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 herbicide (*cry1F, cry34Ab1, cry35Ab1, pat, Zea mays* subsp. *mays* (L.) Iltis) (1507 × 59122, OECD UI : DAS-Ø15Ø7-1 × DAS-59122-7)
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

I. Information concerning preparation of living modified organisms

Stack line 1507×59122 and its parent lines, Cry1F line 1507 and Event DAS-59122-7, were jointly developed by Dow AgroSciences LLC (USA) and Pioneer Hi-Bred International, Inc (USA). Stack line 1507×59122 is a cultivar created by crossbreeding inbred lines of Cry1F line 1507 and Event DAS-59122-7 through the conventional crossbreeding methods.

two genes

The following genes were introduced into the stack line 1507×59122: *cry1F* gene derived from Cry1F line 1507 to confer resistance to Lepidoptera, *cry34/35Ab1* gene derived from Event DAS-59122-7 to confer resistance to Coleoptera, and *pat* gene derived from Cry1F line 1507 and Event DAS-59122-7 to confer tolerance to glufosinate herbicide.

For the parent lines, Cry1F line 1507 and Event DAS-59122-7, applications have been filed for the Type 1 Use of Living Modified Organism, in line with Item 2 of Article 4 of the “Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms” enacted in February 2004. The Conference on Biological Diversity Risk Assessment judged that neither Cry1F line 1507 nor Event DAS-59122-7 would result in Adverse Effect on Biological Diversity when used as Type 1 Use which was described in the application for this stack line 1507×59122. Cry1F line 1507 gained approval on March 2, 2005, and the procedure of public comments for Event DAS-59122-7 have been accepted since February 21, 2005.

To create this evaluation document, we referred to summary documents of Cry1F line 1507 and Event DAS-59122-7, which are both found in the web site of Japan Biosafety Clearing House (J-BCH) provided by the Ministry of the Environment.

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

Table 1 and Table 2 show the composition and the origins of component elements of the donor nucleic acid used to produce Cry1F line 1507 and Event DAS-59122-7, respectively.

Table 1 Composition and origins of component elements of the donor nucleic acid and their origins used for developing Cry1F line 1507

Component elements	Size (kbp)	Origin and function
Cry1F gene expression cassette		
UBIZM1(2) PRO	1.98	Ubiquitin constitutive promoter[1)] derived from *Zea mays* (including intron and 5' untranslated region)
Cry1F	1.82	A gene that encodes Cry1F protein derived from *Bacillus thuringiensis* var. *aizawai* (Optimized to activate the expression in plant body[2)])
ORF25PolyA TERM	0.72	A terminator to terminate transcription from *Agrobacterium tumefaciens* pTi5955
Pat gene expression cassette		
CAMV35S PRO	0.53	35S constitutive promoter[1)] derived from cauliflower mosaic virus (CaMV)
Pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *Streptomyces viridochromogenes* (Optimized to activate the expression in plant body[3)])
CAMV35S TERM	0.21	35S terminator to terminate transcription, which derived from cauliflower mosaic virus (CaMV)

1) Constitutive promoter: A promoter that drives the expression of target genes in all sites in plant body
2) In the produced protein, the residue at the second position from the end of its amino acid sequence is changed from phenylalanine to leucine.
3) The produced protein has none of its amino acids modified.

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

Component elements	Size (kbp)	Origin and function
Cry34Ab1 gene expression cassette		
UBIZM1(2) PRO	1.98	Ubiquitin constitutive promoter[1)] derived from *Zea mays* (including intron and 5' untranslated region)
Cry34Ab1	0.37	A gene that encodes Cry34Ab1 protein derived from *Bacillus thuringiensis* PS149B1 strain
PIN Ⅱ TERM	0.32	A potato-derived protease inhibitor II terminator to terminate transcription
Cry35Ab1 gene expression cassette		
TA Peroxidase PRO	1.30	A peroxidase promoter derived from *Triticum aestivum*, known to be expressed in roots
Cry35Ab1	1.15	A gene that encodes Cry35Ab1 protein derived from *Bacillus thuringiensis* PS149B1 strain
PIN Ⅱ TERM	0.32	A potato-derived protease inhibitor II terminator to terminate transcription
Pat gene expression cassette		
CAMV 35S PRO	0.53	35S constitutive promoter[1)] derived from cauliflower mosaic virus (CaMV)
pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein), derived from *Streptomyces viridochromogenes* (Optimized to activate the expression in plant body[2)])
CAMV 35S TERM	0.21	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
2) The produced protein has none of its amino acids modified.

(2) Functions of component elements

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

Table 1 and Table 2 show functions of individual component elements of donor nucleic acid, including target genes, expression regulation regions, localization signals, selective markers, in Cry1F line 1507 and Event DAS-59122-7.

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

a. Cry1F protein

Cry1F protein is a kind of insecticidal crystal protein (*B.t.* protein) known as δ-endotoxin produced by *Bacillus thuringiensis* (hereinafter referred to as "*B.t.*"), a gram-positive bacterium, universally exists in soil. The Cry1F protein is derived from *Bacillus thuringiensis* var. *aizawai* and possesses an insecticidal activity against European corn borer (*Ostrinia nubilalis*).

