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 tolerant to glufosinate and glyphosate herbicides (*cry1A.105*, modified *cry2Ab2*, *cry1F*, *pat*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034×*B.t.* Cry1F maize line 1507×NK603, OECD UI: MON-89Ø34-3× DAS-Ø15Ø7-1×MON-ØØ6Ø3-6) [including the progeny lines isolated from the maize lines, MON89034, *B.t.* Cry1F maize line 1507 and NK603, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type I Use Regulation)] |
|---|---|
| Content of the Type 1 Use of Living Modified Organism | Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them |
| Method of the Type 1 Use of Living Modified Organism | — |

## Outline of the Biological Diversity Risk Assessment Report

# I. Information collected prior to assessing Adverse Effect on Biological Diversity

### 1. Information concerning preparation of living modified organisms

A cross progeny line (OECD UI: MON-89Ø34-3×DAS-Ø15Ø7-1×MON-ØØ6Ø3-6) (hereinafter referred to as “this stack maize line”) is developed by crossing the following three (3) recombinant maize lines, using the traditional crossbreeding method. The three recombinant maize lines are: i) the maize resistant to Lepidoptera (*cry1A.105,* modified *cry2Ab2*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034, OECD UI: MON-89Ø34-3) (hereinafter referred to as “MON89034”), ii) the maize resistant to Lepidoptera and tolerant to glufosinate herbicide (*cry1F*, *pat*, *Zea mays* subsp. *Mays* (L.) Iltis) (*B.t.* Cry1F maize line 1507, OECD UI: DAS-Ø15Ø7-1) (hereinafter referred to as “Cry1F line 1507”), and iii) the maize tolerant to glyphosate herbicide (modified *cp4 epsps*, *Zea mays* subsp. *Mays* (L.) Iltis) (NK603, OECD UI: MON-ØØ6Ø3-6) (hereinafter referred to as “NK603”). Therefore, this stack maize line possesses the characteristics of these three parent recombinant maize lines, MON89034, Cry1F line 1507 and NK603. 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. Then, the information concerning preparation of MON89034, Cry1F line 1507 and NK603 are explained individually in the following sections.

### (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 MON89034, Cry1F line 1507 and NK603 are shown individually in Figure 1 to Figure 3 (pp.3-5), and Table 1 to Table 3 (pp.6-10).

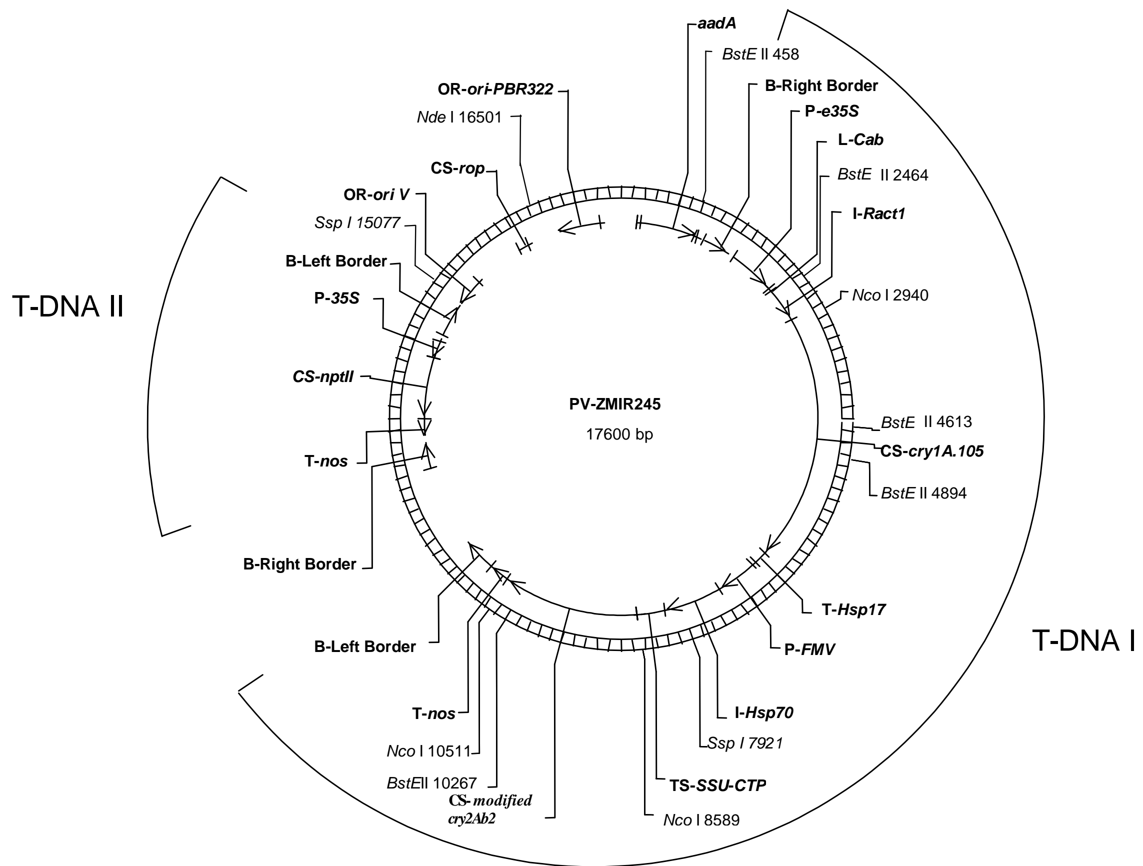


**Figure 1 Map of the plasmid PV-ZMIR245 used for the development of MON89034**[1]

In the development process of MON89034, 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.

