

The plasmid PHP8999 was digested with 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 host organism.

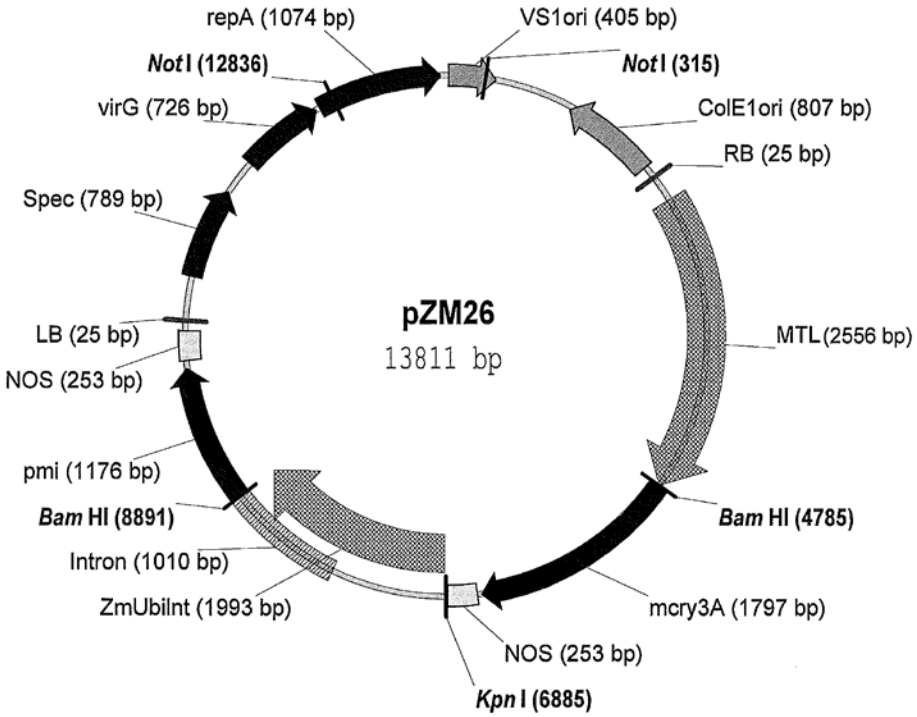

Figure 2 Composition of the plasmid pZM26 region

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

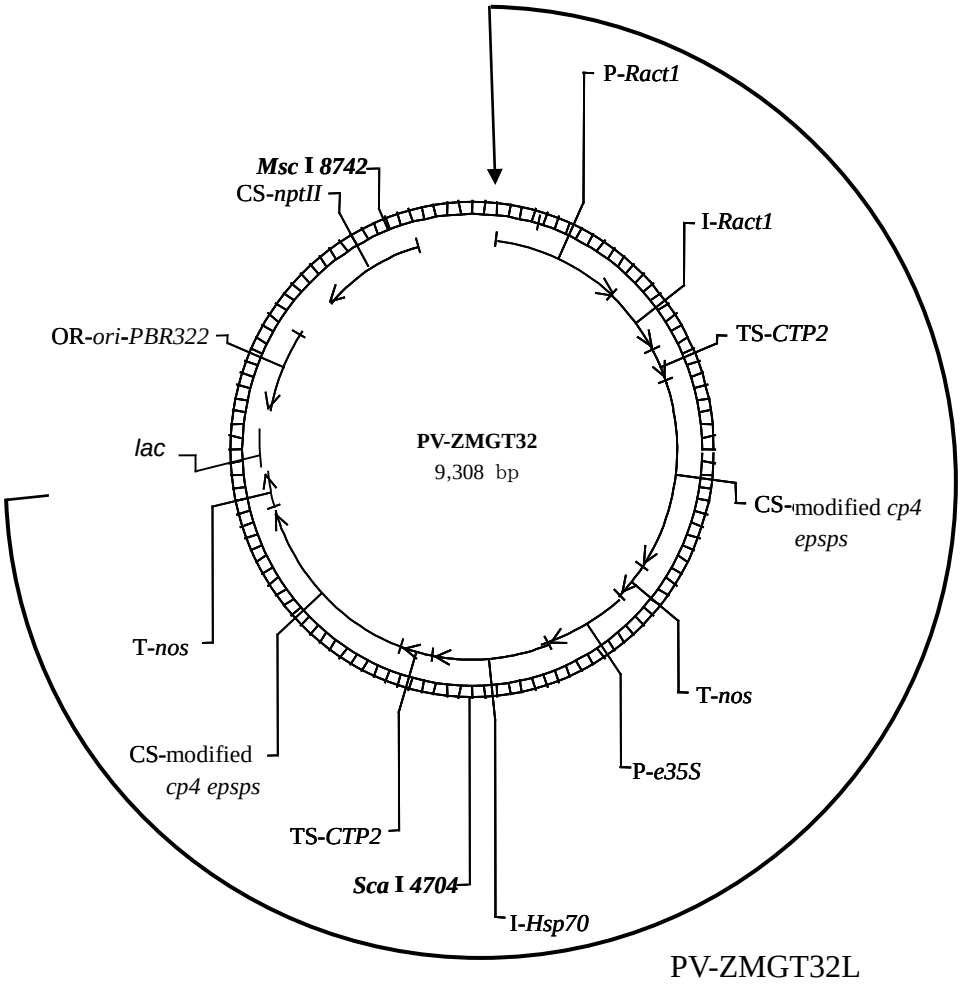

Figure 3 Composition of the plasmids PV-ZMGT32*

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

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

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

The methods employed to transfer nucleic acid into the host organism were the particle gun bombardment for DAS-01507-1 and MON-00603-6, and the *Agrobacterium* method for SYN-IR604-5.

3) Processes of rearing of living modified organisms

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

Selection of nucleic acid-transferred cells was carried out by culturing on the growth medium containing the substances listed below.

DAS-01507-1: Glufosinate
SYN-IR604-5: Mannose
MON-00603-6: Glyphosate

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

For SYN-IR604-5, after transferring of genes, the antibiotic Cefotaxime was added to the mannose medium to remove any residual *Agrobacterium* used for the transformation. Then the PCR was carried out for regenerated plants. Consequently, it is considered that there is no remaining *Agrobacterium.*

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

This stack maize line was developed by cross-breeding between DAS-01507-1, SYN-IR604-5 and MON-00603-6. The process is shown in Figure 4 (p.13; Confidential: Not disclosed to unauthorized person). In addition, the status of approval of these parent lines in Japan is listed in Table 4 (p.14).

(Not made available or disclosed to unauthorized person)

Figure 4 Example of the process of rearing of this stack maize line

Table 4 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	July 8, 2002	March 27, 2003	March 2, 2005
SYN-IR604-5	August 17, 2007	August 22, 2007	August 23, 2007
MON-00603-6	March 30, 2001	March 27, 2003	November 22, 2004
This stack maize line	2011 Pending application	2011 Pending application	2011: Submitted

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

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