The Cry1F protein binds to the specific receptors in the midgut cells of the target pest insects when ingested similarly as other *B.t.* proteins and forms pores in the cells, which leads to the destruction of ion channels and results in the broken midgut cells and successful insecticide activity. In general, *B.t.* proteins are known to have an extremely specific insecticidal activity. Likewise, the Cry1F protein shows toxicity only against Lepidoptera such as European corn borer, Fall armyworm and Beet armyworm, not against other non-target species.

It has not been reported that the Cry1F protein shares amino acid sequence homology with any of the known allergenic proteins.

b. Cry34Ab1 and Cry35Ab1 proteins

Cry34Ab1 and Cry35Ab1 proteins are ones of *B.t.* proteins derived from *Bacillus thuringiensis* PS149B1 strain, having an insecticidal activity against corn rootworm (*Diabrotica spp.*). Since they function in collaboration with each other, they are called binary proteins.

It is suggested that the Cry34Ab1 and Cry35Ab1 proteins work in concert to effect, and, like other *B.t.* proteins including Cry1F protein, destroy midgut cell membrane of target insects. The Cry34Ab1 and Cry35Ab1 proteins also show an extremely specific insecticidal activity, only against larvae of two Coleopteran pests, northern corn rootworm (*Diabrotica barberi*) and western corn rootworm (*Diabrotica virgifera virgifera*).

It has not been reported that the Cry34Ab1 and Cry35Ab1 proteins share amino acid sequence homology with any of the known allergenic proteins.

c. PAT protein

PAT protein (phosphinothricin acetyltransferase) confers the tolerance to glufosinate herbicide. The glufosinate herbicide contains *L*-glufosinate, the active ingredient, which inhibits the activity of glutamine synthase that synthesizes glutamine from glutamic acid and ammonia. As a result, ammonia is accumulated in the plant body, causing the plant to die. The PAT protein acetylates and detoxifies *L*-glufosinate, thereby conferring the glufosinate tolerance to the plant body.

It is reported that the PAT protein shows extremely high substrate specificity against *L*-glufosinate, and that it does not accept other *L*-amino acids or select *D*-glufosinate for substrates.

It has not been reported that the PAT proteins share amino acid sequence homology with any of the known allergenic proteins.

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

Similarly to other Cry proteins, it has not been reported that the Cry1F, Cry34Ab1 and Cry35Ab1 proteins act as enzyme in any plant body. As mentioned above, it has been reported that the PAT protein exhibits high substrate specificity.

Consequently, none of the Cry1F, Cry34Ab1 and Cry35Ab1 proteins possesses enzyme activity, and the PAT protein exhibits very high substrate specificity. Therefore, it is highly unlikely that, in stack line 1507×59122, the inserted genes will have unexpected effects on the recipient organism's metabolic systems, or interact with each other.

2. Information concerning vector

(1) Name and origin

The plasmid PHP8999 derived from *Escherichia coli* plasmid pUC19 was used for a vector to create Cry1F line 1507 (Figure 1). The plasmid PHP17662 derived from *Escherichia coli* plasmid pSB1 was used for a vector to create Event DAS-59122-7 (Figure 2).

(2) Properties

i) The numbers of base pairs and nucleotide sequence of vector

The number of base pairs of the vector used to create Cry1F line 1507 is 9,504 bp.

The number of base pairs of the vector used to create Event DAS-59122-7 is 50,321 bp. The nucleotide sequences of the component elements in each vector have been obtained.

ii) Types of any nucleotide sequence having specific functions

Vector backborn of plasmid PHP8999 contains antibiotic resistant marker (*nptII* gene) to select the microorganisms that contain the transformed plasmid for propagation. The *nptII* gene confers the resistance to antibiotics, kanamycin. Vector backbone of plasmid PHP17662 contains antibiotic resistant markers (*tet* and *spc* genes) outside the T-DNA region. These markers enable us to screen microorganisms containing a transformed plasmid. The *tet* gene confers the resistance to antibiotics, tetracycline; the *spc* gene confers the resistance to antibiotics, spectinomycin. These antibiotic genes are not introduced in the recipient organism.

iii) Presence or absence of infectious characteristics of vector

Neither of 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

Plasmid PHP8999 was treated with the restriction enzyme *Pme*I to produce a linear DNA fragment (PHI8999A) containing the donor nucleic acid only, which was used to create Cry1F line 1507. The antibiotic-resistant marker (*nptII* gene) is present outside the donor nucleic acid region, and therefore is not introduced into the recipient organism. The linear DNA fragment (PHI8999A) consists of the following components; [*UBIZM1(2) Promoter*]-[*cry1F*]-[*ORF25PolyA Terminator*]-[*CAMV35S Promoter*]-[*pat*]-[*CAMV35S Terminator*].

As for plasmid PHP17662 used to create Event DAS-59122-7, its T-DNA region consists of the following components; [*UBIZM1(2) Promoter*]-[*cry34Ab1*]-[*PINII Terminator*]-[*TA Peroxidase Promoter*]-[*cry35Ab1*]-[*PINII Terminator*]-[*CAMV35s Promoter*]-[*pat*]-[*CAMV35s Terminator*].