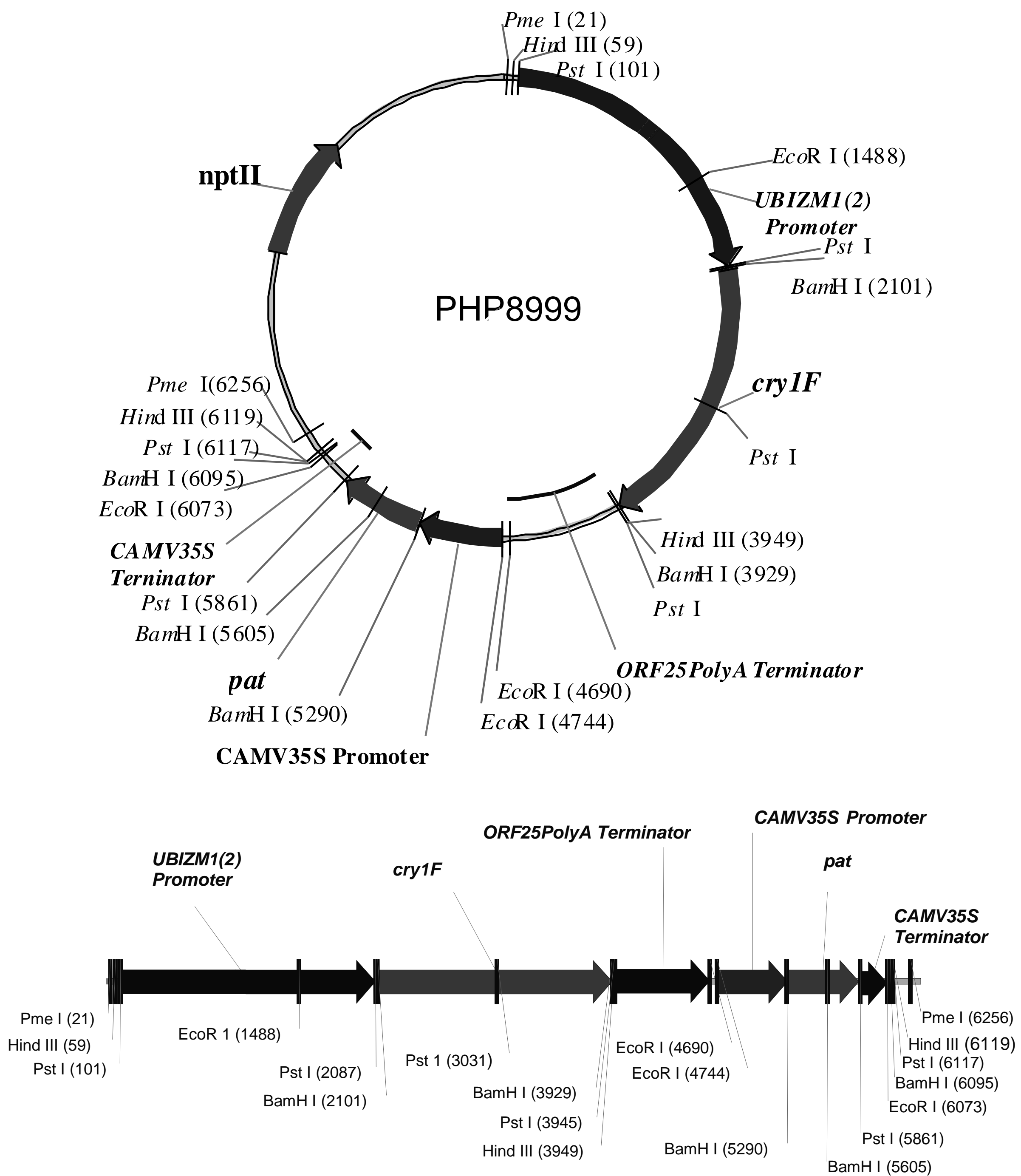


**Figure 2 Map of the plasmid PHP8999 used for the development of Cry1F line 1507 and the transferred DNA region[2]**

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

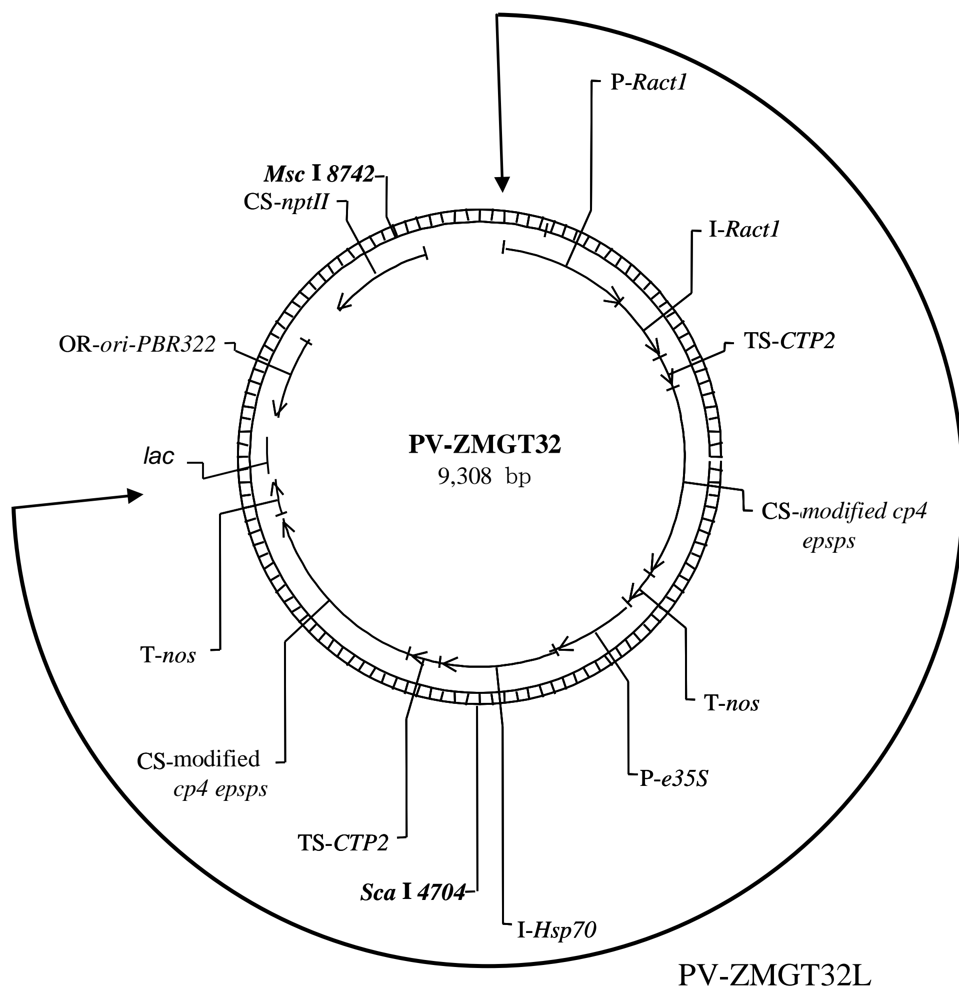


**Figure 3 Map of the plasmid PV-ZMGT32 used for the development of NK603[3]**

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

**Table 1 Origins and functions of component elements of PV-ZMIR245 used for the development of MON89034[4]**

| 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*) gene 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 MON89034 (continued)[4]**

| 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 the neomycin phosphotransferase II and confers kanamycin resistance to plants. 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) (Left border | 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

[4] 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[5]**

| Component elements | Origin and function |
|---|---|
| *cry1F* gene expression cassette | |
| *UBIZM1(2) Promoter*[*1] | Ubiquitin constitutive promoter 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*[*1] | 35S constitutive promoter 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

[5] 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-ZMGT32 used for the development of NK603[6]**

| Component elements | Origin and function |
|---|---|
| | Modified *cp4 epsps* gene cassette (1) |
| P[*1]-*Ract1* | Promoter region of actin 1 gene derived from rice. It makes target genes expressed (Reference 20). Involved in the constant expression of the target gene in the entire tissue of plant body. |
| I[* 2]-*Ract1* | Rice actin gene intron (Reference 37). 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 38). 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 39; Reference 40). 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). |
| | Modified *cp4 epsps* gene cassette (2) |
| 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. |