It has been confirmed that the traits from DAS-01507-1, SYN-IR604-5 and MON-00603-6 are transferred in accordance with the Mendel's law and that the replication product of the transferred nucleic acid exists on the maize genome.

2) 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 blotting analysis confirmed that one copy each of the modified *cry1F* gene expression cassette and the *pat* gene expression cassette are transferred in 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, a part 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 blotting analysis confirmed that these gene fragments were not transcribed into mRNA, thereby not functioning.

SYN-IR604-5:
The result of Southern blotting analysis confirmed that one copy each of the modified *cry3Aa2* gene and the *pmi* gene are transferred in the maize genome and that the transferred genes are inherited stably in offspring.

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

It was found that a 217 bp fragment of *P-ract1* was inserted near the 3'-end of the transferred gene in the reverse direction; however, this fragment was confirmed to be not involved in the production of any new protein by a Western blotting 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 three reasons: that this amino acid is not among the seven amino acids essential for activating the EPSPS protein family, that the change of amino acid does not affect the active site of the protein and three-dimensional structure, and that the traits of enzyme activity and immune response are comparable to those of the original protein.

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

—

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

The transgene-expression stability of the parent lines of this stack maize line was examined 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

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

MON-00603-6: Glyphosate herbicide-spraying test

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

The transferred nucleic acid does not contain any sequence 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, SYN-IR604-5 and MON-00603-6) based on the real-time quantitative PCR analysis is available at the Web site of European Commission (Joint Research Centre, 2005, 2010).