(2) Method of transferring nucleic acid transferred in the recipient organism

The nucleic acid was transferred into the recipient organism by the particle gun bombardment to create Cry1F line 1507, and into the recipient organism by the Agrobacterium method to create Event DAS-59122-7. Each of the nucleic acid

fragments was introduced into the Hi-II callus of the maize cultivar A188×B73 to create Cry1F line 1507 and Event DAS-59122-7.

(3) Processes of rearing of living modified organisms

The parent lines, Cry1F line 1507 and Event DAS-59122-7, were bred based on the conventional F1 hybrid breeding method. This stack line 1507×59122 was bred based on the conventional crossbreeding method by Dow AgroSciences LLC (USA) and Pioneer Hi-Bred International, Inc (USA).

In Japan, it was confirmed in June 2002 that the use of Cry1F line 1507 in an open system met the Guidelines for the Use of Recombinant in Agriculture, Forestry, and Fisheries (hereinafter referred to as "Guidelines"). An application was filed for the Type 1 Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" enacted in February 2004, and then gained approval on March 2, 2005. In addition, its safeties as food and as feed were confirmed in July 2002 and in May 2002, respectively (the safety as feed was reconfirmed in March 2003 in accordance with the legislation of the examination system).

For Event DAS-59122-7, like for Cry1F line 1507, an application was filed for the Type 1 Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms". The procedure of public comments for Event DAS-59122-7 have been completed in February 21, 2005. In addition, applications were filed for its safety as food with the Ministry of Health, Labour and Welfare in May 2004, and for its safety as feed with the Ministry of Agriculture, Forestry and Fisheries in June 2004.

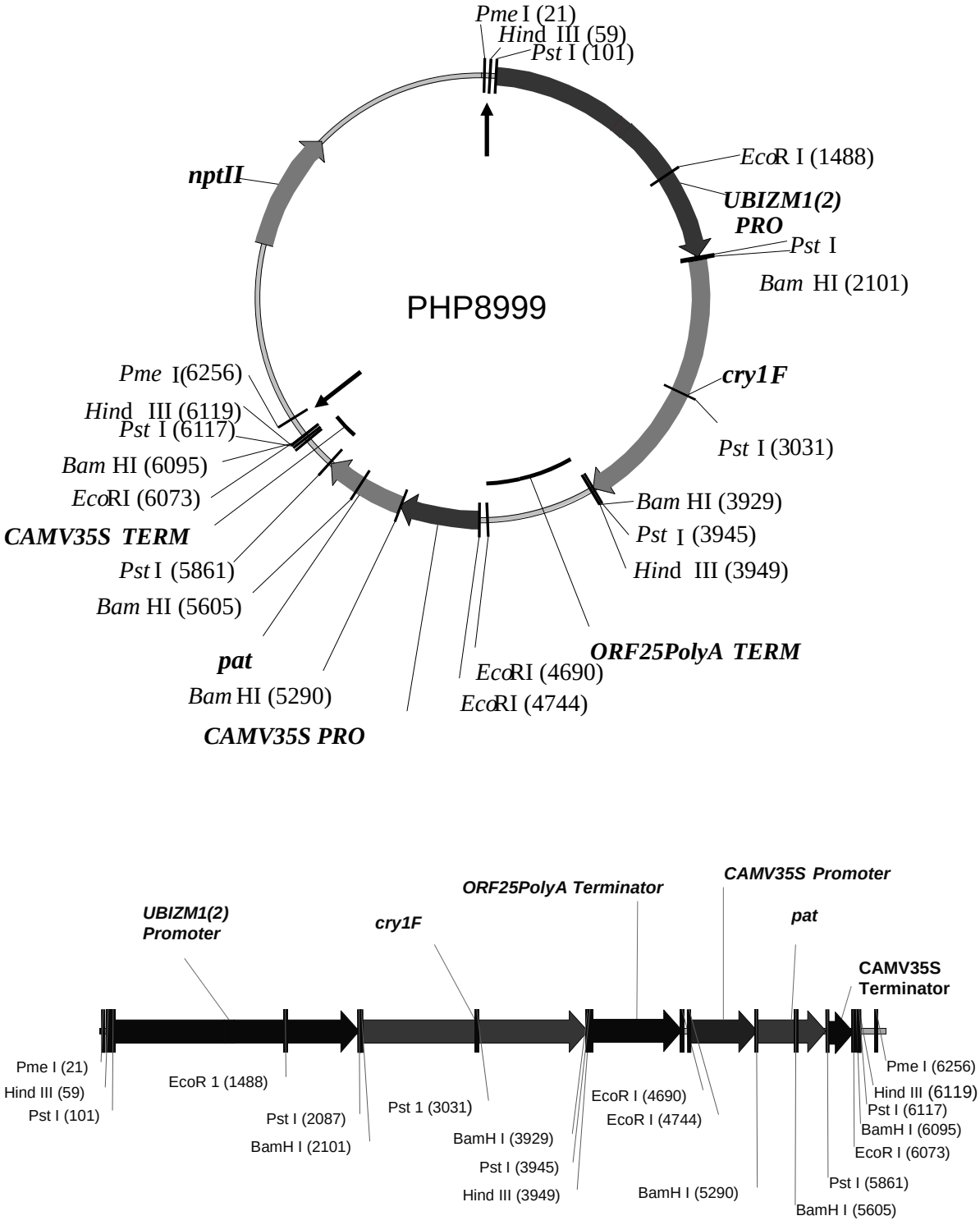

Figure 1 Compositions of plasmid PHP8999 (upper diagram) and inserted DNA region PHI8999A (lower diagram)

