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

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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 (*cry1A.105*, modified *cry2Ab2*, *cry1F*, *pat*, modified *cp4 epsps*, modified *cry3Bb1*, *cry34Ab1*, *cry35Ab1*, *Zea mays* subsp. *mays* (L.) Iltis) (MON 89034 × *B.t.* Cry1F maize line 1507 × MON 88017 × *B.t.* Cry34/35Ab1 Event DAS-59122-7, OECD UI: MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-88Ø17-3 × DAS-59122-7) [including the progeny lines isolated from the maize lines, MON 89034, *B.t.* Cry1F maize line 1507, MON 88017 and *B.t.* Cry34/35Ab1 Event DAS-59122-7, 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)]
Method 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

Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate and glyphosate herbicides (OECD UI: MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-88Ø17-3 × DAS-59122-7) (hereinafter referred to as "this stack maize line") is a cross progeny line developed by crossing the following four (4) recombinant maize lines, using the traditional crossbreeding method. The 4 recombinant maize lines are: i) Maize resistant to Lepidoptera (*cry1A.105*, modified *cry2Ab2*, *Zea mays* subsp. *mays* (L.) Iltis) (MON 89034, OECD UI: MON-89Ø34-3) (hereinafter referred to as "MON 89034"), ii) Maize tolerant to glufosinate herbicide and resistant to Lepidoptera (*cry1F*, *pat*, *Zea mays* subsp. *mays* (L) Iltis) (*B.t.* Cry1F maize line 1507, OECD UI: DAS-Ø15Ø7-1) (hereinafter referred to as "Cry1F line 1507"), iii) Maize tolerant to glyphosate herbicide and resistant to Coleoptera (modified *cp4 epsps*, modified *cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis.) (MON 88017, OECD UI: MON-88Ø17-3) (hereinafter referred to as "MON 88017"), and iv) Maize tolerant to glufosinate herbicide and resistant to Coleoptera (*cry34Ab1, cry35Ab1, pat, Zea mays* subsp. *mays* (L.) Iltis) (*B.t.* Cry34/35Ab1 Event DAS-59122-7, OECD UI: DAS-59122-7) (hereinafter referred to as "Event DAS-59122-7"). Therefore, this stack maize line possesses the characteristics of these four (4) parent recombinant maize lines, MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7. In addition, 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. In the following sections, outlines of preparation of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 and other information are explained.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of MON 89034, Cry1F line 1507, MON 88017 and

Event DAS-59122-7 are shown individually in Figure 1 to Figure 4 (pp.3-6) and Table 1 to Table 4 (pp.7-12).

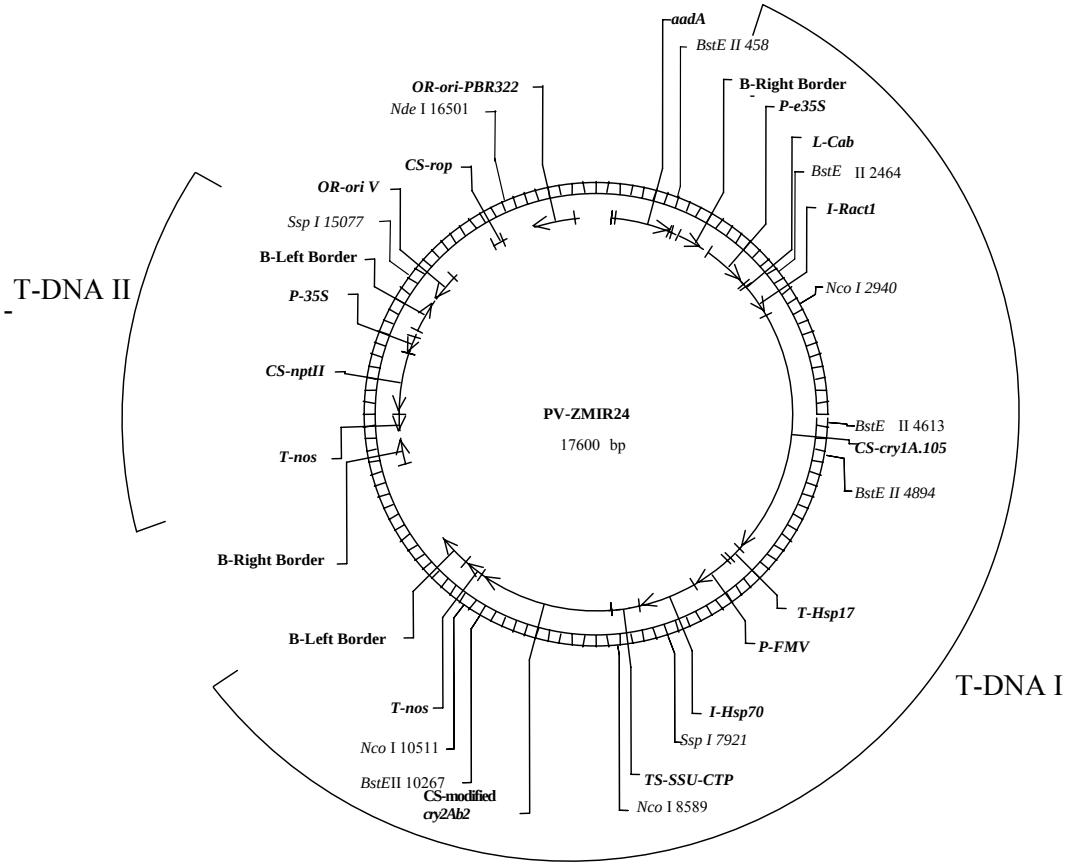

Figure 1 Map of the plasmid PV-ZMIR245 used for the development of MON 89034[1]

In the development process of MON 89034, those individuals were selected that contain T-DNA I region shown above but not contain T-DNA II region.

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

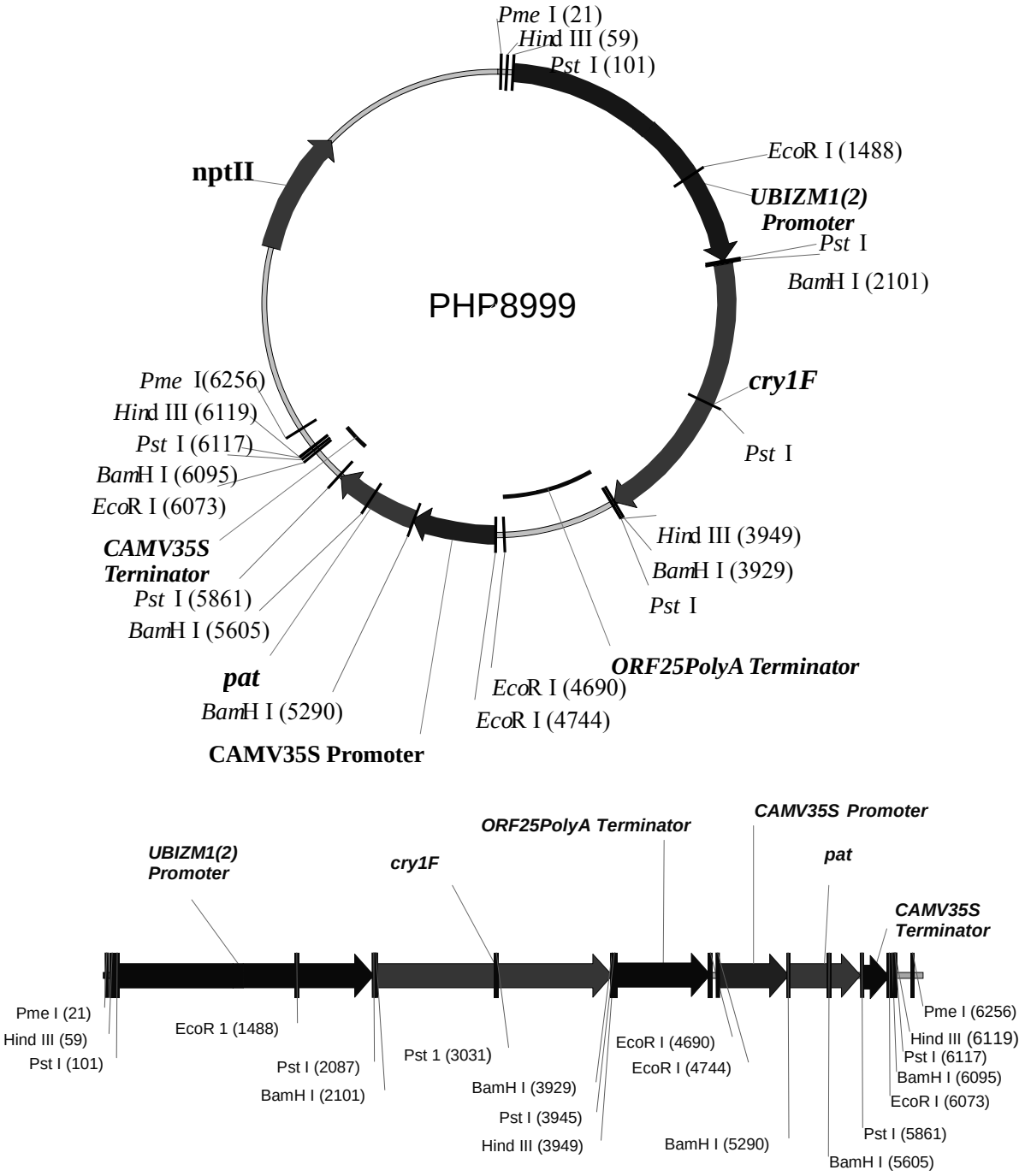

Figure 2 Map of the plasmid PHP8999 used for the development of Cry1F line 1507 and the transferred DNA region[2]

[2] All the rights pertinent to the information in the diagram above and the responsibility for the content remain with Dow Chemical Japan Limited.