| I-*Hsp70* | Intron of heat shock protein 70 gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants (Reference 41). |
| TS-*CTP2* | N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 38). Transfers target proteins from cytoplasm to chloroplast. |
| CS-modified *cp4 epsps* | 5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 39; Reference 40). 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-*nos* | 3' untranscribed region of nopaline synthase (*nos*) gene derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26). |

**Table 3 Origins and functions of component elements of PV-ZMGT32 used for the development of NK603 (continued)**[6]

| Others (not existing in plant body) | |
|---|---|
| *lac* | Consists of partial coding sequence for *lacI* (Reference 42), *lac* promoter (Reference 43), and partial coding sequence for *lacZ*. Hydrolyzes lactose and expresses β-galactosidase used as a selective marker (Reference 44). |
| OR[*6]-*ori- PBR 322* | The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Reference 32). |
| *nptII* | Encodes phosphotransferase type II (NPT II) derived from *E.coli* transposon Tn5 (Reference 28) and confers resistance to neomycin and kanamycin. The region contains the partial *ble* gene derived from Tn5 (Reference 45), and is regulated by *nptII* promoter and β-lactamase termination sequence. Used as marker to select the transgenic plant during the gene transfer (Reference 29). |

[*1] P – promoter
[*2] I – intron
[*3] TS – targeting sequence
[*4] CS – coding sequence
[*5] T – transcript termination sequence
[*6] OR – origin of replication

[6] 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 selectable marker

Functions of individual component elements of donor nucleic acid used for the development of MON89034, Cry1F line 1507 and NK603 are shown in Table 1 to Table 3 (pp.6-10). Details of target genes; the *cry1A.105* gene, the modified *cry2Ab2* gene, the *cry1F* gene, the *pat* gene and the modified *cp4 epsps* gene are shown in Table 1 to Table 3 (pp.6-10).