Plasmid PHP8999 was treated with the restriction enzyme *Pme* I (cleaved at the two points indicated by arrows in the upper diagram) and the resulting linear DNA fragment PHI8999A (lower diagram) was used to introduce genes into recipient organism.

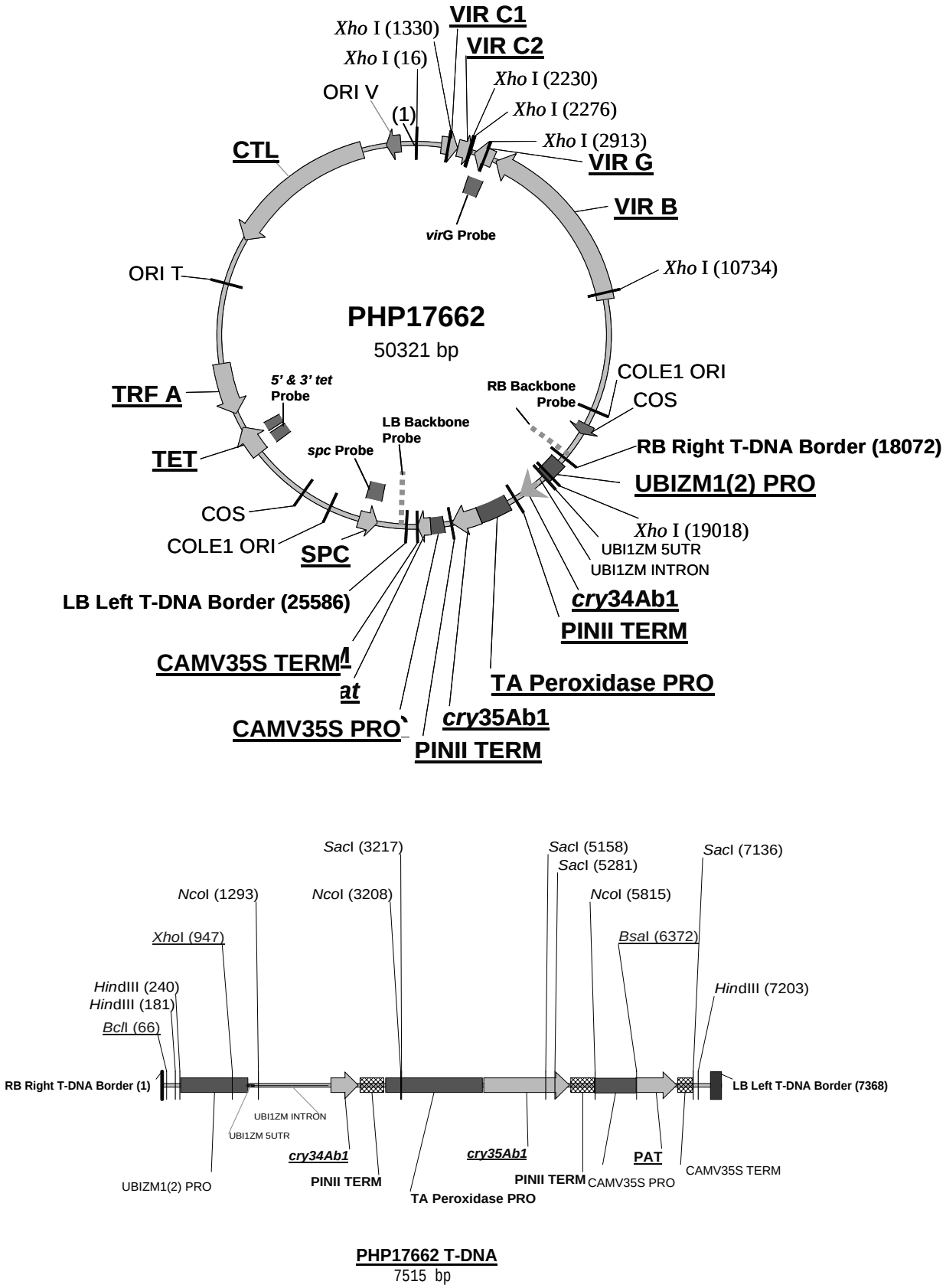

Figure 2 Compositions of plasmid PHP17662 and inserted T-DNA region

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

(1) Location of the copy of transferred nucleic acid

It was confirmed by Southern blotting analysis that, in both Cry1F line 1507 and Event DAS-59122-7, a copy of transferred nucleic acid was introduced into the maize genome.