Sensitivity:
The quantification limits of DAS-01507-1, SYN-IR604-5 and MON-00603-6 are ≤0.08%, <0.09%, and 0.1%, respectively.

Reliability:
As for the methods for detection of DAS-01507-1, SYN-IR604-5 and MON-00603-6, their reliability have been verified as a result of tests performed at 14 (repeated twice), 14 (repeated 4 times) and 12 (repeated twice) 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

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

This stack maize line is given the traits as described below: resistance to Lepidoptera due to the modified *cry1F gene* and tolerance to glufosinate herbicide due to *pat* gene both of which are derived from DAS-01507-1, resistance to Coleoptera due to the modified *cry3Aa2* gene which is derived from SYN-IR604-5, and tolerance to glyphosate herbicide due to the modified *cp4 epsps* gene which is derived from MON-00603-6. As for SYN-IR604-5, *pmi* gene is also inserted as a selective marker.

The possibility of functional interaction between proteins produced by these genes is discussed below from the 3 categories: between the pest insects-resistant proteins, between the herbicide-tolerant proteins, and between these two types of proteins.

Functional interaction between the pest insect-resistant proteins

The modified Cry1F protein exhibits the insecticidal activity against pest insects of the order Lepidoptera. The modified Cry3Aa2 protein exhibits the insecticidal activity against pest insects of the order Coleoptera. The modified Cry1F protein does not show the insecticidal activity against pest insects of the order Coleoptera to which the modified Cry3Aa2 offers the insecticidal activity. Similar things could be said for the insecticidal activity of the modified Cry3Aa2 protein against pest insects of the order Lepidoptera, to which the modified Cry1F offers the insecticidal activity. These observations suggest that the specificity of 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. As mentioned above, the insecticidal spectrum of the modified Cry1F protein and the modified Cry3Aa2 protein differ from each other; therefore, it is quite unlikely that functional interaction between them would occur.

Functional interaction between the herbicide-tolerant proteins

The *L*-glufosinate, an active ingredient of glufosinate herbicide, inhibits the activity of glutamine synthase. The PAT protein detoxifies *L*-glufosinate by acetylation. *L*-glufosinate is the sole substrate of the protein, and the protein does not use *D*-glufosinate or other amino acids as substrate (OECD, 1999). The modified CP4 EPSPS protein possesses the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway for biosynthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine). The substrates of the modified CP4 EPSPS protein are limited to phosphoenolpyruvate (PEP), shikimate-3-phosphate (S3P) and shikimic acid. Therefore the production of unexpected metabolites are unlikely, since that the PAT protein and the modified CP4 EPSPS protein differ from each other in their respective substrates and mechanism of action, and that the metabolic pathways they affect are independent of each other;.

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

The pest insect-resistant 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 a pest

insect-resistant protein and herbicide-tolerant protein among the previously approved stack lines.

In order to examine whether the expressed proteins, which are originally 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 the resistance to European corn borer (*Ostrinia nubilalis*), to the pest insect of the order Lepidoptera, to Corn rootworm, and to the pest insect of the order Coleoptera, as well as the tolerance to herbicides glufosinate and glyphosate.

The PMI protein, used 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 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, a parent line DAS-01507-1 and the non-recombinant control maize (Annex 4; Confidential: Not disclosed to unauthorized person). No statistically significant difference between this stack maize line and the parent line was found regarding the levels of feeding damage to leaves by European corn borer larvae (Table 5, p.17).

Table 5 Levels of feeding damage to leaves by the larvae of European corn borer larvae (*Ostrinia nubilalis*)

Samples tested *	Levels of feeding damage
This stack maize line	0.0193 ± 0.0414 a
Control; Parent line DAS-01507-1	0.0222 ± 0.0297 a
(Reference) Non-recombinant control maize	0.4321 ± 0.1598 b

n=20, mean value ± standard deviation

* All genetic background = PHEED×SYNZSYTAF

Test condition: Each line was cultivated in growth chambers in the US in 2010. At the 6-leaf stage (V6 stage), leaves were collected, and the larvae of 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.