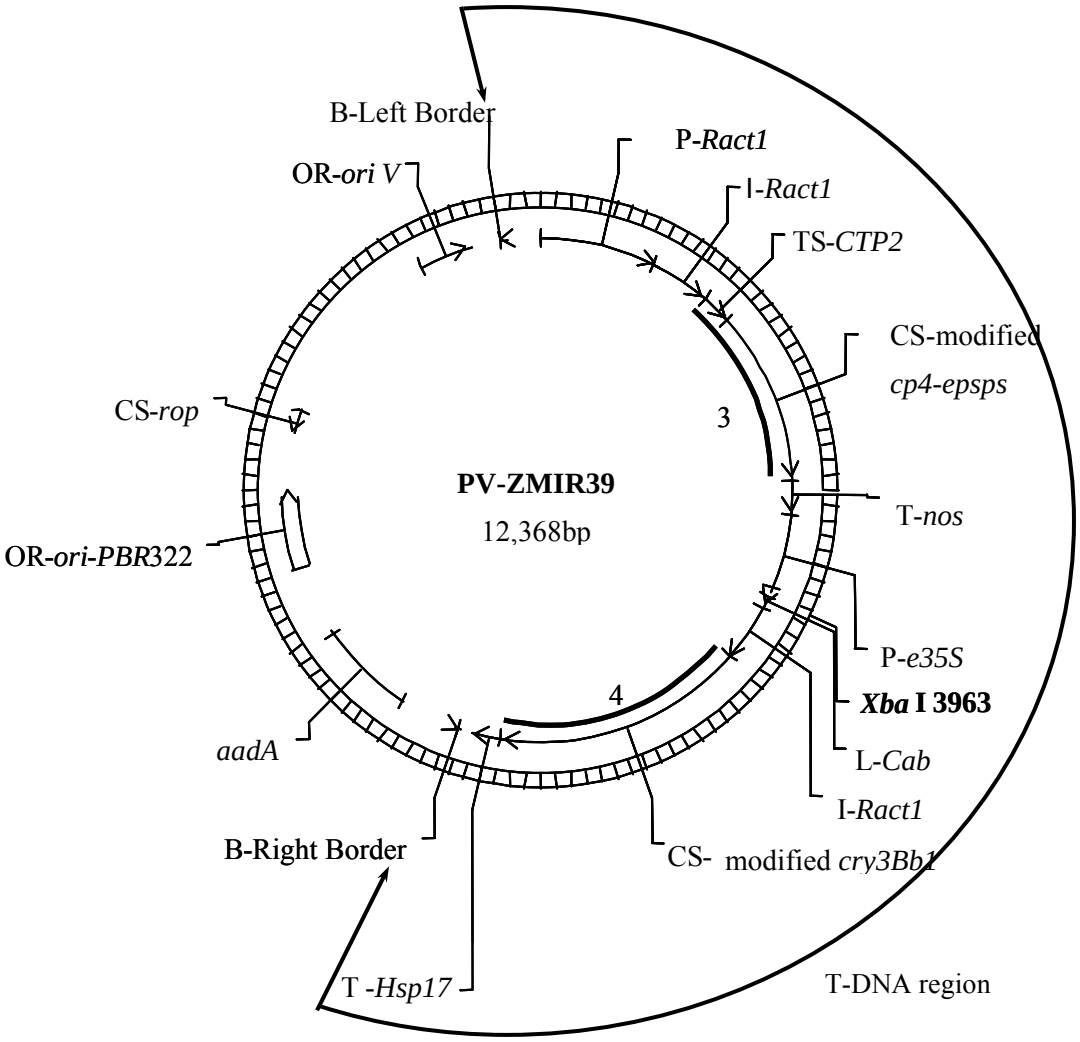

Figure 3 Map of the plasmid PV-ZMIR39 used for the development of MON 88017[3]

[3] All the rights pertinent to the information in the diagram above and the responsibility for the content remain with Monsanto Japan Limited.

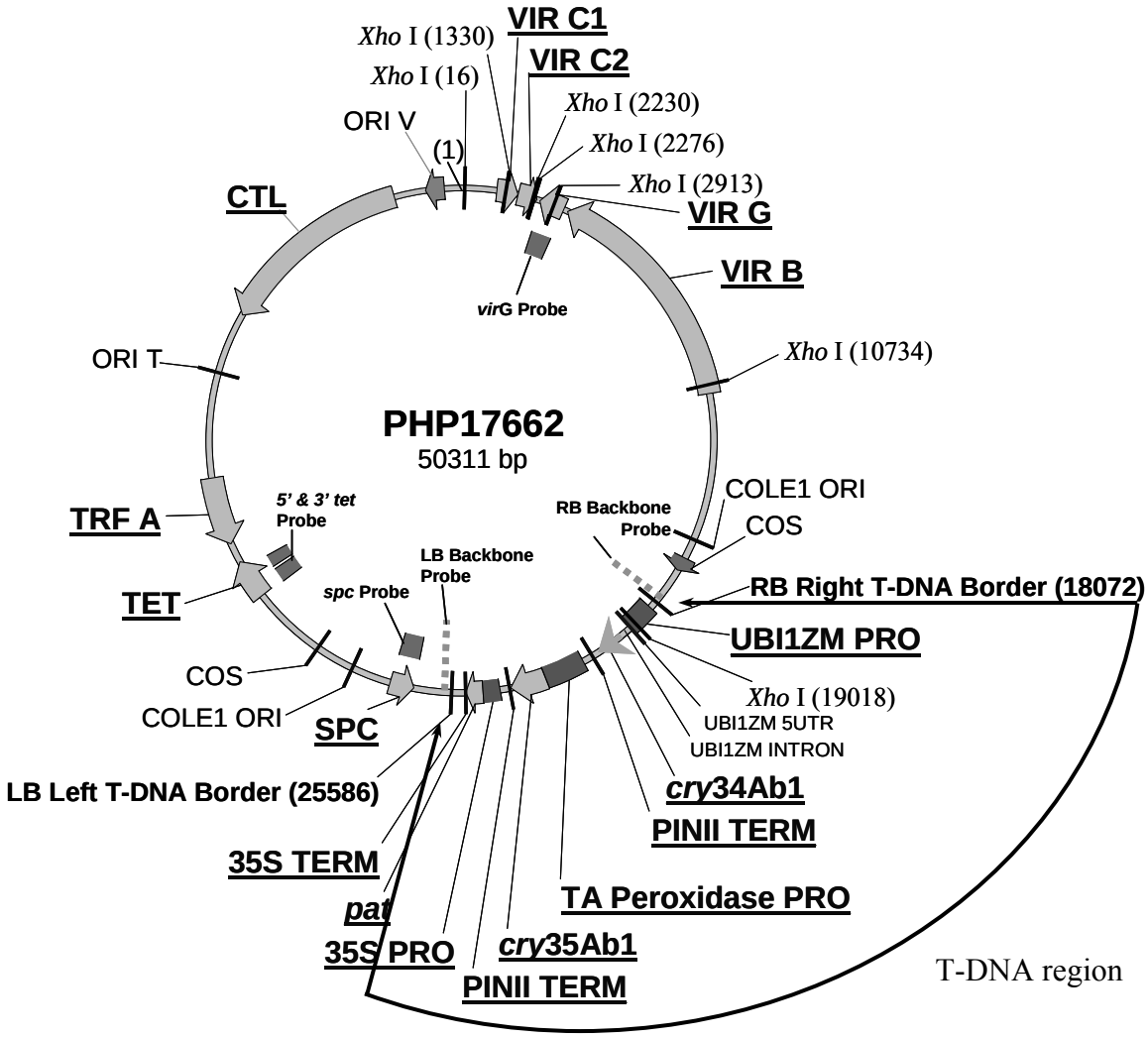

Figure 4 Map of the plasmid PHP17662 used for the development of Event DAS-59122-7[4]

[4] All the rights pertinent to the information in the diagram above and the responsibility for the content remain with Dow Chemical Japan Limited.

Table 1 Origins and functions of component elements of PV-ZMIR245 used for the development of MON 89034[5]

Component elements	Origin and function
T-DNA I region	
B*[1]-Right Border (Right border region)	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 16).
P*[2]-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Reference 18) promoter and 9bp leader sequence, containing double enhancer regions (Reference 17). Involved in the constant expression of the target gene in the entire tissue of plant body.
L*[3]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Reference 19).
I*[4]-*Ract1*	Rice actin gene intron (Reference 20). Activates the expression of target gene.
CS*[5]-*cry1A.105*	A gene that encodes the Cry1A.105 protein. Details are described in I-2-(1)-2)-(a).
T*[6]-*Hsp17*	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (Reference 21).
P-*FMV*	35S promoter derived from Figwort Mosaic Virus (Reference 22). Involved in the constant expression of the target gene in the entire tissue of plant body.
I-*Hsp70*	First intron of maize heat shock protein 70 gene (Reference 23). Activates the expression of target gene.
TS*[7]-*SSU-CTP*	Transit peptide of small subunit of ribulose 1,5-carboxylase diphosphate of maize, including the first intron sequence (Reference 24). Transfers downstream-connected protein to plastid.
CS-modified *cry2Ab2*	A gene that encodes the modified Cry2Ab2 protein derived from *Bacillus thuringiensis* (Reference 25). It has the site broken by the restriction enzyme transferred during the cloning and then, a single aspartic acid is transferred after the methionine at the N-terminal compared to the wild-type Cry2Ab2 protein.
T-*nos*	3' untranscribed region of nopaline synthase (*nos*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).
B-Left Border (Left border region)	A DNA fragment containing the left border sequence (25bp) derived from *A. tumefaciens.* It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 27).

