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 glyphosate herbicide (*cry1A.105*, modified *cry2Ab2*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034×NK603, OECD UI: MON-89Ø34-3×MON-ØØ6Ø3-6)
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

Maize resistant to Lepidoptera and tolerant to glyphosate herbicide (*cry1A.105*, modified *cry2Ab2*, modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (OECD UI: MON-89Ø34-3×MON-ØØ6Ø3-6) (hereinafter referred to as "this stack line maize") was developed through the crossing of the following two recombinant maize lines with the use of traditional crossbreeding method. The two recombinant maize lines are; i) 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"), and ii) maize tolerant to glyphosate herbicide (modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (NK603, OECD UI: MON-ØØ6Ø3-6) (hereinafter referrd to as "NK603"). Therefore, this stack line maize possesses the both characteristics of these two parent recombinant maize lines, MON89034 and NK603. Then, the information concerning preparation of MON89034 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 used for the development of MON89034 and NK603 and the origins of component elements are shown in Figure 1, and Table 1, and Figure 2 and Table 2.

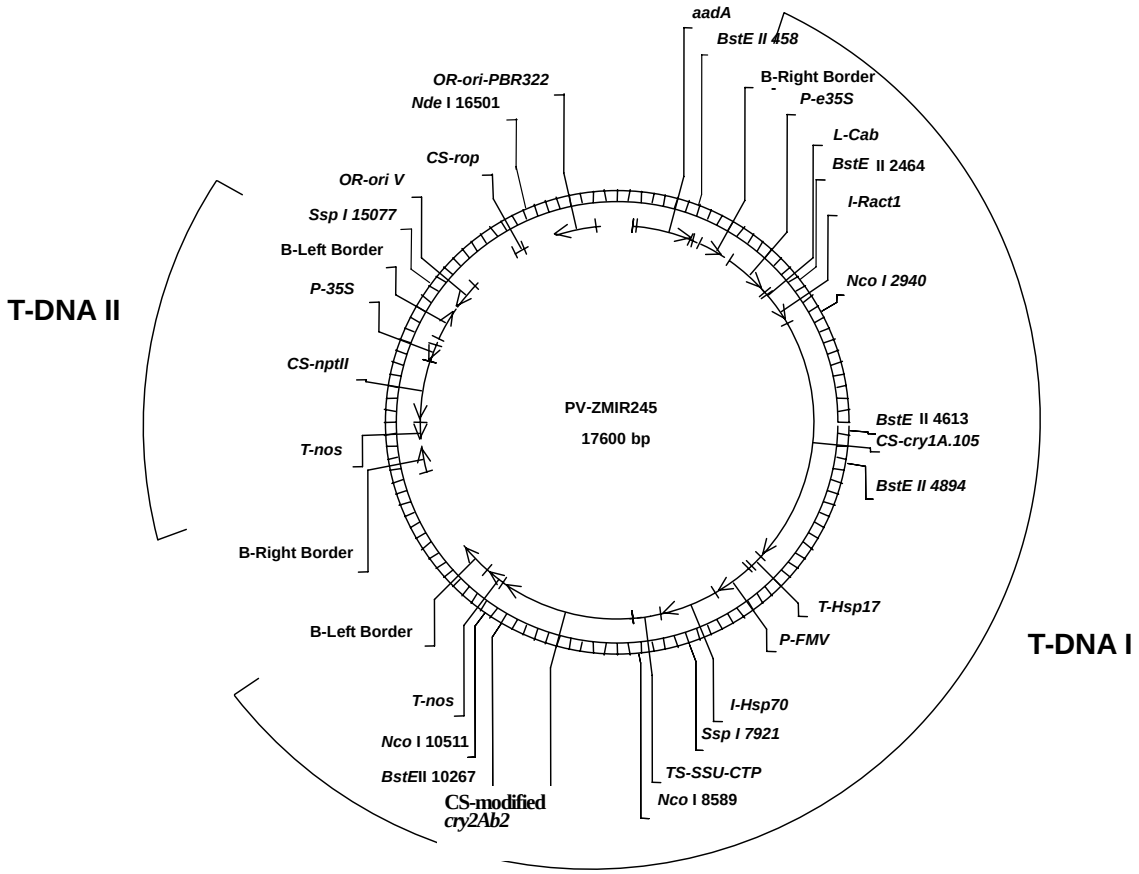

Figure 1 Map of the plasmid PV-ZMIR245 used for the development of MON89034[1]

In the process of rearing 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 rest upon Monsanto Japan Limited.