Evaluation method: Level of feeding damage was determined through digital images of the leaves fed to the larvae. The damage-free part of a leave 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 using the inverse sine transformed values and paired comparison based on the 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, parent line SYN-IR604-5 and the non-recombinant control maize (Annex 4; Confidential: Not disclosed to unauthorized person). No statistically significant difference was observed between this stack maize line and the parent line regarding the levels of feeding damage to roots of by Western corn rootworm (Table 6, p18).

Table 6 Levels of feeding damage to root by Western corn rootworm

Samples tested *	Nodal Injury score (NIS)
This stack maize line	0.9750 ± 0.3881 a
Control; Parent line SYN-IR604-5	0.9079 ± 0.3652 a
(Reference) Non-recombinant control maize	2.0375 ± 0.3561 b

n=20, mean value ± standard deviation

* All genetic background = PHEED×SYNZSYTAF

Test condition: Each line was cultivated in growth chambers and greenhouses in the US in 2010. At the 4-leaf stage (V4 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 3.8cm form 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 root was damaged at 1 node.
Score 2.00: All the root was damaged at 2 nodes.
Score 3.00: All the root was damaged at three nodes or more.
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 the Tukey's Test, $P<0.05$).

Tolerance to glufosinate herbicide

Glufosinate herbicide was sprayed to this stack maize line, a parent line DAS-01507-1 and the non-recombinant control maize, and their severity of herbicide injury were examined (Annex 5; Confidential: Not disclosed to unauthorized person). No statistically significant difference on the severity of herbicide injury between this stack maize line and the control parent line was found (Table 7, p18).

Table 7 Levels of herbicide injury to this stack maize line and parent line 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 ± 0 a[3)]	0 ± 0 a	9.33 ± 2.58 a
Control; Parent line DAS-01507-1	0 ± 0	0 ± 0 a	2.00 ± 4.14 a	10.0 ± 5.35 a
(Reference) Non-recombinant control maize	0 ± 0	4.67 ± 6.40 a	96.7 ± 4.88 b	97.3 ± 4.58 b

n=15, mean value ± standard deviation

Test condition: Each line was cultivated in greenhouses in the US in 2010. 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 7th day after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis or bleaching) was visually evaluated by a scale of 0% (intact) to 100% (complete death).

1) All genetic background = PHEED×SYNZSYTAF
2) The concentration of glufosinate sprayed (actual value):
   Dosage tested includes normal dosage of 0.453 kg active ingredient (a.i.)/ha, x16 dosage of 7.22 kg a.i./ha, and x32 dosage of 14.5 kg a.i./ha. The spraying at the concentration at 16-times or higher was intended for the evaluation of herbicide tolerance levels, and spraying of herbicides at such concentrations is not intended in the commercial cultivation.
3) Different letters in a given column indicate that 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

Glufosinate herbicide was sprayed to this stack maize line, parent line MON-00603-6, parent line SYN-IR604-5 (reference) and the non-recombinant control maize (reference), and their severity of herbicide injury were examined (Annex 5; Confidential: Not disclosed to unauthorized person). No statistically significant difference on the severity of herbicide injury between this stack maize line and individual control parent lines was found (Table 8, p20).

Table 8 Levels of herbicide injury to this stack maize line and parent lines by spraying of 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 ± 0 a[3)]	9.33 ± 2.58 a	10.0 ± 0 a
Control; Parent line MON-00603-6	0 ± 0	0 ± 0 a	10.0 ± 3.78 a	8.00 ± 7.75 a
(Reference) Parent line SYN-IR604-5	0 ± 0	86.7 ± 9.76 b	94.3 ± 5.14 b	93.3 ± 4.88 b
(Reference) Non-recombinant control maize	0 ± 0	84.0 ± 11.8 b	93.3 ± 4.88 b	90.0 ± 0 b

n=15, mean value ± standard deviation

Test condition: Each line was cultivated in a greenhouse in the US in 2010.At the 2-leaf stage (V2 stage), the glufosinate herbicide was sprayed. Tests were repeated 3 times with 5 plants per repeat (excluding 5 individuals of normal dosage of MON-00603-6 due to wrong spray).