Table 1 Origins and functions of component elements of PV-ZMIR245 used for the development of MON 89034 (continued)[5]

Component elements	Origin and function
T-DNA II region	
B-Right Border (Right border region)	A DNA fragment containing the right border sequence (24 bp) of nopaline type T-DNA, derived from *A. tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 16).
T-*nos*	3' transcription region of nopaline synthase (*nos*) gene derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).
CS-*nptII*	A gene derived from *E. coli* transposon Tn5 (Reference 28). Encodes neomycin phosphotransferase type II (*NPT II*) and confers resistance to kanamycin. Used as marker to select the transgenic plant during the gene transfer (Reference 29).
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 18). Involved in the constant expression of the target gene in the entire tissue of plant body.
B-Left Border (Left border region)	A DNA fragment containing the left border sequence (25bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 27).
Plasmid backbone region	
OR[*8]-*ori V*	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *A. tumefaciens* (Reference 30).
CS-*rop*	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Reference 31)
OR-*ori- PBR 322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Reference 32).
aadA	Bacteria promoter, code region and terminator for the 3'(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Reference 33).

[*1] B – border

[*2] P – promoter

[*3] L – leader

[*4] I – intron

[*5] CS – coding sequence

[*6] T – transcript termination sequence

[*7] TS – targeting sequence

[*8] OR – Origin of Replication

[5] All the rights pertinent to the information in the table above and the responsibility for the content remain with Monsanto Japan Limited.

Table 2 Origins and functions of component elements of PHP8999 used for the development of Cry1F line 1507[6]

Component elements	Origin and function
cry1F gene expression cassette	
UBIZM1(2) Promoter	Ubiquitin constitutive promoter [1)] derived from *Z. mays* (including intron and 5' untranslated region) (Reference 34)
cry1F	A gene that encodes Cry1F protein derived from *B. thuringiensis* var. *aizawai*. Optimized to activate the expression in plants (GenBank AAA22347).
ORF25PolyA Terminator	A terminator to terminate transcription from *A. tumefaciens* pTi5955 (Reference 27)
pat gene expression cassette	
CAMV35S Promoter	35S constitutive promoter [1)] derived from cauliflower mosaic virus (CaMV) (Reference 35)
Pat	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *Streptomyces viridochromogenes*. Optimized to activate the expression in plants (Reference 36).
CAMV35S Terminator	35S terminator to terminate transcription from cauliflower mosaic virus (CaMV) (Reference 35)

[1)] Constitutive promoter: A promoter that drives the expression of target genes in all sites in plant body

[6] All the rights pertinent to the information in the table above and the responsibility for the content remain with Dow Chemical Japan Limited.

Table 3 Origins and functions of component elements of PV-ZMIR39 used for the development of MON 88017[7]

Component elements	Origin and function
T-DNA region	
P*[1]-*Ract1*	Promoter region of actin 1 gene derived from rice. It makes target genes expressed (Reference 20).
I*[2]-*Ract1*	Rice actin gene intron (Reference 20). Activates the expression of target gene.
TS*[3]-*CTP2*	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 37). Transfers target proteins from cytoplasm to chloroplast.
CS*[4]-modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 38; Reference 39). 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.
T*[5]-*nos*	3' untranscribed region of nopaline synthase (*nos*) gene derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).
P-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Reference 18) promoter and 9bp leader sequence, containing double enhancer regions (Reference 17). Involved in the constant expression of the target gene in the entire tissue of plant body.
L*[6]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Reference 19).
I-*Ract1*	Rice actin gene intron (Reference 20). Activates the expression of target gene.
CS-modified *cry3Bb1*	The gene which encodes Cry3Bb1 protein of *B. thuringiensis* (Reference 40). It differs from the modified Cry3Bb1 protein in MON 863 at one site in the amino acid sequence; the 166th amino acid sequence is aspartic acid in MON 88017 whereas glycine in MON 863.
T-*Hsp*17	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (Reference 21).

Table 3 Origins and functions of component elements of PV-ZMIR39 used for the development of MON 88017 (continued)[7]

Component elements	Origin and function
Plasmid backbone region	
B[*7]-Right Border (Right border region)	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 16).
aadA	Bacteria promoter, code region and terminator for the 3'(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Reference 33).
OR[*8]-*ori-PBR322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Reference 32).
CS-*rop*	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Reference 31)
OR-ori-V	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *A. tumefaciens* (Reference 30).
B-Left Border (Left border region)	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Reference 31)

[*1] P – promoter

[*2] I – intron

[*3] L – leader

[*4] TS – targeting sequence

[*5] CS – coding sequence

[*6] T – transcript termination sequence

[*7] B – border

[*8] OR – Origin of Replication

[7] All the rights pertinent to the information in the table above and the responsibility for the content remain with Monsanto Japan Limited.

Table 4 Origins and functions of component elements of PHP17662 used for the development of Event DAS-59122-7[8]

Component elements	Origin and function
cry34Ab1 gene expression cassette	
UBI1ZM PRO	Ubiquitin constitutive promoter [1)] derived from *Z. mays* (including intron and 5' untranslated region)
cry34Ab1	A gene that encodes Cry34Ab1 protein derived from *B. thuringiensis* PS149B1 strain
PIN II TERM	Protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum* (including intron and 5' untranslated region)
cry35Ab1 gene expression cassette	
TA Peroxidase PRO	Peroxidase promoter (nucleotide sequence 45-1342 of GenBank X53675) derived from *Triticum aestivum* known to express in roots
cry35Ab1	A gene that encodes Cry35Ab1 protein derived from *B. thuringiensis* PS149B1 strain
PIN II TERM	Protease inhibitor II terminator to terminate transcription derived from *S. tuberosum* (including intron and 5' untranslated region)
pat gene expression cassette	
35S PRO	35S constitutive promoter [1)] derived from cauliflower mosaic virus (CaMV)
pat	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *S. viridochromogenes*
35S TERM	35S terminator to terminate transcription from cauliflower mosaic virus (CaMV)

[1)] Constitutive promoter: A promoter that drives the expression of target genes in all sites in plant body

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

2) Functions of component elements

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

Functions of individual component elements of donor nucleic acid used for the development of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 are shown in Table 1 to Table 4 (pp.7-12). Details on the target genes, *cry1A.105* gene, modified *cry2Ab2* gene, *cry1F* gene, *pat* gene, modified *cp4 epsps* gene, modified *cry3Bb1* gene, *cry34Ab1* gene and *cry35Ab1* gene, are shown in Table 1 to Table 4 (pp.7-12).

(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

— Proteins resistant to pest insect [9] —

[Cry1A.105 protein]

The Cry1A.105 protein, which is encoded by the *cry1A.105* gene used for the development of MON 89034, is a chimeric Bt protein composed of Domains I and II of the Cry1Ab protein, Domain III of the Cry1F protein, and the C-terminal Domain of the Cry1Ac protein, and it has been developed in order to enhance the insecticidal activity

[9] The Bt protein, produced by *Bacillus thuringiensis*, a gram-positive bacterium existing universally in soil, is known to bind to the specific receptors in the midgut epithelium of the target pest insects and form cation-selective pores in the cells and as a result, inhibit the digestive process, thereby providing insecticidal activity (Reference 41; Reference 42; Reference 43). In addition, several studies have shown that the Bt protein is composed of several Domains and what functions individual Domains possess. For example, it has been shown that the Bt protein is composed of Domains I, II, and III and the C-terminal Domain, and Domain I is involved in the formation of cation-selective pores to inhibit the digestive process, Domain II is involved in the recognition of specific receptors, Domain III is involved in the binding to receptors, and the C-terminal Domain is involved in the crystal structure of the Bt protein (Reference 44; Reference 45).

against target pest insects by combining the different Domains of the Bt protein.

In order to investigate the insecticidal spectrum of the Cry1A.105 protein, the Cry1A.105 protein was added to artificial feeds, which were given to 15 different kinds of insects including five (5) species of insects of the order Lepidoptera. As a result, the Cry1A.105 protein exhibited the insecticidal activity against the larvae of Corn earworm (*Helicoverpa zea*) (Reference 46), Black cutworm (*Agrotis ipsilon*) (Reference 47), Fall armyworm (*Spodoptera frugiperda*) (Reference 47), Southwestern corn borer (*Diatraea grandiosella*) (Reference 47), and European corn borer (*Ostrinia nubilalis*) (Reference 48), which are the major pest insects for maize, though it did not exhibit any insecticidal activity against honeybee (Reference 49, Reference 50), ladybug (Reference 51) and other beneficial insects except the insects of order Lepidoptera.

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

[Modified Cry2Ab2 protein]

The modified Cry2Ab2 protein, which is encoded by the modified *cry2Ab2* gene used for the development of MON 89034, had the site which was broken by the restriction enzyme transferred during the cloning and then, a single aspartic acid is transferred after the methionine at the N-terminal compared to the wild-type Cry2Ab2 protein.