(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 conferring pest resistance[7]—

**[Cry1A.105 protein]**

The Cry1A.105 protein, which is encoded by the *cry1A.105* gene used for the development of MON89034, 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 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 51), Black cutworm (*Agrotis ipsilon*) (Reference 52), Fall armyworm (*Spodoptera frugiperda*) (Reference 52), Southwestern corn borer (*Diatraea grandiosella*) (Reference 52), and European corn

[7] 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 46; Reference 47; Reference 48). 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 49; Reference 50).

borer (*Ostrinia nubilalis*) (Reference 53), which are the major pest insects of the order Lepidoptera for maize, though it did not exhibit any insecticidal activity against honeybee (Reference 54, Reference 55), ladybug (Reference 56) and other beneficial insects except the insects of order Lepidoptera.

Based on the above understanding, it was confirmed that the Cry1A.105 protein exhibits a selective insecticidal activity against only the insects of the order Lepidoptera, similarly to the Cry1Ab protein, the Cry1F protein and the Cry1Ac protein, which are component elements for the recombinant maize, and it does not exhibit toxicity against the other non-target insects tested.

**[Modified Cry2Ab2 protein]**

The modified Cry2Ab2 protein, which is encoded by the modified *cry2Ab2* gene used for the development of MON89034, has 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 53), Fall armyworm (Reference 57) and European corn borer (Reference 53) among the four species of major pest insects of the order Lepidoptera used in the investigation and not against Black cutworm (Reference 57). Also, the modified Cry2Ab2 protein did not exhibit any insecticidal activity against honeybee (Reference 58, Reference 59), ladybug (Reference 60) 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 non-target insects tested.

**[Cry1A.105 protein ＋ Modified Cry2Ab2 protein]**

MON89034 is given the resistance to the target insects of the order Lepidoptera with simultaneous expression of the Cry1A.105 protein and the modified Cry2Ab2 protein. Actually, as a result of tests of MON89034 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 MON89034 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 MON89034, could not acquire any resistance to MON89034 unless they become insensitive to both Bt proteins. This raises expectations that MON89034 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 MON89034 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]**

The *cry1F* gene used for the development of Cry1F line 1507 is a gene isolated from *B. thuringiensis* var. *aizawai,* and expresses the Cry1F protein of the Bt 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 61), 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, fishes, 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 62).

— Proteins conferring tolerance to herbicides —

**[PAT protein]**

The *pat* gene used for the development of Cry1F line 1507 is a gene isolated from *S. viridochromogenes,* and expresses the PAT protein which confers the tolerance to glufosinate herbicide.

Glufosinate herbicide is a nonselective herbicide, and possesses herbicidal activity against a variety of weeds even with a single agent. It is used in Japan, the US and many countries around the world. 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.

**[Modified CP4 EPSPS protein]**

The modified *cp4 epsps* gene expressed in NK603 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 *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. Two modified *cp4 epsps* gene cassettes were transferred to NK603 in order to enhance tolerance to the herbicide glyphosate.

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 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 PAT protein and the modified CP4 EPSPS protein expressed in the parent lines share any functionally important amino acid sequences with known allergens, it was 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 and the Cry1F 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 63), 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 64). 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 65). Consequently, for its high substrate specificity, the PAT protein is considered unlikely to affect the metabolic system of the recipient organism.

The EPSPS protein, functionally identical to the modified CP4 EPSPS protein, 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 (Reference 66; Reference 67). In addition, the EPSPS protein is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 68), and the only shikimate that is known to react with the EPSPS protein other than these offers the reactivity of only one-two millionth that of S3P (calculated based on the article written by Gruys, *et al*); 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.

Consequently, it is considered to be extremely unlikely that the production of the modified CP4 EPSPS protein, which is functionally equivalent to the plant EPSPS protein, has an effect on the metabolic pathways of plants.

## (2) Information concerning vector

1) Name and origin

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

| | |
|---|---|
| MON89034: | 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* |
| NK603: | PV-ZMGT32 assembled from plasmids including pUC119 derived from *E. coli* |

2) Properties

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

The total number of base pairs in the plasmid vectors used for the development of parent lines is as follows.