Evaluation method: On the 7th day after spraying, severity of herbicide injury (level of leaf chlorosis, necrosis or bleaching) was visually evaluated based on the scale from 0% (intact) to 100% (complete death).

1) All genetic background = PHEED×SYNZSYTAF
2) The concentration of glyphosate sprayed (experimental value):
Dosage tested are: normal dosage of 1.26 kg acid equivalent (glyphosate free acid equivalent: a.e.)/ha, x16 dosage of 20.2 kg a.e./ha, and x32 dosage of 40.3 kg a.e./ha. The spraying at the concentrations at 16-times or higher was intended for evaluation of herbicide tolerance levels, and spraying of herbicides at such concentrations is not intended in the commercial cultivation.
3) Different letters in a given column indicate that 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 of the possibility of functional interaction between the pest insect-resistant proteins, between the herbicide-tolerant proteins, and between the pest insect-resistant proteins and herbicide-tolerant proteins, as well as the examinations of the characteristics of each protein leads to a conclusion that any interaction between these proteins is unlikely to take place in this stack line. In addition, this conclusion was supported by the result of bioassays which comfirmed 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 parental lines do not interact with each other and that the traits acquired by the transferred genes remain unchanged in this stack maize line.

Consequently, as this stack line and maize, the taxonomic species the host organism belongs were evaluated on their differences in physiological or ecological characteristics, the evaluation was based on the results of individual examinations of the parent lines at the time of their approval (Annex 1 – Annex 3).

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

(a) Morphological and growth characteristics

The parent lines (DAS-01507-1, SYN-IR604-5 and MON-00603-6) and their non-recombinant control maize were examined on their morphological and growth characteristics listed in Table 9 (p.22). No significant difference was observed between the parent lines and their controls except germination rate and ear diameter of DAS-01507-1, and 100-kernel weight of MON-00603-6. However, the differences were found in only one of the two varieties with different genetic background 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, SYN-IR604-5 and MON-00603-6 to wither, wilt or die as their non-recombinant control maize 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. Neither does it re-grow after harvest and propagate vegetatively, nor produce seeds. In fact, DAS-01507-1 was confirmed to wither when it was cultivated and harvested in test fields in the US. Furthermore, during the isolated field tests of SYN-IR604-5 and MON-00603-6, 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 pollens were stained and observed under a microscope, no difference was observed in fertility, size or shape of the pollen between DAS-01507-1, SYN-IR604-5 and MON-00603-6, and their non-recombinant control maize.

Table 9 Investigational results of morphological and growth characteristics

Item for investigation	DAS-01507-1	SYN-IR604-5	MON-00603-6
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	○	○	○

○: Tested
—: Not tested
*: 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 2)-(a) (p.21), ear length, ear diameter, row number per ear, grain number per row and 100-kernel weight were examined. Statistically significant difference from non-recombinant maize was observed in ear diameter of DAS-1507-1 and 100-kernel weight of MON-00603-6; however, the difference was observed in only one out of two non-recombinant maize varieties.

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, SYN-IR604-5 and MON-00603-6, seed shedding has not been observed under natural conditions.

Dormancy and germination rate: Maize exhibit almost no seed dormancy (CFIA, 1994). In the germination rate tests referred to above, DAS-01507-1, SYN-IR604-5 and MON-00603-6

showed high germination rates, and there was no difference from their non-recombinant control maize.

(f) Crossability

Crossability test was not performed, since no wild relatives (Teosinte: *Euchlaena mexicana*) capable of naturally crossed with maize, the host organism, grows wild in Japan.

(g) Productivity of harmful substances

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

Assessment Result by the Committee for Review on the Biological Diversity Risk Assessment

Name of the type of Living Modified Organism: Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides (modified *cry1F*, modified *cry3Aa2*, *pat*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (1507×MIR604×NK603, OECD UI: DAS-Ø15Ø7-1×SYN-IR6Ø4-5×MON-ØØ6Ø3-6) [including the progeny lines isolated from the maize lines, *B.t.* Cry1F maize line 1507, MIR604 and NK603, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type 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