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

Southern blotting analysis confirmed that, in Cry1F line 1507, one copy of the following gene expression cassettes was each inserted in the maize genome in the intact form; the *cry1F* gene expression cassette, which confers the resistance to Lepidoptera such as corn borer, and the *pat* gene expression cassette, which confers the tolerance to glufosinate herbicide. It was also confirmed that they were inherited stably in offspring. In addition, as a result of sequencing of the introduced DNA, it was confirmed that the introduced DNA 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.

Southern blotting analysis confirmed that, in Event DAS-59122-7, one copy of the following gene expression cassettes was each inserted in the maize genome in the intact form; the *cry34Ab1*gene expression cassette and the *cry35Ab1*gene expression cassette, which confer the resistance to Coleoptera such as corn rootworm, and the *pat* gene expression cassette, which confers the tolerance to glufosinate herbicide. It was also confirmed that they are inherited stably in offspring.

(3) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid

A glufosinate herbicide spraying test and a biological test using target insects were conducted. These tests confirmed the stable expression of the stack line 1507×59122's acquired traits derived from Cry1F line 1507 and Event DAS-59122-7. The followings show the results.

Glufosinate herbicide spraying test

A glufosinate herbicide spraying test was conducted in a greenhouse of Pioneer Hi-Bred International, Inc (USA) in March 2005. This test was for the purpose of confirming whether the stack line 1507×59122's tolerance to glufosinate herbicide was equivalent to those of the parent lines, Cry1F line 1507 and Event DAS-59122-7.

Fifteen seeds from each of the stack line 1507×59122, parent lines—Cry1F line 1507 and Event DAS-59122-7—and non-recombinant maize were sowed on a vat (vat size: length × width × depth = about 35 cm × 50 cm × 10 cm). These plants were thinned out on the 8th day after the sowing, whereby ten plants were left in each vat. The glufosinate herbicide was sprayed onto them on the 15th day after sowing, on which farmers usually spray the glufosinate. The glufosinate herbicide was tested at a dosage accepted under the pesticide registration system (i.e. standard dosage), as well as at 16 and 32 times higher ones than the standard dosage. Then the levels of pesticide-induced damage (growth inhibition, color fading and spots) were visually evaluated for each vat on the 8th day after spraying (the 23rd day after sowing), and scored on a percentage basis (0% = no pesticide-induced damage, 100% = plant death). This test was performed in triplicate.

As a result, the levels of pesticide-induced damage showed no statistically significant differences between the stack line 1507×59122 and the parent lines—Cry1F line 1507 and Event DAS-59122-7—at any of the dosages tested (Table 3). This result confirmed that the stack line 1507×59122's tolerance to glufosinate herbicide was equivalent to those of parent lines, Cry1F line 1507 and Event DAS-59122-7.

Table 3 Levels of pesticide-induced damage by spraying of glufosinate herbicide

Tested plants	Levels of pesticide-induced damage (%)			
	No spraying	Normal dosage (0.5kg ai/ha)	16 times (8.0kg ai/ha)	32 times (16.0kg ai/ha)
Stack line 1507×59122	0.0±0.0 b	0.0±0.0 b	6.7±3.3 ab	13.3±3.3 a
Cry1F line 1507	0.0±0.0 b	0.0±0.0 b	6.7±3.3 ab	13.3±3.3 a
Event DAS-59122-7	0.0±0.0 b	6.7±3.3 ab	6.7±3.3 ab	10.0±0.0 ab
Non-recombinant maize	0.0±0.0 b	40.0±0.0 c	[redacted]	

For each maize type, the levels of pesticide-induced damage were compared with data from a non-spraying plot. Symbols written with damage levels indicate that damage levels showed no statistically significant differences within the same groups (a/b/c) (Tukey's multiple test, $P<0.05$). Standard deviation is given to the right of ±. Herbicide dosages are expressed in the amount of active ingredient per hectare (kg ai/ha).

Actually, a sufficient herbicidal effect is expected with the standard dosage although the herbicide was sprayed specially in this test at 16 and 32 times higher ones than the standard dosage. At ordinary fields, the glufosinate herbicide will never be sprayed at such high dosages as it was in this test.

Biological test using European corn borer

A biological test using a target insect, European corn borer, was conducted in a growth chamber at Pioneer Hi-Bred International, Inc (USA) in March 2005. This test was for the purpose of confirming whether the stack line 1507×59122's resistance to Lepidoptera was equivalent to that of one of its parent lines, Cry1F line 1507.

The stack line 1507×59122, parent lines—Cry1F line 1507 and Event DAS-59122-7—and non-recombinant maize were cultivated in a greenhouse. Leaves at the V6 stage (6th foriage leaf stage) were collected, punched into a circle about 1 cm in diameter. Subsequently, five newly hatched European corn borer larvae were released on each sample. They were incubated at 27°C in a growth chamber for three days. Then the fertility rate of larvae and the levels of feeding damage were observed. Four discs from each sample were used in each test. This test was performed in pentaplicate.

As a result, the fertility rate of European corn borer larvae and the levels of feeding damage showed no statistically significant differences between the stack line 1507×59122 and Cry1F line 1507. This result confirmed that obtained trait of resistance to Lepidoptera was expressed in the stack line 1507×59122 as stably as one of its parent line, Cry1F line 1507 (Table 4).