MON89034: PV-ZMIR245; 17,600 bp
Cry1F line 1507: PHP8999; 9,504 bp
NK603: PV-ZMGT32; 9,308 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.

| | |
|---|---|
| MON89034: | The *nptII* gene to confer the resistance to aminoglycoside antibiotics such as kanamycin and neomycin, and the *aadA* gene to confer the resistance to spectinomycin and streptomycin |
| Cry1F line 1507: | The *nptII* gene to confer the resistance to aminoglycoside antibiotics such as kanamycin and neomycin |
| NK603: | The *nptII* gene to confer the resistance to aminoglycoside antibiotics such as kanamycin and neomycin |

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

Neither the PV-ZMIR245, PHP8999 nor PV-ZMGT32 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 and PV-ZMGT32 transferred in the recipient organism for development of MON89034, Cry1F line 1507 and NK603 are listed in Table 1 to Table 3 (pp.6-10). 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 3 (pp.3-5).

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

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

MON89034: *Agrobacterium* method
Cry1F line 1507: Particle gun method
NK603: Particle gun method

3) Processes of rearing 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.

MON89034: Paromomycin
Cry1F line 1507: Glufosinate
NK603: Glyphosate

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

In the development of MON89034, *Agrobacterium* was removed by adding carbenicillin to the medium (Reference 69). Absence of remaining *Agrobacterium* in MON89034 was confirmed by transferring MON89034 to the carbenicillin-free medium and then observing that no colony of *Agrobacterium* is formed on the medium. For Cry1F line 1507 and NK603, the particle gun 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 MON89034, 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. MON89034 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.

For NK603, commercial cultivars of yellow dent corn were crossed with a wide variety of cultivars, evaluation for line selection started in 1997, and field experiments were carried out regarding morphological and growth characteristics at a total of 103 field sites from 1997 to 1999. In addition, the analysis for expression of the modified CP4 EPSPS protein and the transferred gene were conducted, and the final excellent line was selected.

The status of application for approval of MON89034, Cry1F line 1507, NK603 and this stack maize line in Japan is the following (Table 4, p.19).

**Table 4 Status of application for approval of MON89034, Cry1F line 1507, NK603 and this stack maize line 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 |
| NK603 | March, 2001: Approved safety of use as food | March, 2003: Approved safety of use as feed | November, 2004: Approved for Type I Use Regulation |
| This stack maize line | Scheduled for application | Scheduled for notification | November, 2009: Pending application |

**[Process of rearing of MON89034×Cry1F line 1507×NK603]**

This stack maize line is an F1 hybrid developed from the inbred lines of MON89034, Cry1F line 1507 and NK603 as the parents (Figure 4, p.19).

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

**Figure 4 Development process of MON89034×Cry1F line 1507×NK603**

### (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 MON89034, Cry1F line 1507 and NK603 exist on the maize genome.

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

**[MON89034]**

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 MON89034 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.

As a result of the nucleotide sequence analysis of transferred genes in MON89034, 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.

**[Cry1F line 1507]**

As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy of individual target genes exists in the maize genome of Cry1F line 1507 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.

Moreover, as a result of the nucleotide sequence analysis of transferred nucleic acid in 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.

**[NK603]**

As a result of analysis by Southern blot, it was confirmed that both copies of the modified *cp4 epsps* gene found in plasmid PV-ZMGT32 exist at a single site in the maize genomic DNA of NK603. 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.

For NK603, it was confirmed that 217bp fragment of ract1 promoter exists in the reverse direction near the 3'-terminal of the transferred gene by Southern blotting analysis and nucleotide sequence analysis of the 3'-terminal.

Regarding 217bp fragment which is ract1 promoter near the 3'-terminal of the transferred gene in NK603, the strand-specific RT-PCR was conducted and as a result, transcription product was found that was considered to start from either ract1 promoter of the transferred gene or E35S promoter and to read through NOS 3' terminator. However, only the modified CP4 EPSPS protein was detected in NK603. This is likely because a static codon that is preserved upstream of the transcription termination signal in the transcription product reads through the terminator of the transferred gene of NK603. However, it was concluded that this reading through does not affect the safety evaluation. Therefore, it was approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment of Japan in November 2004, concerning the use in accordance with the Type I Use Regulation for Living Modified Organism (provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms.

In addition, in the transferred gene of NK603, the 456th base and the 641st base from 5'-terminal of coding region in the modified *cp4 epsps* gene induced by the E35S promoter was changed from thymine (T) to cytosine (C) compared to the base in plasmid for expression of plant. It was determined that the change of the 456th base is not connected with the change of amino acid, but in the modified CP4 EPSPS protein which is expressed by the E35S promoter by the change of the 641st base, leucine changes to proline in the 214th amino acid from N-terminal in the original CP4 EPSPS protein (hereinafter this protein is referred to as "L214P").

Regarding L214P, the following are considered: 1) Proline which is the 214th amino acid from N-terminal is not included in the seven amino acids essential for activating the EPSPS protein family; 2) This change of the amino acid does not affect the active side of the EPSPS protein and three-dimensional structure; and 3) As the traits of enzyme activity and immune response of the L214P protein and the modified CP4 EPSPS protein are substantially comparable, the structure and function of the L214P protein and the modified CP4 EPSPS protein are substantially comparable.