In order to investigate the insecticidal spectrum of the modified Cry2Ab2 protein, the modified Cry2Ab2 protein was added to artificial feeds, which were given to 15 different species of insects including four (4) insects of the order Lepidoptera. As a result, the modified Cry2Ab2 protein exhibited the insecticidal activity against the larvae of Corn earworm (Reference 48), Fall armyworm (Reference 52), and European corn borer (Reference 48) among the 4 species of major pest insects of the order Lepidoptera used in the investigation and not against Black cutworm (Reference 52). Also, the modified Cry2Ab2 protein did not exhibit any insecticidal activity against honeybee (Reference 53, Reference 54), ladybug (Reference 55) and other beneficial insects except the insects of the order Lepidoptera; therefore, it was confirmed that the modified Cry2Ab2 protein offers specific insecticidal activity against only the insects of

the order Lepidoptera and not against the other species of insects.

[Cry1A.105 protein ＋ Modified Cry2Ab2 protein]

MON89034 is given the resistance to the target insects of the order Lepidoptera with simultaneous expression of both Cry1A.105 protein and modified Cry2Ab2 protein. Actually, as a result of tests of MON 89034 for resistance to major pest insects of the order Lepidoptera [European corn borer, Southwestern corn borer, Corn earworm, Sugarcane borer (SCB; *Diatraea saccharalis*), and Fall armyworm] conducted from 2003 to 2004 in the US, Puerto Rico and Argentina, it was confirmed that MON 89034 exhibited resistance to all the Lepidopteran insects examined.

In addition, it has been also confirmed that the Cry1A.105 protein and the modified Cry2Ab2 protein both possess insecticidal activity against Corn earworm, Fall armyworm and European corn borer. However, with simultaneous expression of two proteins offering insecticidal spectrum which overlaps to some extent with each other, the target Lepidopteran insects, which exhibit sensitivity to MON 89034, could not acquire any resistance to MON 89034 unless they become insensitive to both Bt proteins. This raises expectations that MON 89034 would be able to substantially reduce the probability of occurrence of insensitive pest insects compared to the Bt maize in which only one kind of Bt protein is independently expressed.

It has been confirmed during the biological diversity risk assessment of MON 89034 that the Cry1A.105 protein and the modified Cry2Ab2 protein do not offer any synergistic insecticidal activity against the target insects of the order Lepidoptera which show sensitivity to both Bt proteins.

[Cry1F protein]

In order to investigate the insecticidal spectrum of the Cry1F protein, the Cry1F protein produced in the *Pseudomonas fluorescens* was added to artificial feeds, which were given to 15 different kinds of insects of the order Lepidoptera which are considered typical insect pests for the farming in the US. Among the 15 kinds of insects of the order Lepidoptera, six (6) are regarded as insect pests for maize grown in the US and the other nine (9) for cotton, soybean, canola and other crops. Among the 6 kinds of insect pests for the cultivation of maize, the Cry1F protein showed higher insecticidal activity against European corn borer, Fall armyworm and Beet armyworm (*Spodoptera*

exigua), the target insect pest of Cry1F line 1507, though, against the other three (3) kinds of insect pests (Southwestern corn borer, Black cutworm and Bollworm), it showed lower insecticidal activity. Also for *Danaus plexippus*, which is not regarded as an agricultural insect pest, a test was conducted, though, even at the maximum dose obtained in the test, the death rate of *Danaus plexippus* was found equivalent to that in the control plot. Based on the results, it was found that the Cry1F protein has highly specific insecticidal spectrum similar as for other Bt proteins (Reference 56) and thus it offers insecticidal activity against limited insects.

In addition to the insects of the order Lepidoptera, tests were also conducted to the mammals, birds, fish, and Coleoptera, Hymenoptera, Neuroptera, Collembola and other insects. As a result, it was confirmed that the Cry1F protein exhibits no toxicity against any of the non-target organisms tested (Reference 57).

[Modified Cry3Bb1 protein]

The modified Cry3Bb1 protein exhibits the insecticidal activity against Corn rootworm (*Diabrotica* sp.), which is one of the major pest insects of the order Coleoptera to maize cultivation in the US and eats and damages the roots of maize.

The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow, and the modified Cry3Bb1 protein exhibits the insecticidal activity only against the Colorado potato beetle (CPB; *Leptinotarsa decimlineata*) and CRW (Corn rootworm), which are classified into two genera, *Leptinotarsa* and *Diabrotica* respectively, of the family Chrysomelidae, among the order Coleoptera (Table 10 of Annex 2, p.54). It was indicated by literature search that there was no report that related species of the same genera of these two insects have ever inhabited Japan (Reference 58).

The modified Cry3Bb1 protein has amino acids at six (6) sites substituted compared to the wild-type Cry3Bb1 protein. One of the 6 sites was added with the restriction enzyme that broke the site during the cloning and then, the other 5 sites were modified to enhance the insecticidal activity.

[Cry34Ab1 protein ＋Cry35Ab1 protein]

As a result of test conducted to investigate the functions of the Cry34Ab1 protein and

the Cry35Ab1 protein, it was suggested that the Cry34Ab1 protein acts as a protein to form pores to the phospholipids membrane and the Cry35Ab1 protein expands the pores and increases the permeability of the membrane (in-house data of Pioneer Hi-Bred International, Inc.). In the *in vivo* test, it was confirmed that the Cry34Ab1 protein alone offers the insecticidal activity against Corn rootworm, though, in the presence of Cry35Ab1 protein, the Cry34Ab1 protein works in concert to exhibit approximately eight (8) times higher effect compared to when it works alone (Reference 59). The Cry35Ab1 protein alone does not exhibit any insecticidal activity against Corn rootworm. In order to examine any morphological changes in the midgut tissue based on the immunohistochemical method, the larvae of Corn rootworm were fed with the recombinant maize which produces the Cry34Ab1 protein and the Cry35Ab1 protein. As a result, the larvae fed with the non-recombinant maize exhibited no abnormality, though the larvae fed with the recombinant plant showed the phenomena implying cell death such as swollen and/or vacuolated midgut cells, and foamy and/or dissolved cell membranes. The results demonstrate that the Cry34Ab1 protein and the Cry35Ab1 protein destroy the midgut as the target organ, like other Bt proteins (Reference 60).

In general, Bt proteins are known to have an extremely specific insecticidal activity (Reference 56). In fact, in the test of feeding a mixture of the Cry34Ab1 protein and the Cry35Ab1 protein conducted to examine the insecticidal spectrum against the six (6) kinds of insect pests for the cultivation of maize in the US, it was found that the proteins show high specific insecticidal activity against limited insect pests (Reference 61). Among the 6 kinds of insect pests tested, the larvae of two (2) kinds of pest insects of the order Coleoptera, Northern corn rootworm (*Diabrotica barberi*) and Western corn rootworm (*D. virgifera virgifera*), exhibited especially high insecticidal activity. For Southern corn rootworm (*D. undecimpunctata howardi*), which belongs to the same family of Corn rootworm, both proteins exhibit lower insecticidal activity than against Northern corn rootworm and Western corn rootworm. For the adults of European corn borer, Corn earworm and Black cutworm, which are pest insects of the order Lepidoptera, and for Western corn rootworm, a pest insect of the order Coleoptera, no mortality was observed even at the maximum dose obtained in the test.

In order to investigate any effects on the non-target insects of the order Coleoptera other than Corn rootworm, a bioassay was conducted with two (2) species of ladybug (*Hippodamia convergens* and *Coleomegilla maculata*) (Reference 61). For the 2 species of ladybug tested, *H. convergens* is a relative of the species *H. tredecimpunctata*

timberlakei Capra which occurs in Japan (Reference 62). *C. maculata* is a beneficial insect occurring throughout North America and is known to eat maize pollen (Reference 63), though there is no report that it has related species inhabiting Japan. As a result of the bioassay, even at the maximum dose obtained in the test, no effect on the adults of *H. convergens* was observed. For the larvae of *C. maculata*, a decrease in live weight was observed, though no mortality was observed even at the maximum dose obtained in the test.

In addition to the insects of the order Coleoptera, tests were conducted to mammals (Reference 64; Reference 65; Reference 66), birds (Reference 67), fish (Reference 68), and Lepidoptera (Reference 61), Hymenoptera (Reference 61), Neuroptera (Reference 61), Collembola (Reference 61) and other insects. As a result, it was confirmed that the Cry34Ab1 protein and the Cry35Ab1 protein exhibit no toxicity against any of the non-target organisms tested.

― Proteins conferring tolerance to herbicides ―

[PAT protein]

The PAT protein (phosphinothricin acetyltransferase) confers tolerance to glufosinate herbicide. The glufosinate herbicide inhibits the glutamine synthase enzyme that synthesizes glutamine from glutamic acid and ammonia, which causes the ammonia to be accumulated in the plant body and causes the plant to die. The PAT protein acetylates glufosinate herbicide to transform it to nontoxic acetylglufosinate, thereby conferring glufosinate tolerance to the plant. Glufosinate herbicide is a nonselective herbicide, and possesses herbicidal activity against a variety of weeds even with a single agent. It has been safely applied throughout the world including in Japan and the US. Transfer of the *pat* gene into corn has made it possible to apply this herbicide to corn fields for weed control, which would provide farmers with an option for weed control.