Table 4 Resistance to European corn borer larvae

Tested plants	Mortarity rates of larvae (%)	Levels of feeding damage to foriage leaves (%)
Stack line 1507×59122	98±2	1±0
Cry1F line 1507	94±3	1±0
Event DAS-59122-7	9±3	96±2
Non-recombinant maize	13±3	92±4

Standard deviation is given to the right of ±.

Biological test using Western corn rootworm

A biological test using a target insect, Western corn rootworm, was conducted in a greenhouse of Pioneer Hi-Bred International, Inc (USA) in March 2005. This test was for the purpose of confirming whether the stack line 1507×59122's resistance to Coleoptera was equivalent to that of one of its parent lines, Event DAS-59122-7.

The stack line 1507×59122, parent lines—Cry1F line 1507 and Event DAS-59122-7—and non-recombinant maize were cultivated (one maize individual per pot, pot size: 24 cm in diameter, 22 cm in height, 6.4 L in volume). At the V4 stage

(4th foriage leaf stage), 100 eggs of Western corn rootworm were each placed at their roots four times every other day (a total of 400 eggs). Then the levels of feeding damage at roots were observed at the time hatched larvae pupated (about two weeks after eggs were placed). Four plants from each sample were used in each test. This test was performed in pentaplicate.

As a result, the levels of feeding damage by Western corn rootworm showed no statistically significant differences between the stack line 1507×59122 and the Event DAS-59122-7. This result confirmed that the obtained trait of resistance to Coleopteran pests was expressed in the stack line 1507×59122 as stably as one of its parent line, Event DAS-59122-7 (Table 5).

Table 5 Resistance to Western corn rootworm

Tested plants	Score of feeding damage at roots *
Stack line 1507×59122	0.08±0.01
Event DAS-59122-7	0.09±0.01
Cry1F line 1507	2.65±0.08
Non-recombinant maize	2.64±0.08

* Node-Injury Scale developed by Iowa State University
(0-3: 0 = no feeding damage, 3 = three or more root nodes eaten)
(http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html)
Standard deviation is given to the right of ±.

Conclusion

These results confirmed the stable expression of the stack line 1507×59122's acquired characters derived from Cry1F line 1507 and Event DAS-59122-7.

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

Transmission of this item is absence, because the transferred nucleic acid does not contain any sequence allowing transmission.

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

A polyclonal antibody-based ELISA method has been developed to detect Cry1F and PAT proteins, and detection kits are available for each protein. Detection kits for Cry34Ab1 and Cry35Ab1 proteins are now under development, and will appear on the market in August 2005. Applying these three methods to every maize grain will enable this stack line

1507×59122 to be detected.

The Cry1F protein detection kit can detect a maize grain containing the Cry1F protein out of 600 grains. Likewise, the PAT protein detection kit can detect a maize grain containing the PAT protein out of 500 grains. Several tests have demonstrated the reliability of each detection kit.

6. Difference from the recipient organism or the taxonomic species to which the recipient organism belongs

(1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acids

i) Resistance to Lepidoptera

The stack line 1507×59122 produces the Cry1F protein by the introduction of the *cry1F* gene derived from *B.t.* var. *aizawai* into one of its parent lines, Cry1F line 1507. As a result, the stack line is given resistance to European corn borer, which causes feeding damage to maize.

ii) Resistance to Coleoptera

The stack line 1507×59122 produces the Cry34Ab1 and Cry35Ab1 proteins by the introduction of the *cry34Ab1* and *cry35Ab1* genes derived from *B.t.* PS149B1 strain into one of its parent lines, Event DAS-59122-7. As a result, the stack line is given resistance to corn rootworm, which causes feeding damage to maize.

iii) Tolerance to glufosinate herbicide

The stack line 1507×59122 is given tolerance to glufosinate herbicide by the introduction of the *pat* gene derived from *Streptomyces viridochromogenes* into Cry1F line 1507 and Event DAS-59122-7. The PAT protein produced by the expression of the *pat* gene acetylates and transforms the glufosinate herbicide to nontoxic acetylglufosinate, thereby conferring the glufosinate tolerance to the plant body.

(2) Presence or absence of difference between recombinant plant and the species to which recipient organism belongs, and the degree of difference, if any

The stack line 1507×59122 is a cultivar created from inbred lines of Cry1F line 1507 and Event DAS-59122-7 with a conventional crossbreeding method. This stack line

has the obtained traits of both Cry1F line 1507 and Event DAS-59122-7. The Cry1F, Cry34Ab1 and Cry35Ab1 proteins expressed in this stack line 1507x59122 are believed to possess no enzyme activity, and the PAT protein has high substrate specificity. It is therefore unlikely that these proteins conferring to the traits will interact with each other. It has actually been confirmed that each trait conferred from both the two parent lines is stably expressed in the stack line 1507×59122, according to herbicide spray tests and biological examinations, as stated in (3)-4-2-I; "The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid".