In order to investigate whether the L214P shares functionally important amino acid sequences with known contact allergens, it was compared with contact allergens in the database. As a result, the L214P did not share structurally related homologous sequences with any of the known allergens examined.

It is confirmed that the change of the base was observed in multiple generations and inherited stably in offspring.

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

This item is not applicable as there is only one copy of MON89034, Cry1F line 1507 and NK603.

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.

| | |
|---|---|
| MON89034: | Confirming the expression of the Cry1A.105 protein and the modified Cry2Ab2 protein in multiple generations by Western blotting analysis |
| Cry1F line 1507: | Confirming the expression of proteins by ELISA method, the bioassay using pest insects of the order Lepidoptera, and glufosinate herbicide-spraying test to confirm the expression of the Cry1F protein and the PAT protein in multiple generations |
| NK603: | Confirming the expression of the modified CP4 EPSPS protein in multiple generations by glyphosate-herbicide spraying test during the growth |

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 MON89034 and the plasmid PV-ZMGT32 used for the production of NK603, 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 does not contain any sequence allowing transmission and thus, transmission of this item is absent.

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

A specific method for the detection and identification of MON89034 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 NK603 is available using the DNA sequences of the transferred genes and the nearby regions of the plant genome as primers.

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 between the modified organism and the recipient organism or the species to which the recipient organism belongs**

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

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

| | |
|---|---|
| MON89034: | Resistance to the insects of the order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein, which are derived from the transferred genes |
| Cry1F line 1507: | Resistance to the insects of the order Lepidoptera due to the Cry1F protein and tolerance to glufosinate herbicide due to the PAT protein, which are derived from the transferred genes |
| NK603: | Tolerance to glyphosate herbicide due to the modified CP4 EPSPS protein, which is derived from the transferred genes |

The Bt proteins (the Cry1A.105 protein, the modified Cry2Ab2 protein, the Cry1F 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 46; Reference 48). For the mechanism in which Bt proteins develop the insecticidal activity, a number of studies have been made (Reference 63), 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, the Bt proteins (the Cry1A.105 protein, the modified Cry2Ab2 protein, the Cry1F protein) bind to the specific receptors on the cell membranes of midgut epithelium of the insects of the order Lepidoptera such as European corn borer and Fall armyworm, and exhibit insecticidal activity. Therefore, the Bt proteins are considered to have selective insecticidal spectrum against the insects of the order Lepidoptera. There is no report so far that combination of the Bt proteins or Bt preparations which have the insecticidal activity against the insects classified in the same order lead to expansion of insecticidal spectrum to the other order. Therefore, it is considered unlikely that insecticidal spectrum would expand in this stack maize line.

In addition, it has been reported that the PAT protein expressed in Cry1F line 1507 possesses extremely high substrate specificity against the *L*-glufosinate, an active ingredient of glufosinate herbicide (Reference 65).

Moreover, the modified CP4 EPSPS protein expressed in NK603 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 (Reference 66; Reference 67).

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 action mechanism and thus function independently from each other.

In addition, the Bt proteins, the PAT protein, and the modified CP4 EPSPS protein expressed in this stack maize line 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 was considered unlikely that the proteins expressed in this stack maize line from individual parent lines 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.

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 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, MON89034, Cry1F line 1507 and the non-recombinant control maize (XE6001), were cultivated in pots (2 experiments x 2 replicates x 3 plants per replicate), and at the 5th to 6th leaf stage, the first instar larvae of Fall armyworm (FAW, *Spodoptera frugiperda*), the representative Lepidopteran insect for maize cultivation were inoculated (25 larvae/individual). On the 9th day after inoculation of Fall armyworm, Leaf Damage Rating (LDR; the severity of insect damage to leaf) was determined based on 10-step scales from 0 (no damage) to 9 (serious damage: a greater part of leaf is damaged) (Reference 70) (Table 5, p.26). 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 MON89034 and Cry1F line 1507 (t-test, $\alpha=0.05$) (Table 6, p. 26).

**Table 5 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)[8]**

| Samples tested | Leaf Damage Rating (LDR) ± Standard error |
|---|---|
| This stack maize line | 1.08 ± 0.08 |
| MON89034 | 1.08 ± 0.08 |
| Cry1F line 1507 | 1.42 ± 0.16 |
| Non-recombinant control maize | 7.25 ± 0.25 |

[1] 0: No damage
1: Needle-tip like worm bores are observed on developing leaves.
2: Needle-tip and small round-shape worm bores are observed on developing leaves.
3: Needle-tip, small round-shape and small eaten worm bores (0.5 inch or less) are observed on developing and developed leaves.
4: Small eaten worm bores are observed on developing leaves, and 2 to 3 medium eaten worm bores (0.5 to 1 inch) are observed on developing or developed leaves.
5: Small eaten worm bores and some medium eaten worm bores are observed on developing and developed leaves.
6: Small eaten worm bores and medium eaten worm bores (1 inch and over) are observed on developing or developed leaves.
7: Many small and medium eaten worm bores are observed on developing leaves, and some large eaten worm bores are seen on developed leaves.
8: Many small and medium eaten worm bores are observed on developing leaves, and many large eaten worm bores are seen on developed leaves.
9: Many various sizes of worm bores are observed on developing and developed leaves, and roots of developing or developed leaves are damaged.