Applicant: Du Pont Kabushiki Kaisha

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

Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides [including the progeny lines isolated from the maize lines, *B.t.* Cry1F maize line 1507, MIR604 and NK603, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type I Use Regulation)] (hereinafter referred to as "this stack maize line") is a cross progeny line developed by crossing the following three (3) recombinant maize lines, using the traditional crossbreeding method; i) maize resistance to Lepidoptera and tolerant to glufosinate herbicide (DAS-01507-1) 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 (SYN-IR604-5) due to the *cry3Aa2* gene that encodes the modified Cry3Aa2 protein, and iii) maize tolerant to glyphosate herbicide (MON-00603-6) due to the modified *cp4 epsps* gene that encodes the modified CP4 EPSPS protein (5-enol-pyruvyl-shikimate-3-phosphate synthase derived from CP4 strain).
These three 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 cell 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 and the modified Cry3Aa2 protein) were unlikely to interact with each other and to affect the specificity of another. Furthermore, the PAT protein and the modified CP4 EPSPS protein differ in their substrate and mechanism of action from the other. There is no report of a 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 their host's metabolic system and producing an unexpected metabolite.

In addition, based on a bioassay, the resistance to Lepidoptera and Coleoptera and the tolerance to glufosinate and glyphosate herbicides in this stack maize line were found at

similar levels as exhibited by the individual parent lines.

Consequently, it is considered unlikely that the proteins expressed in this stack maize line 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 (*Zea mays* subsp. *mays* (L) Iltis), the taxonomical species to which the recipient organism belongs, has been long used in Japan, though there is no report that it has become self-seeding in the natural environment in Japan.

As a result of investigation for various characteristics referring to competitiveness of DAS-01507-1, SYN-IR604-5 and MON-00603-6, the parent lines of this stack maize line, a significant difference was observed between this stack maize line and its non-recombinant maize in some items examined. However, the differences were judged not to be so large as enhancing the competitiveness of this stack maize line.

The resistant trait to pest insects of the order Lepidoptera is given by the modified *cry1F* gene, and that of the order Coleoptera is given by the modified *cry3Aa2* gene in this stack maize line. However, the insect damage by Lepidopteran and Coleopteran insect pests is not the major factor to inhibit the growth of maize under the natural environment in Japan. Therefore, it is considered unlikely that these traits cause maize, a crop plant, to become self-seeding in the natural environment and enhance the competitiveness.

In addition, this stack maize line is given traits to be tolerant to glufosinate and glyphosate herbicides due to the *pat* gene and the modified *cp4 epsps* gene. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of these herbicides, the tolerances to glufosinate and glyphosate would increase the competitiveness of this stack maize line.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: 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 (*Zea mays* subsp. *mays* (L) Iltis), the taxonomical species to which the recipient organism belongs, has been long used in Japan, though it is not generally known that the maize produces any harmful substances that could affect wild animals and wild plants.

It has been confirmed that the proteins expressed in this stack maize line (the modified Cry1F protein, the modified Cry3Aa2 protein, the PAT protein and the modified CP4 EPSPS protein) do not have any homology with any of the known allergens. In addition, for the ability of the parent lines of this stack maize line to produce any harmful substances (the substances secreted from the roots which can affect other plants and microorganisms in soil, the substances existing in the plant body which can affect other plants after dying), plow-in tests, succeeding crop tests and soil microflora tests were conducted. As a result, there was no difference observed in all tests suggesting that the productivity of harmful substances of the

parent lines might have increased. Consequently, it is considered unlikely that this stack maize line possesses productivity of unintended harmful substances.

The modified Cry1F protein and the modified Cry3Aa2 protein expressed in this stack maize line exhibit the insecticidal activity against the insects of the order Lepidoptera and 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 Lepidopteran and Coleopteran insects which could eat directly this stack maize line or eat pollens dispersed from this stack maize line by eating with dietary plants. However, it is considered unlikely that the Lepidopteran and Coleopteran insects inhabit locally near the fields for cultivation of this stack maize line and then, it is considered extremely low that they could be affected in the level of population.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line would pose no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances.

(3) Crossability

In the Japanese natural environment, there are no wild plants which can cross with maize. Therefore, it was judged that there are no specific wild plants that are possibly affected by this 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 I Use Regulation causes Adverse Effect on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.

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