For these reasons, the differences between this stack line and the taxonomic maize species to which recipient organism belongs are evaluated based on the results of individual examinations for traits of Cry1F line 1507 and Event DAS-59122-7. The individual examinations were conducted for Cry1F line 1507 in 2001 and for Event DAS-59122-7 in 2003, at isolated fields of the National Institute for Agro-Environmental Sciences, an Independent Administrative Institution (Tsukuba City, Ibaraki Prefecture).

a) Morphological and growth characteristics

Morphological and growth characteristics of Cry1F line 1507 was evaluated for the following examination items: germination rate, the uniformity of germination, time of tasseling, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, the number of tillers, the total number of ears, the number of effective ears, grain color and grain shape, culm length, ear height, ear length, ear diameter and the fresh weight of the above-ground part. As a result, no statistically significant differences were observed between the Cry1F line 1507 and the non-recombinant maize in any of the examination items except germination rate and ear diameter. One of the two recombinant plants tested showed statistically significant differences in germination rate and ear diameter (germination rate in Cry1F line 1507: 96.7%; germination rate in the non-recombinant maize: 92.8%) (ear diameter in Cry1F line 1507: 4.60 cm; ear diameter in the non-recombinant maize: 4.32 cm), while the other did not.

Similarly, morphological and growth characteristics of Event DAS-59122-7 was evaluated for the following examination items: germination rate, the uniformity of germination, time of tasseling, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, the number of tillers, the number of effective ears, grain color and grain shape, culm length, ear height, ear length, ear diameter, the fresh weight of the above-ground part and flower shape. As a result, no statistically significant differences were observed

between the Event DAS-59122-7 and the non-recombinant maize in any of the examination items except culm length. One of the two recombinant plants tested showed statistically significant differences in culm length (Event DAS-59122-7: 192.0 cm; the non-recombinant maize: 212.3 cm), while the other did not.

It is therefore unlikely that there are differences in morphological and growth characteristics between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

Additionally according to the germination test for the stack line 1507×59122 carried out in the US, the germination rate was 98% in this stack line, which is virtually equal to 98 % in Cry1F line 1507 and 96% in Event DAS-59122-7. Their germination rates were all over 90%, which is the standard germination rate determined by OECD.

b) Chilling-tolerance at the early stage of growth

The chilling tolerance of seedlings was evaluated for Cry1F line 1507. All the samples faded and wilted after about three weeks after placing them in a growth chamber (set to every 12 hour cycle of 12-14°C [when the light is on] and 2°C [when the light is off]). The degree of wilting showed no differences between Cry1F line 1507 and the non-recombinant maize.

Similarly, the chilling tolerance of seedlings was evaluated for Event DAS-59122-7. The plant bodies in the fourth leaf stage having a plant height of 16-19 cm were left out in the open in the winter season at a minimum temperature below 10°C to observe the growth. When the minimum temperature dropped down to 1.5°C, all the plant bodies tested died, and no difference was observed in the sensitivity to low temperatures between Event DAS-59122-7 and the non-recombinant maize.

It is therefore unlikely that there are differences in chilling tolerance between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

c) Wintering ability and summer survival of the matured plant

It is well known that 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. For these reasons, no wintering ability tests were conducted for matured plants of Cry1F line 1507 and Event DAS-59122-7. Instead, it was actually confirmed that there were no surviving plants observed the following year at fields used for the trial cultivation of Cry1F line 1507 and Event DAS-59122-7

in the USA..

It is therefore unlikely that there are differences in wintering ability between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

d) Fertility and size of the pollen

Pollen was sampled during the flowering period of Cry1F line 1507 to evaluate the fertility and its size. No differences were observed in either of the examination items between Cry1F line 1507 and the non-recombinant control maize.

Similarly, no differences were observed in either of the fertility and size of pollen between Event DAS-59122-7 and the non-recombinant control maize.

It is therefore unlikely that there are differences in the fertility and size of pollen between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

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

As examination items relevant to the production of seeds, the number of rows per ear, the number of grains per row, and 100-kernel weight were examined for Cry1F line 1507. As a result, no statistically significant differences were observed in any of the examination items between Cry1F line 1507 and the control non-recombinant maize. Also, both seeds (F2) collected from Cry1F line 1507 and the non-recombinant maize showed a high germination rate and no differences between them.

Maize is acultivated species, and its dormancy has not been reported. Also, Cry1F line 1507 showed as high a germination rate as did the non-recombinant maize. It is therefore unlikely that Cry1F line 1507 will exhibit dormancy.

Similarly, the number of rows per ear, the number of grains per row, and 100-kernel weight were examined for Event DAS-59122-7. As a result, no significant differences were observed between Event DAS-59122-7 and the non-recombinant maize in any of the examination items. No dormancy was observed in either of Event DAS-59122-7 or the non-recombinant maize. No differences were observed in the germination rate of collected seeds (F2) between Event DAS-59122-7 and the non-recombinant maize.

Maize ears are covered with husk at harvest time. Shedding habit under the

natural conditions was not observed in any of Cry1F line 1507, Event DAS-59122-7 or their non-recombinant control maize.