[Modified CP4 EPSPS protein]

The modified *cp4 epsps* gene expressed in MON 88017 is a gene isolated from the *Agrobacterium* CP4 strain, which encodes 5-enol-pyruvylshikimate-3-phosphate synthase (CP4 EPSPS) and expresses the modified CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The nucleotide sequence of the wild-type *cp4*

epsps gene was modified in the modified *cp4 epsps* gene to enhance the expression level in plants without changing the functional activity of the wild-type CP4 EPSPS protein. Only a single modification was introduced to the amino acid sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine.

Plants treated with glyphosate wither away and die, since their 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19) is inhibited and the synthesis of aromatic amino acids that are essential for the synthesis of proteins fails. The modified *cp4 epsps* gene, gene of interest in MON 88017, produces the modified CP4 EPSPS protein which has high tolerance to the glyphosate herbicide. The activity of the modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene is not inhibited under the presence of glyphosate, thus the recombinant plants that express this protein have normal functions of shikimate synthesis pathway and continue to grow in the presence of glyphosate.

In order to investigate whether the Cry1A.105 protein, the modified Cry2Ab2 protein, the Cry1F protein, the modified Cry3Bb1 protein, the Cry34Ab1 protein, the Cry35Ab1 protein, the PAT protein, and the modified CP4 EPSPS protein expressed in the parent lines share any functionally important amino acid sequences with known allergens, they were compared with the allergens in the database (GenBank, EMBL, PIR, PBD, SwissProt, etc.). As a result, they did not share structurally related sequences with any known allergens.

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

The Cry1A.105 protein, the modified Cry2Ab2 protein, the Cry1F protein, the modified Cry3Bb1 protein, the Cry34Ab1 protein, and the Cry35Ab1 protein are all crystalline insecticidal proteins (Bt proteins) derived from *B. thuringiensis*. Many studies have been conducted on the mechanism of the insecticidal activity of the Bt proteins, (Reference 69), and there is no report available to date that the Bt protein possess any other functions. Therefore, it is considered unlikely that the Bt proteins possess any enzymatic activity affecting the metabolic system of the recipient organism.

The PAT protein acetylates the *L*-phosphinothricin (classified into *L*-amino acids), an active ingredient of glufosinate herbicide, though it does not acetylate any other *L*-amino acids, and it has little affinity for the glutamic acid, which is especially

resembling structurally (Reference 70). In addition, even in the presence of excessive amount of various amino acids, the reaction of the PAT protein to acetylate glufosinate is not inhibited and furthermore, it is reported that the PAT protein has extremely high substrate specificity to glufosinate (Reference 70). Consequently, for its high substrate specificity, the PAT protein is considered unlikely to affect the metabolic system of the recipient organism.

The EPSPS, functionally identical to the modified CP4 EPSPS, is an enzyme protein that catalyzes the shikimate pathway for the biosynthesis of aromatic amino acid. However, it is not a rate-determining enzyme in the pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In addition, EPSPS is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 71), and the only shikimate that is known to react with EPSPS other than S3P offers the reactivity of only one-two millionth that of S3P; therefore, it is considered unlikely to react as the substrate of EPSPS in any living organisms. Therefore, it is considered unlikely that the modified CP4 EPSPS protein would affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

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

MON 89034: PV-ZMIR245 assembled from plasmids including the vector pBR322 derived from *E. coli*

Cry1F line 1507: PHP8999 assembled from plasmids including the vector pUC19 derived from *E. coli*

MON 88017: PV-ZMIR39 assembled from plasmids including the vector pBR322 derived from *E. coli*

Event DAS-59122-7: PHP17662 assembled from plasmids including the vector pSB1 derived from *A. tumefaciens*

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 production of parent lines is as follows.

MON 89034:	PV-ZMIR245; 17,600 bp
Cry1F line 1507:	PHP8999; 9,504 bp
MON 88017:	PV-ZMIR39; 12,368 bp
Event DAS-59122-7:	PHP17662; 50,311 bp

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

The antibiotic resistant genes used as selectable markers are as follows. None of these antibiotic resistance marker genes have been transferred in the recipient organism.

MON 89034:	*aadA* gene to confer resistance to spectinomycin and streptomycin
Cry1F line 1507:	*nptII* gene to confer resistance to kanamycin
MON 88017:	*aadA* gene to confer resistance to spectinomycin and streptomycin
Event DAS-59122-7:	*tet* gene to confer resistance to tetracycline and *spc* gene to confer resistance to spectinomycin

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

Neither PV-ZMIR245, PHP8999, PV-ZMIR39, nor PHP17662 is known to be infectious.

(3) Method of preparing living modified organisms

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

The component elements of the plasmid vectors PV-ZMIR245, PHP8999,

PV-ZMIR39 and PHP17662 transferred in the recipient organism for development of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 are listed in Table 1 to Table 4 (pp.7-12). In addition, for the component elements from the donor nucleic acid used in the vectors, the location and section broken by restriction enzymes are shown in Figure 1 to Figure 4 (pp.3-6).

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

Transferring nucleic acid into the recipient organism was based on the following method.

MON 89034: *Agrobacterium* method
Cry1F line 1507: Particle gun bombardment method
MON 88017: *Agrobacterium* method
Event DAS-59122-7: *Agrobacterium* method

3) Processes of rearing of living modified organisms

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

Selection of transformed cells was made on the medium containing the following.

MON 89034: Paromomycin
Cry1F line 1507: Glufosinate
MON 88017: Glyphosate
Event DAS-59122-7: Glufosinate

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

In the development of MON 89034, MON 88017 and Event DAS-59122-7, *Agrobacterium* was removed by adding carbenicillin to the medium (Reference 72). Absence of remaining *Agrobacterium* in MON 89034 and MON 88017 was confirmed by transferring MON 89034 or MON 88017 to the carbenicillin-free medium and then observing that no colony of *Agrobacterium* is formed on the medium. In addition, absence of remaining *Agrobacterium* in Event

DAS-59122-7 was confirmed by transferring Event DAS-59122-7 to the carbenicillin-free medium and then observing the plant body under a microscope. For Cry1F line 1507, the particle gun bombardment method was used for transferring the nucleic acid into the recipient organism and thus *Agrobacterium* was not used.

(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

For MON 89034, in the LH172BC0F1 generation, which was obtained by crossing of regenerated individuals of R0 generation with other conventional cultivar of maize LH172, those individuals were selected (based on the PCR method) that contained only the T-DNA I region with the T-DNA II region separated. The individuals that contained T-DNA II region were discarded.

Regarding the selected individuals, further selection was carried out based on the analysis of transferred genes and the expression level of the Cry1A.105 protein and the modified Cry2Ab2 protein. Tests in the climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters (morphological and growth characteristics, yield and productivity, pest insect sensitivity, etc.) were examined in outdoor field tests. MON 89034 was selected upon the comprehensive evaluation of these results.

For Cry1F line 1507, leaf samples were taken from regenerated plant body to identify the presence or absence of the transferred genes based on the PCR method and then check that the Cry1F protein is successfully produced based on the ELISA method. In addition, the entire plant body was examined to identify the resistance to larvae of European corn borer is successfully available. The plant that was found to possess the resistance based on the examination was crossed with the line of the same propagation lines as the plant to obtain the seeds of the recombinant of the current generation (T0). Cry1F line 1507 was selected upon the comprehensive evaluation of the resistance to European corn borer and agronomic characters examined in outdoor field tests.

In the development of MON 88017, field experiments were carried out at a total of 169 field sites from 2000 to 2001. The strain for the final commercial cultivation was selected, and its environmental safety was evaluated.

For Event DAS-59122-7, leaf samples were taken from regenerated plant body to identify the presence or absence of the transferred genes based on the PCR method and then checked using the ELISA method to ensure that the Cry34Ab1 protein and the Cry35Ab1 protein were successfully produced. In addition, the entire plant body was examined to confirm that the resistance to Corn rootworm was successfully available. The plant that was found to possess the resistance based on the examination was crossed with the line of the same propagation lines as the plant to obtain the seeds of the recombinant of the current generation (T0). Event DAS-59122-7 was selected upon the comprehensive evaluation of the resistance to Corn rootworm and agronomic characters examined in outdoor field tests.

The status of application for approval of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 in Japan is summarized below (Table 5, p. 25).

Table 5 Status of application for approval of MON89034, Cry1F line 1507, MON88017, and Event DAS-59122-7 in Japan

	Safety as food	Safety as feed	Environmental safety
MON89034	November, 2007: Approved safety of use as food	October, 2007: Approved safety of use as feed	January, 2008: Approved for Type I Use Regulation
Cry1F line 1507	July, 2002: Approved safety of use as food	March, 2003: Approved safety of use as feed	March, 2005: Approved for Type I Use Regulation
MON88017	October, 2005: Approved safety of use as food	March, 2006: Approved safety of use as feed	April, 2006: Approved for Type I Use Regulation
Event DAS-59122-7	October, 2005: Approved safety of use as food	March, 2006: Approved safety of use as feed	April, 2006: Approved for Type I Use Regulation
This stack maize line	December, 2008: Approved safety of use as food	November, 2008: Approved safety of use as feed	October, 2008: Pending application

[Development process for MON 89034 × MON 88017 × Cry1F line 1507 × Event DAS-59122-7]

This stack maize is an F1 hybrid developed from the inbred lines of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 as the parents (Figure 5, p. 27).