**Table 6 Statistical treatment result of the severity of damage on leaves by Fall armyworm (FAW; *S. frugiperda*), order Lepidoptera to this stack maize line, MON89034 and Cry1F line 1507[9]**

| Compared lines | P-value |
|---|---|
| This stack maize line vs. MON89034 | 1.0000 |
| This stack maize line vs. Cry1F line 1507 | 0.0628 |
| This stack maize line vs. non-recombinant control maize | <0.0001 |

**[Bioassay using glufosinate herbicide]**

Regarding tolerance to glufosinate herbicide, five (5) individuals each of this stack maize line, Cry1F line 1507 and the non-recombinant control maize (XE6001) were cultivated in pots in a greenhouse (5 replicates x 1 plant/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

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[9] 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.

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 of 17.0 kg a.i./ha refers to 32-times higher dosage than the normal dosage of 0.54 kg active ingredient (a.i.)/ha. The severity of injury in Table 7 (p.27) refers to mean value ± standard error.

As a result of statistical treatment, regarding the severity of injury by spraying of herbicide, no statistically significant difference was observed between this stack maize line and Cry1F line 1507 (t-test, $\alpha$=0.05) (Table 8, p.27).

**Table 7 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)[9]**

| Samples tested | Concentration | |
|---|---|---|
| | 0.54 kg a.i./ha[2] | 17.0 kg a.i./ha[3] |
| This stack maize line | 0.2 ± 0.2 | 1.8 ± 0.2 |
| Cry1F line 1507 | 0.0 ± 0.0 | 1.6 ± 0.2 |
| Non-recombinant control maize | 8.0 ± 0.3 | 8.0 ± 0.5 |

[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] Normal dosage
[3] 32-times higher dosage

**Table 8 Statistical treatment result of the severity of injury by spraying of herbicide glufosinate to this stack maize line and Cry1F line 1507[10]**

| Compared lines | P-value | |
|---|---|---|
| | 0.54 kg a.i./ha dosage [1] | 17.0 kg a.i./ha dosage [2] |
| This stack maize line vs. Cry1F line 1507 | 0.5466 | 0.6063 |
| This stack maize linevs. non-recombinant control maize | <0.0001 | <0.0001 |

[1] Normal dosage
[2] 32-times higher dosage

**[Bioassay using glyphosate herbicide]**

Regarding tolerance to glyphosate herbicide, five (5) individuals each of this stack maize line, NK603 and the non-recombinant control maize (XE6001) were cultivated in pots in a greenhouse (5 replicates x 1 plant/replicate), and at the 4th to 5th leaf state, 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 11-step scales from 0 (no injury) to 10 (nearly the entire plant body withered and died due to the injury). The concentration

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of glyphosate sprayed of 27.0 kg a.e./ha refers to 32-times higher dosage than the normal dosage of 0.84 kg acid equivalent(a.e.)/ha. The severity of injury in Table 9 (p.28) refers to mean value ± standard error.

As a result of statistical treatment, 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 NK603 (t-test, $\alpha$=0.05) (Table 10, p.28).

**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)[10]**

| Samples tested | Concentration | |
|---|---|---|
| | 0.84 kg a.e./ha[2] | 27.0 kg a.e./ha[3] |
| This stack maize line | 0.4 ± 0.2 | 1.8 ± 0.4 |
| NK603 | 0.4 ± 0.2 | 1.6 ± 0.2 |
| Non-recombinant control maize | 9.4 ± 0.4 | 10.0 ± 0.0 |

[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] Normal dosage
[3] 32-times higher dosage

**Table 10 Statistical treatment result of the severity of injury by spraying glyphosate herbicide to this stack maize line and NK603[11]**

| Compared lines | P-value | |
|---|---|---|
| | 0.84 kg a.e./ha dosage [1] | 27.0 kg a.e./ha dosage [2] |
| This stack miaze line vs. NK603 | 1.0000 | 0.5034 |
| This stack maize line vs. non-recombinant control maize | <0.0001 | <0.0001 |

[1] Normal dosage
[2] 32-times higher dosage

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, regarding the differences in physiological or ecological characteristics between this stack maize line and the taxonomic species to which the recipient organism belongs, evaluation was conducted based on the results of individual examinations on the MON89034, Cry1F lines 1507 and

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NK603.

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[12]

(a) Morphological and growth characteristics

For the morphological and growth characteristics of MON89034, Cry1F line 1507, NK603 and their non-recombinant control maize, examination was conducted for the items listed in Table 11 (p.30).

As a result, a statistically significant difference was observed between MON89034 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, and between NK603 and its non-recombinant control maize in 100-kernel weight. However, the average values in ear diameter and grain number per ear of MON89034 were found to fall within the variable ranges for conventional maize. In addition, statistically significant differences in germination rate and ear diameter of Cry1F line 1507 and 100-kernel weight of NK603 were observed but limited to only one of the two hybrid varieties tested (Table 2 in p.8 of Annex 1; Table 2 to Table 11 in pp.5-9 of Annex 2; Table 3-2 in p.17 of Annex 3).