It is therefore unlikely that there are differences in the production and other relevant characters between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

f) Crossability

In Japan, the growth of wild relatives (teosinte) that can be crossed with maize in natural environment has not been reported.

g) Productivity of harmful substances

It has not been reported that maize roots secrete harmful substances that have adverse effects on other plants and soil microorganisms, or that maize produces allelochemicals that have adverse effects on other plants after the death of the plant body.

Cry1F and PAT proteins are newly produced in Cry1F line 1507 due to the introduction of *cry1F* and *pat* genes, while Cry34Ab1, Cry35Ab1 and PAT proteins are newly produced in Event DAS-59122-7 due to the introduction of *cry34Ab1*, *cry35Ab1* and *pat* genes. Like other Cry proteins derived from *B.t.*, however, it is not reported that Cry1F, Cry34Ab1 and Cry35Ab1 proteins have enzyme activities in plant bodies. In addition, it is reported that the PAT protein shows extremely high substrate specificity. It is therefore unlikely that these proteins have effects on the metabolic systems of recipient maize, newly producing unexpected harmful substances.

A succeeding cropping test, a soil microflora test and a plow-in test were carried out for Cry1F line 1507 in isolated fields. In the US, moreover, the productivity of harmful substances was evaluated by the sandwich method using the roots, leaves and stems of Cry1F line 1507 and the non-recombinant maize. Also, their effects on succeeding crops were visually observed in 46 field tests. As a result, no differences were observed between Cry1F line 1507 and the non-recombinant control maize in the productivity of unexpected harmful substances.

For Event DAS-59122-7, weeds growing in the isolated field were examined to evaluate their species composition (DCA score), total population size and dry weight; and a succeeding cropping test and a soil microflora test were carried out in a screening house. In addition, a soil microflora test was carried out at the test field (volcanic ash soil) in Hawaii State. The results indicated that there were no

differences between Event DAS-59122-7 and the non-recombinant control maize in the productivity of unexpected harmful substances.

It is therefore unlikely that there are differences in the productivity of unexpected harmful substances between this stack line 1507×59122 and the taxonomic maize species to which the recipient organism belongs.

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 applied 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 was created by crossbreeding maize resistant to Lepidoptera and tolerant to glufosinate herbicide (DAS-01507-1) and maize resistant to Coleoptera and tolerant to glufosinate herbicide (DAS-59122-7). The Committee on Adverse Effect on Biological Diversity judged that each of these parent lines would not result in Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stacked line maize.

It is reported that the Cry1F protein encoded by the Lepidoptera-resistant gene (*cry1F*) derived from DAS-01507-1 and the Cry34Ab1 and Cry35Ab1 proteins encoded by the Coleoptera-resistant gene (*cry34Ab1* and *cry35Ab1*) derived from DAS-59122-7 possess no enzyme activity. Besides, it is reported that the PAT protein encoded by the glufosinate-tolerant gene (*pat*) derived from DAS-01507-1 and DAS-59122-7 exhibits high substrate specificity. It is therefore unlikely that traits conferred by *pat* as well as *cry1F* and *cry34Ab1*, *cry35Ab* will interact with each other.

It has been confirmed that this stack maize expresses tolerance to glufosinate herbicide, resistance to Lepidoptera and resistance to Coleoptera, respectively by an herbicide spray test, a biological test using European corn borer (*Ostrinia nubilalis*) and a biological test using Western corn rootworm (*Diabrotica virgifera virgifera*).

Based on the above understanding, it is unlikely that notable changes in traits have occurred in this stacked line maize, except for the traits conferred by both the parent lines.

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

(1) Competitiveness

This stack maize has resistance to Lepidoptera derived from DAS-01507-1 and resistance to Coleoptera derived from DAS-59122-7, as well as tolerance to glufosinate herbicide derived from both lines. However, it is hard to consider that feeding damage by Lepidoptera and Coleoptera is a major factor that hinders the growth of maize in Japan in the natural environment, and that the glufosinate tolerance becomes a selection

pressure in the natural environment. For these reasons, none of these traits will enhance the competitiveness of the stack maize. It is therefore unlikely that this stack maize will be more dominant in competition than its parent lines. These mentioned above demonstrate the validity of the conclusion by the applicant who judged that use of this maize line would pose no risk of Adverse Effect on Biological Diversity attributable to competitiveness.

(2) Productivity of harmful substances

This stack maize has the Cry1F protein productivity derived from DAS-01507-1 and the Cry34Ab1 protein and Cry35Ab1 protein productivity derived from DAS-59122-7, as well as the PAT protein productivity derived from both lines. It has been reported that the Cry1F protein shows an insecticidal activity against Lepidoptera, and Cry34Ab1 and Cry35Ab1 proteins against Coleoptera, while the PAT protein is not a harmful substance to animals and plants. For these reasons, even though this stack maize has all of these proteins, it is unlikely that the productivity of harmful substance will be greater in this stack line than its parent lines. These mentioned above demonstrate the validity of the conclusion by the applicant who judged that use of this maize line would pose no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances.

(3) Crossability

In Japan, the growth of wild relatives (teosinte) that can be crossed with maize in natural environment has not been reported.

Based on the above understanding, no wild species can be specified as having some effects, the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability is valid.

2. Conclusion

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of the stack line 1507 x 59122 in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is valid.