[Confidential: Not made available or disclosed to unauthorized person]

Figure 5 Development process for MON 89034 × Cry1F line 1507 × MON 88017 × Event DAS-59122-7

(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 was confirmed that the transferred genes in MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 exist in the maize genome.

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

As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy of the individual target genes exists in the maize genome of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 at one site. In addition, it was also confirmed as a result of Southern blotting analysis on multiple generations that the transferred genes are inherited stably in offspring.

In addition, as a result of the nucleotide sequence analysis of transferred genes in MON 89034, it was found that, due to the homologous recombination, the 5'-terminal region of P-*e35S* to control the expression of the *cry1A.105* gene and the neighboring right border region have been replaced by the left border region in the T-DNA II region and the 5'-terminal region of P-*35S* to control the expression of the *nptII* gene. However, this homologous recombination did not take place in the protein encoding regions, and it has been confirmed that even in the Cry1A.105 protein encoding region, which is the nearest open reading frame, the Cry1A.105 protein is expressed normally in individual tissues. Consequently, it was concluded that this homologous recombination could not cause formation of any new open reading frame.

Moreover, as a result of the nucleotide sequence analysis of transferred nucleic acid in the Cry1F line 1507, it was confirmed that the transferred nucleic acid contained a part of the *cry1F* gene sequence in the 5'-terminal region, a part of the *pat* gene sequence in the 5'-terminal and 3'-terminal regions, and a part of *ORF25PolyA Terminator* sequence in the 3'-terminal region. However, Northern blotting analysis confirmed that these gene fragments were not transcribed into mRNA, thereby not functioning.

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

This item is not applicable because there is one copy for all of MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7.

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

The stability of expression was identified as follows.

MON 89034: Confirmed the expression of proteins by Western blotting analysis

Cry1F line 1507: Confirmed the expression of proteins by ELISA method, the bioassay using pest insects of the order Lepidoptera, and glufosinate herbicide-spraying test

MON 88017: Confirmed the expression of proteins by herbicide glyphosate spraying test and ELISA method

Event DAS-59122-7: Confirmed the expression of proteins by ELISA method, the bioassay using pest insects of the order Coleoptera, and glufosinate 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

Regarding the plasmid PV-ZMIR245 used for the production of MON 89034 and the plasmid PV-ZMIR39 used for the production of MON 88017, the region of recipient organism, which allows autonomous replication, is limited to gram-negative bacteria such as *E. coli*. Therefore, there is no possibility that the plasmids might be transmitted to any wild animals and wild plants under natural environment. In addition, the transferred nucleic acid in Cry1F line 1507 and Event DAS-59122-7 does not contain any sequence allowing transmission and thus, transmission of this item is absence.

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

A specific method for the detection and identification of MON 89034 is available using the DNA sequences of the transferred genes and the nearby regions of the plant genome primers.

For the detection and identification of Cry1F line 1507, a quantitative analysis kit is available from GeneScan Europe AG (Freiberg, Germany) (Cat. No.: 512 12023 10) applying a RT (Real Time)-PCR method using the nucleotide sequence specific to Cry1F line 1507 as primers. In addition, the ELISA method using the polyclonal antibodies respectively for the Cry1F protein and the PAT protein has been developed. The analysis kit for detection of the Cry1F protein is also commercially available from Strategic Diagnostics Inc. (Newark, DE, USA) (Cat. No.: 7000018). Moreover, the PAT protein detection kit is available from EnviroLogix (Portland, ME, USA) (Cat. No.: AP 014).

A specific method for the detection and identification of MON 88017 is available using the DNA sequences of the transferred genes and the nearby regions of the plant genome as primers.

For detection and identification of Event DAS-59122-7, a quantitative ELISA method using the polyclonal antibodies respectively for the Cry34Ab1 protein, the Cry35Ab1 protein, and the PAT protein has been developed.

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

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

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

This stack maize line contains the following traits derived from individual parent lines.

MON 89034:	Resistance to insects of the order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein which are produced from the transferred genes
Cry1F line 1507:	Resistance to insects of the order Lepidoptera due to the Cry1F protein, and tolerance to glufosinate herbicide due to the PAT protein, which are produced from the transferred genes
MON 88017:	Tolerance to glyphosate herbicide due to the modified CP4 EPSPS protein, and resistance to insects of the order Coleoptera (corn rootworm) due to the modified Cry3Bb1 protein, which are produced from the transferred genes
Event DAS-59122-7:	Resistance to insects of the order Coleoptera due to the Cry34Ab1 protein and the Cry35Ab1 protein, and tolerance to glufosinate herbicide due to the PAT protein, which are produced from the transferred genes

The Bt proteins (Cry1A.105 protein, modified Cry2Ab2 protein, Cry1F protein, modified Cry3Bb1 protein, Cry34Ab1 protein and Cry35Ab1 protein) expressed in the respective parent lines are crystalline insecticidal proteins (Cry) derived from *B. thuringiensis*. The Bt proteins are partially digested in the midgut of sensitive species of insects to form core proteins. The core proteins bind to the specific receptors on the cell membranes of midgut epithelium to form cation-selective pores on the cell membranes of midgut epithelium, causing destruction of midgut epithelium cells and inhibition of digestive process in the sensitive species of insects, leading to death of the insects (Reference 41; Reference 43). For the mechanism in which Bt proteins develop the insecticidal activity, a number of studies have been made (Reference 69), and there is no report published to date that Bt proteins possess any other functions. Therefore, Bt proteins are considered unlikely to exhibit any enzymatic activity.

In addition, it has been reported that the PAT protein expressed in Cry1F line 1507 and Event DAS-59122-7 possesses extremely high substrate specificity against *L*-glufosinate, the active ingredient in glufosinate herbicide (Reference 70).

Moreover, the modified CP4 EPSPS protein expressed in MON 88017 has high substrate specificity and it is not the rate-determining enzyme in the pathway of

shikimate synthesis and thus, enhanced EPSPS activity would not increase the concentrations of aromatic amino acids, the end products of this pathway.

Based on the above understanding, it is considered that the Bt proteins, the PAT protein, and the modified CP4 EPSPS protein expressed in this stack maize line differ from each other in the mechanism of action and thus function independently from each other, Consequently, these proteins are considered not to fall under the proteins referred to in Reference 73 as requiring examinations on possible interaction.

In addition, the Bt proteins, the PAT protein, and the modified CP4 EPSPS protein expressed in this stack line maize are also considered not to affect the metabolic pathway of plants based on the facts that either they do not possess any enzyme activity or they have high substrate specificity. Therefore, it is considered low that the proteins expressed from the individual parent lines in this stack line maize would additionally affect the metabolic pathway of plants.

Based on the above understanding, it is unlikely that the proteins expressed in this stack maize line from the individual parent lines would interact with each other, affecting the safety of this maize.

To confirm in practice that the proteins expressed in this stack maize line from individual parent lines do not interact with each other in terms of the resistance of this stack maize line to insects of the order Lepidoptera, insects of the order Coleoptera and tolerance to glufosinate herbicide and glyphosate herbicide, bioassays were carried out in 2007 at the US Monsanto Company using this stack maize line as described below.

[Bioassay using insects of the order Lepidoptera]

Regarding resistance to Lepidoptera, twelve (12) individuals each of this stack maize line, MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7, and the non-recombinant control maize (XE6001), were cultivated in pots (2 experiments x 2 replicates per experiment x 3 plants per replicate; n =4), and at the 5 to 6 vegetative stage, the first instar larvae of Fall armyworm (FAW, *Spodoptera frugiperda*) were inoculated (25 larvae/individual). On the 9th day after inoculation of Fall armyworm, the Leaf Damage Rating (LDR; the severity of insect damage to leaf) was determined based on a 10-step scale from 0 (no damage) to 9 (serious damage: a greater part of leaf

is damaged) (Reference 74). For the heteroscedasticity, data was translated by rank before statistical treatment, and the translated data was subject to statistical treatment.

As a result of the investigation, regarding the severity of insect damage to leaf (LDR), no statistically significant difference was observed between this stack maize line and MON 89034 and Cry1F line 1507 (Tukey's Test, significance level 5%) (Table 6, p. 33). Therefore, it was confirmed that the resistance of this stack maize line to pest insects of the order Lepidoptera remains unchanged by crossing of parent lines.

Table 6 Investigational result of the severity of damage [1] by Fall armyworm (FAW; *S. frugiperda*), order Lepidoptera, based on bioassay of this stack maize line (n=4 replicates)[10]

Samples tested	Leaf Damage Rating (LDR) ± Standard error	
This stack maize line	1.08 ± 0.08	A[2]
MON 89034	1.08 ± 0.08	A
Cry1F line 1507	1.42 ± 0.16	A
MON 88017	7.50 ± 0.17	B
Event DAS-59122-7	7.58 ± 0.08	B
Non-recombinant control maize	7.25 ± 0.25	B

[1] 0: No damage, 1: A small number of needle tip like holes and worm holes observed on a small number of leaves, 2: A small number of worm holes observed on a small number of leaves, 3: Worm holes frequently observed on several leaves, 4: Worm holes and extending bite marks observed on several leaves, 5: Extending bite marks observed on several leaves, 6: Bite marks extending about 2.5 cm observed on several leaves, 7: A half of a leaf area found damaged, 8: Two-thirds of a leaf area found damaged, 9: A greater part of leaves found damaged.

[2] Different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (Tukey's Test, significance level 5%).