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

MON89034, Cry1F line 1507 and NK603 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 2; Table 3-4 in p.24 of Annex 3).

(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, it was confirmed that MON89034 withered or died at the end of the isolated field test (Figure 7, p.15 of Annex 1).

For Cry1F line 1507, it was confirmed that no remaining plants exist in the field used for cultivation tests in the US when observing the field in the following year.

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[12] All the rights pertinent to the information of MON89034 and NK603 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 in (a) to (g) of this item and the responsibility for the content remain with Dow Chemical Japan Limited.

For NK603, at the end of isolated field tests, withering had begun and death after ripening was observed.

(d) Fertility and size of the pollen

MON89034, Cry1F line 1507 and NK603 exhibited high fertility of the pollen similarly to their non-recombinant control maize, and no difference was observed also 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.10-12 of Annex 2; Table 3-3 and Photo in pp.21-22 of Annex 3).

**Table 11 Investigational results of morphological and growth characteristics of MON89034, Cry1F line 1507 and NK603**

| | MON89034 | Cry1F line 1507 | NK603 |
|---|---|---|---|
| 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.

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

Regarding seed production, comparisons were conducted between MON89034, Cry1F line 1507, NK603 and their non-recombinant control maize for the characteristics. As a result, a statistically significant difference was observed between MON89034 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 ear diameter, and between NK603 and its non-recombinant control maize in 100-kernel weight. However, the average values in ear diameter and grain number per ear of MON89034 were found to fall within the variable ranges for conventional maize. In addition, observed statistically significant difference in ear diameter of Cry1F line 1507 was limited to only one of the two hybrid varieties tested (Table 6 in p.20 of Annex 1; Table 6 to Table 10 in pp.8-9 of Annex 2; Tables 3-2 in p.17 Annex 3).

Regarding shedding habit of the seed, shedding habits of the seed were not observed in natural environment, since the ears of all of MON89034, Cry1F line 1507, NK603 and their non-recombinant control maize were covered with bracts at the time of harvesting.

Regarding germination rate, comparison was made between MON89034, Cry1F line 1507, NK603 and their non-recombinant control maize. As a result, no difference was observed and then, 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 2; Table 3-4 in p.24 of Annex 3).

(f) Crossability

Crossability test of the parent lines MON89034, Cry1F line 1507 and NK603 was not performed, since no wild relatives that can be crossed grow in Japan.

(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 MON89034, Cry1F line 1507 and NK603, 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. However, observed statistically significant difference was limited to one of the two hybrid varieties tested (Tables 7, 8, and 9 in p.23 of Annex 1; Table 14-1 to 14-4, and Tables 15 to 16 in p.16 and p.18 of Annex 2;

Table 3-5 to Table 3-7 in pp.26-28 of Annex 3).

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

This stack maize line was produced by crossing of maize resistance to Lepidoptera (MON89034), maize resistant to Lepidoptera and tolerant to glufosinate herbicide (*B.t.* Cry1F maize line 1507) and maize tolerant to glyphosate herbicide (NK603). 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.

It is considered that the Bt proteins (the Cry1A.105 protein, the modified Cry2Ab2 protein and the Cry1F protein), the PAT protein and the modified CP4 EPSPS protein differ from each other in the action mechanism and thus they are considered to function independently from each other.

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 in the similar levels as offered by the individual parent lines. Consequently, it is considered of low probability 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.

## 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 and NK603, 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, the tolerance to glufosinate herbicide due to the expression of the PAT protein expressed in Cry1F line 1507, and the tolerance to glyphosate herbicide due to the

expression of the modified CP4 EPSPS protein expressed in NK603. However, the insect damage by Lepidopteran 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 the characteristics to be resistant to the insects of order Lepidoptera cause maize, a crop plant, to become self-seeding in the natural environment and enhance the competitiveness. In addition, 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 the progeny lines of stack maize line isolated from the parent lines (MON89034, Cry1F line 1507 and NK603) of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, 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 the proteins expressed in this stack maize line (the Cry1A.105 protein, the modified Cry2Ab2 protein, the Cry1F protein, the PAT protein and the modified CP4 EPSPS protein) 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. Actually, succeeding crop tests, plow-in tests and soil microflora tests were conducted to examine the ability of the parent lines (MON89034, Cry1F line 1507 and NK603) 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). As a result, 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 MON89034 and the Cry1F protein expressed in Cry1F line 1507 exhibit the insecticidal activity against the insects of the order Lepidoptera. For these findings, there is a concern about the possibility that the pollen of this stack maize line dispersed or directly eaten would affect the non-target insects of the order Lepidoptera. 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 the progeny lines of stack maize line isolated from the parent lines (MON89034, Cry1F line 1507 and NK603) of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, 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 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 the progeny lines of stack maize line isolated from the parent lines (MON89034, Cry1F line 1507 and NK603) of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, in accordance with 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.