[Bioassay using insects of the order Coleoptera]

Regarding resistance to the insects of the order Coleoptera, twelve (12) individuals each of this stack maize line, MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7 and the non-recombinant control maize (XE6001) were cultivated in pots (4 replicates x 3 plants per replicate), and at the 3 to 4 vegetative stage, eggs of Western

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corn rootworm (WCRW, *Diabrotica virgifera virgifera*) (930 eggs/pot) were inoculated. In the 3rd week after inoculation of Western corn rootworm, the severity of insect damage by Western corn rootworm was evaluated by using the Nodal Injury Score (NIS: the severity of damage to root) developed by Reference 75. In this method, the severity of insect damage is represented in the successive values ranging from 0.00 (no damage) to 3.00 (serious damage). The scores of 0.25 or less (25% or less of the first node is damaged) refer to the minimum damage, while the score of 3.00 indicates that three (3) or more nodes are all damaged. For the heteroscedasticity, data was translated by rank before statistical treatment, and the translated data was subject to statistical treatment.

As a result of the investigation, a statistically significant difference was observed between this stack maize line and MON 88017 and between this stack maize line and Event DAS-59122-7 (Tukey's Test, significance level 5%) (Table 7, p.34). However, differences in the observed severity of insect damage to the roots were found slight, and this was considered to result from the fact that the individual Bt proteins worked properly.

Table 7 Investigational result of the severity of damage [1] by Western corn rootworm (WCRW; *D. virgifera virgifera*), order Coleoptera, based on bioassay of this stack maize line (n=4 replicates)[11]

Samples tested	Nodal Injury score (NIS) ± Standard error	
This stack maize line	0.04 ± 0.00	A[2]
MON 88017	0.24 ± 0.04	B
Event DAS-59122-7	0.26 ± 0.05	B
MON 89034	1.89 ± 0.01	C
Cry1F line 1507	2.10 ± 0.07	D
Non-recombinant control maize	1.97 ± 0.03	CD

[1] 0.00: No damage, 1.00: The first node or the root equivalent to the first node is damaged, 2.00: The 2nd or the root equivalent to the 2nd node is damaged, 3.00: The 3rd and subsequent nodes of roots are damaged.

[2] Different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (Tukey's Test, significance level 5%).

[Bioassay using glufosinate herbicide]

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Regarding tolerance to glufosinate herbicide, five (5) individuals each of this stack maize line, MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7 and the non-recombinant control maize (XE6001) were cultivated in pots in a greenhouse (5 replicates x 1 plant per replicate), and at the 4 to 5 vegetative stage, glufosinate herbicide (Product name: Liberty) was sprayed. On the 10th day after spraying glufosinate herbicide, the severity of injury by spraying of glufosinate herbicide to plant bodies was evaluated based on a 11-step scale from 0 (no injury) to 10 (nearly the entire plant body withered and died due to the injury). The concentration of glufosinate sprayed was 80 L Liberty/ha (17.0 kg a.i./ha) and corresponds to a 32-times higher dosage than the normal dosage of 2.5 L Liberty/ha (0.54 kg active ingredient (a.i.)/ha). The severity of injury in the table refers to the mean value ± standard error.

As a result of the investigation, regarding the severity of injury by the spraying of herbicide, no statistically significant difference was observed between this stack maize line and Cry1F line 1507 and Event DAS-59122-7 (Tukey's Test, significance level 5%) (Table 8, p.35). Therefore, it was confirmed that the tolerance of this stack maize line to glufosinate herbicide remains unchanged by the crossing of the parent lines.

Table 8 Investigational result of the severity of injury by spraying glufosinate herbicide to this stack maize line (mean value ± standard error of the severity of injury by the herbicide to plant body [1]) (n=5 replicates)[12]

Samples tested	Concentration			
	2.5 L/ha		80 L/ha	
This stack maize line	0.0 ± 0.0	A[2]	1.8 ± 0.2	A
Cry1F line 1507	0.0 ± 0.0	A	1.6 ± 0.2	A
Event DAS-59122-7	0.0 ± 0.0	A	2.0 ± 0.0	A
MON 89034	7.6 ± 0.5	B	8.8 ± 0.4	B
MON 88017	9.0 ± 0.3	C	8.4 ± 0.2	B
Non-recombinant control maize	8.0 ± 0.3	BC	8.0 ± 0.5	B

[1] 0: No injury, 1 to 9: Approx. 10 to 90% of a leaf area turned white, yellow and/or decayed, 10: 100% of a leaf area died due to the injury.

[2] In a given line, different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (Tukey's Test, significance level 5%).

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[Bioassay using glyphosate herbicide]

Regarding tolerance to glyphosate herbicide, five (5) individuals each of this stack maize line, MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7 and the non-recombinant control maize (XE6001) were cultivated in pots in a greenhouse (5 replicates x 1 plant per replicate), and at the 4 to 5 vegetative stage, herbicide glyphosate (Product name: Roundup WeatherMAX) was sprayed. On the 10th day after spraying herbicide glyphosate, the severity of injury by spraying of herbicide glyphosate to plant bodies was evaluated based on a 11-step scale from 0 (no injury) to 10 (nearly the entire plant body withered and died due to the injury). The concentration of glyphosate sprayed of 68 L Roundup/ha (27.0 kg acid equivalent/ha) refers to 32-times higher dosage than the normal dosage of 2.1 L Roundup/ha (0.84 kg acid equivalent/ha). The severity of injury in the table refers to mean value ± standard error.

As a result of investigation, regarding the severity of injury by spraying of herbicide at the normal dosage, no statistically significant difference was observed between this stack maize line and MON 88017 (Tukey's Test, significance level 5%) (Table 9, p.36). Therefore, it was confirmed that the tolerance of this stack maize line to glyphosate herbicide remains unchanged by crossing of parent lines.

Table 9 Investigational result of the severity of injury by spraying glyphosate herbicide to this stack maize line (mean value ± standard error of the severity of injury by the herbicide to plant body [1]) (n=5 replicates)[13]

Samples tested	Concentration			
	2.1L/ha		68 L/ha	
This stack maize line	0.4 ± 0.2	A[2]	2.0 ± 0.0	A
MON 88017	0.2 ± 0.2	A	1.2 ± 0.4	A
MON 89034	8.6 ± 0.2	B	9.8 ± 0.2	B
Cry1F line 1507	9.8 ± 0.2	C	10.0 ± 0.0	B
Event DAS-59122-7	9.8 ± 0.2	C	10.0 ± 0.0	B
Non-recombinant control maize	9.4 ± 0.4	BC	10.0 ± 0.0	B

[13] All the rights pertinent to the information in the table above and the responsibility for the content remain with Dow Chemical Japan Limited and Monsanto Japan Limited.

[1] 0: No injury, 1 to 9: Approx. 10 to 90% of a leaf area turned white, yellow and/or decayed, 10: 100% of a leaf area died due to the injury.

[2] In a given line, different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (Tukey's Test, significance level 5%).

Based on the above results, it was concluded that the individual proteins expressed in the relevant parental lines do not interact with each other and that the traits obtained from the transferred genes remain unchanged in this stack maize line.

Consequently, with respect to the differences in physiological or morphological characteristics between this stack maize line and maize, the taxonomic species to which the recipient organism belongs, the evaluation was conducted based on the results of the individual examinations of the parental lines MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7.

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 [14]

(a) Morphological and growth characteristics

For the morphological and growth characteristics of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 and their non-recombinant control maize, an examination was conducted for the items listed in Table 10 (p.40).

As a result, a statistically significant difference was observed between MON 89034 and its non-recombinant control maize in ear diameter and grain number per ear, between Cry1F line 1507 and its non-recombinant control maize in germination rate and ear diameter, between MON 88017 and its non-recombinant control maize in culm length and ear diameter, and between Event DAS-59122-7 and its non-recombinant control

[14] All the rights pertinent to the information of MON 89034 and MON 88017 in (a) to (g) of this item and the responsibility for the content remain with Monsanto Japan Limited. All the rights pertinent to the information of Cry1F line 1507 and Event DAS-59122-7 in (a) to (g) of this item and the responsibility for the content remain with Dow Chemical Japan Limited.

maize in culm length. However, these items were fell within the variable ranges for conventional maize or observed statistically significant differences were limited to only one of the two sibling-line hybrid varieties tested (Table 2 in p.8 of Annex 1; Table 2 to Table 11 in pp.11-15 of Annex 3; Tables 2-1 and 2-2 in pp.30-31 of Annex 2; Table 2 to Table 21 in pp.13-24 of Annex 4).

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

MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 withered or died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control maize (Figure 6-2 in p.14 of Annex 1; Photo 6 in p.13 of Annex 3; Table 4 in p.42 of Annex 2; Table 25 and Photo 11 in p.32 and p.33 of Annex 4).

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not re-grow and propagate vegetatively, or produce seeds. Actually, at the end of isolated field tests, the start of withering and death after ripening was observed.

(d) Fertility and size of the pollen

MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 exhibited high fertility of the pollen similarly to their non-recombinant control maize. Also, no difference was observed regarding the shape and size of pollen (Figures 8-1 and 8-2 in p.19 of Annex 1; Photos 4 and 5 in pp.11-12 of Annex 3; Table 3 and Photo in pp.38-40 of Annex 2; Photos 8 and 9 in pp.26-27 of Annex 4).

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

Regarding seed production, comparisons were conducted between MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 and their respective non-recombinant control maize for the characteristics referring to the production of seeds listed in I-2-(6)-2)-(a) (p.36). As a result, a statistically significant difference was observed between MON 89034 and its non-recombinant control maize regarding ear diameter and grain number per ear, between Cry1F line 1507 and its non-recombinant control maize

regarding ear diameter, and between MON 88017 and its non-recombinant control maize regarding ear diameter. However, these items were fell within the variable ranges for conventional maize or observed statistically significant differences were limited to one of the two sibling-line hybrid varieties tested (Table 6 in p.20 of Annex 1; Table 6 to Table 10 in pp.8-9 of Annex 3; Tables 2-1 and 2-2 in pp.30-31 of Annex 2; Table 10 to Table 18 in pp.18-22 of Annex 4).

Regarding the shedding habit of the seed, shedding habits of the seed were not observed in the natural environment, since the ears of all of MON 89034, Cry1F line 1507, MON 88017, Event DAS-59122-7 and their non-recombinant control maize were covered with bracts at the time of harvesting.

Regarding the germination rate in order to identify the dormancy of seeds, a germination test was carried out for the grain harvested from MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 and their non-recombinant control maize. As a result, no difference was observed and no dormancy of the seed was identified (Tables 3 and 4 in p.16 of Annex 1; Tables 2 and 13 in p.5 and p.12 of Annex 3; Table 4 in p.42 of Annex 2; Table 2 and Table 23 in p.13 and p.29 of Annex 4).

(f) Crossability

A crossability test for the parental lines MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7 was not performed, since no wild relatives grow in Japan that can be crossed with maize.

Table 10 Investigational results of morphological and growth characteristics of MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7

	MON 89034	Cry1F line 1507	MON 88017	Event DAS-59122-7
Uniformity of germination	○	○	○	○
Number of germinated plants	○	—	—	—
Germination rate	○	○*	○	○
Time of tasseling	○	○	○	○
Time of silking	○	○	○	○
Flowering time	○	—	—	—
Time of flower initiation	—	○	—	○
Time of flower completion	—	○	—	○
Flowering period	—	○	—	○
Shape of flower organ	—	—	—	○
Culm length	○	○	○*	○*
Culm diameter	○	—	—	—
Plant type (Plant shape)	○	○	○	○
Tiller number	○	○	○	○
Height of ear	○	○	○	○
Maturation time	○	○	○	○
Number of ears (Total number of ears)	○	○	○	—
Number of productive ears	○	○	—	○
Grain number per ear	○*	—	○	—
Row number per ear	○	○	○	○
Grain number per row	○	○	○	○
100-kernel weight	○	○	○	○
Weight of above-ground parts at the harvest time (Plant weight) (Fresh weight of above-ground parts at the harvest time)	○	○	○	○
Ear length	○	○	○	○
Ear diameter	○*	○*	○*	○
Grain color	○	○	○	○
Grain shape	○	○	○	○

○: Examined

－: Not examined

* A statistically significant difference was observed. However, these items were fell within the variable ranges for conventional maize or observed statistically significant difference was limited to one of the two sibling-line hybrid varieties tested (Table 2 in p.8 of Annex 1; Table 2 to Table 11 in pp.5-9 of Annex 3; Tables 2-1 and 2-2 in pp.30-31 of Annex 2; Table 2 to Table 21 in pp.13-24 of Annex 4).

(g) Productivity of harmful substances

For maize, the secretion of any harmful substances from roots, which affect the surrounding plants or microorganisms in soil, has not been reported. In addition, the production of any allelochemicals, which affect other plants after they die, has not been reported.

As a result of succeeding crop tests, soil microflora tests, and plow-in tests conducted for MON 89034, Cry1F line 1507, MON 88017 and Event DAS-59122-7, a statistically significant difference was observed in the fresh weight of lettuce in the succeeding crop tests and plow-in tests for Cry1F line 1507 and in the fresh weight of radish in the plow-in tests for MON 88017. However, these items fell within the variable ranges for conventional maize or observed statistically significant differences were limited to one of the two sibling-line hybrid varieties tested (Tables 7, 8, and 9 in p.23 of Annex 1; Table 14-1 to Table 14-4 and Tables 15 and 16 in p.16 and p.18 of Annex 3; Table 5 to Table 7 in pp.44-45 and p.47 of Annex 2; Table 1 to Table 6 in pp.6-7 of Annex 5).

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

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

This stack maize line was produced by crossing of maize resistance to Lepidoptera (MON 89034), maize tolerant to glufosinate herbicide and resistant to Lepidoptera (*B.t.* Cry1F maize line 1507), maize tolerant to glyphosate herbicide and resistant to Coleoptera (MON 88017), and maize tolerant to glufosinate herbicide and resistant to Coleoptera (*B.t.* Cry34/35Ab1 Event DAS-59122-7). These parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied for Type I Use same as this stack maize line.

The Bt proteins (Cry1A.105 protein, modified Cry2Ab2 protein, Cry1F protein, modified Cry3Bb1 protein, Cry34/35Ab1 protein), the PAT protein and the modified CP4 EPSPS protein differ from each other in their mechanism of action and function independently from each other and thus, these proteins are considered not to fall under the proteins referred to by Schrijver *et al.* (2007) as requiring examinations for possible interaction. In addition, these proteins either do not possess any enzyme activity or have high substrate specificity and thus, they are also considered not to affect the metabolic pathway of plants. Therefore, it was considered unlikely that the proteins expressed in this stack maize line from individual parent lines would additionally affect the metabolic pathway of plants.

As a result of actually conducted bioassays, the resistance to Lepidoptera, the tolerance to glufosinate herbicide, and the tolerance to glyphosate herbicide expressed in this stack maize line were found at similar levels as exhibited by the individual parent lines. The resistance to Coleoptera expressed in this stack maize line was found higher than that exhibited by the parent lines individually, though an observed difference was small

and thus considered to result from the proper functioning of the individual Bt proteins. Consequently, it is considered unlikely that the proteins expressed in this stack maize line from individual parent lines would interact with each other 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.

In addition, based on the above-mentioned findings that in this stack maize line, that was produced by the crossing of all of the parent lines, the proteins expressed from individual parent lines do not interact with each other, it is considered that also in the stack maize lines that contain a any combination of the transferred genes present in the individual parent lines of this stack maize line, no interaction would occur among the expressed proteins and no changes in the obtained traits would occur.

Based on the above understanding, it is considered unlikely that notable changes in traits have occurred in this stack maize line, except for the traits it received from both the parent lines.

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

(1) Competitiveness

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

As a result of investigation for various characteristics referring to competitiveness of MON89034, Cry1F line 1507, MON88017 and Event DAS-59122-7, the parent lines of this stack maize line, the individual parent lines exhibited a statistically significant difference from their non-recombinant control plants regarding some items examined. However, the differences were judged not to be so large as enhancing the competitiveness.

This stack maize line is given the traits to be resistant to the insects of order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein expressed in MON89034 and the Cry1F protein expressed in Cry1F line 1507, and to be resistant to

the insects of order Coleoptera due to the modified Cry3Bb1 protein expressed in MON88017 and the Cry34Ab1 protein and the Cry35Ab1 protein expressed in Event DAS-59122-7. However, the insect damage by these insect pests is not the major factor to inhibit the growth of maize under the natural environment in Japan and then, it is considered unlikely that these characteristics cause maize, a crop plant, to become self-seeding in the natural environment and enhance the competitiveness. In addition, this stack maize line possesses the tolerance to glufosinate herbicide due to the expression of the PAT protein and the tolerance to glyphosate herbicide due to the expression of the modified CP4 EPSPS protein. However, it is not generally considered that, in the natural environment less expected to suffer spraying of glufosinate and glyphosate, the tolerances to glufosinate and glyphosate would increase the competitiveness.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and stack maize lines that contain a any combination of the transferred genes present in the individual parent lines of this stack maize line, would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

(2) Productivity of harmful substances

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

It has been confirmed that all the proteins expressed in this stack maize line have no sequence that is structurally homologous with any known allergens. In addition, based on the findings that the Bt proteins offer no enzyme activity and then they are considered to function independently from the metabolic system of the recipient organism and that the PAT protein and the modified CP4 EPSPS protein possess high substrate specificity, it was considered unlikely that this stack maize line would act on the metabolic system of the recipient organism and produce any harmful substances.

Moreover, as a result of succeeding crop tests, plow-in tests and soil microflora tests conducted to examine 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), a statistically significant difference from the non-recombinant control plants was observed regarding some items. However, these differences did not suggest that the productivity of harmful substances has been increased in any of the parent maize line.

It has been reported that the Cry1A.105 protein and the modified Cry2Ab2 protein expressed in MON 89034 and the Cry1F protein expressed in Cry1F line 1507 exhibit the insecticidal activity against the insects of the order Lepidoptera and also that the modified Cry3Bb1 protein expressed in MON 88017 and the Cry34Ab1 protein and the Cry35Ab1 protein expressed in Event DAS-59122-7 exhibit the insecticidal activity against the insects of the order Coleoptera.

For these findings, there is a concern about the possibility that the pollens of this stack maize line would affect the non-target insects of the order Lepidoptera and the order Coleoptera. However, it is considered unlikely that these non-target insects inhabit locally near the fields for cultivation of this stack maize line and then, it is considered extremely low that they could be affected in the level of population.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and stack maize lines that contain a any combination of the transferred genes present in the individual parent lines of 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 recombinant maize, and that the use of such maize poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by 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 and stack maize lines that contain a any combination of the transferred genes present in the individual parent lines 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.