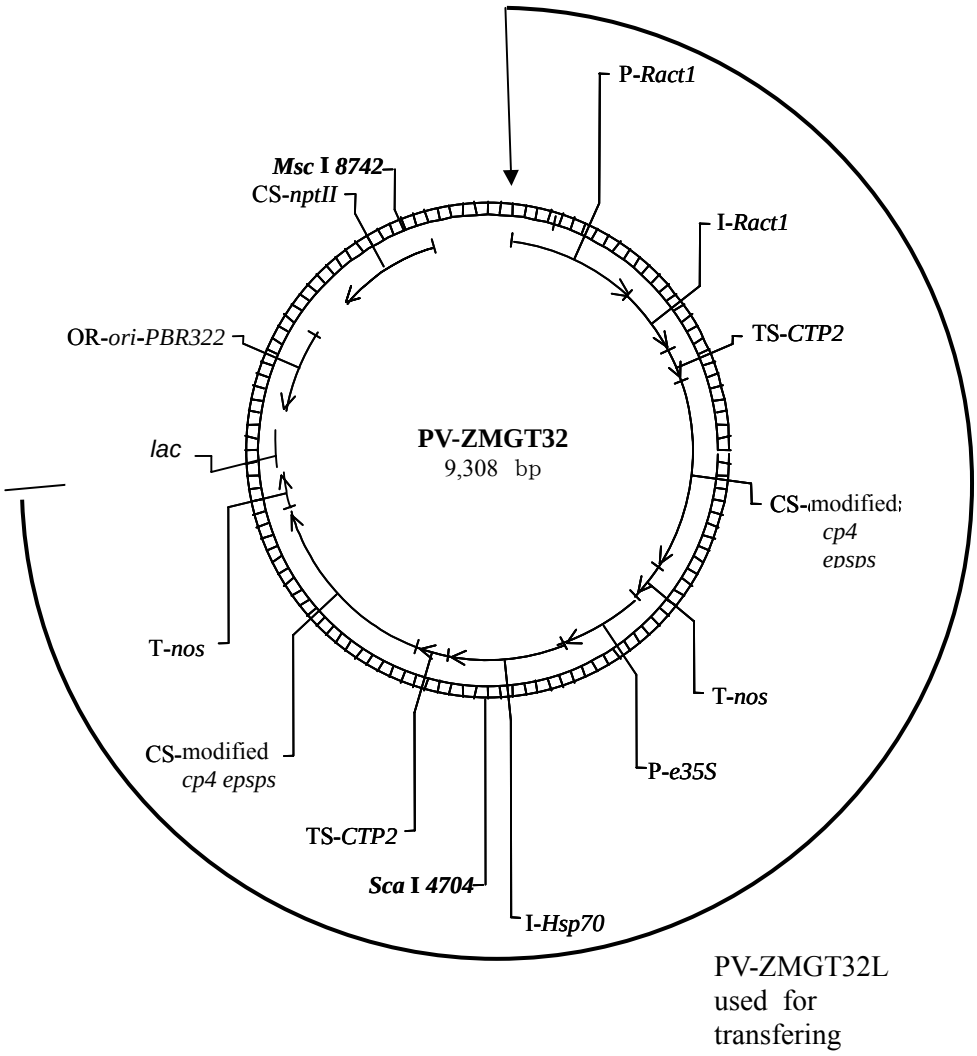

Figure 2 Map of the plasmid PV-ZMGT32 used for the development of NK603[2]

[2] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 1 Origins and functions of component elements of PV-ZMIR245 used for the development of MON89034[3]

Component elements	Origin and function
T-DNA I region	
B[Note 1]-Right Border	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[Note 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[Note 3]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Reference 19).
I[Note 4]-*Ract1*	Rice actin gene intron (Reference 20). Activates the expression of target gene.
CS[Note 5]-*cry1A.105*	A gene that encodes the Cry1A.105 protein. A synthetic Bt protein composed of Domains I and II of Cry1Ab protein, Domain III of Cry1F protein, and C-terminal Domain of Cry1Ac protein.
T[Note 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[Note 7]-*SSU-CTP*	Transit peptide of small subunit of ribulose 1,5-carboxylase diphosphate of maize, including the first intron sequence (Reference 24). Transfers downstream-connected protein to plastid.
CS-modified *cry2Ab2*	A gene that encodes the modified Cry2Ab2 protein derived from *Bacillus thuringiensis* (Reference 25). It has the site broken by the restriction enzyme transferred during the cloning and then, a single aspartic acid is transferred after the methionine at the N-terminal compared to the wild-type Cry2Ab2 protein.
T-*nos*	3' untranscribed region of nopaline synthase (*nos*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).
B-Left Border	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)[3]

Component elements	Origin and function
T-DNA II region	
B-Right Border	A DNA fragment containing the right border sequence (24 bp) of nopaline type T-DNA, derived from *A. tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Reference 16).
T-*nos*	3' transcription region of nopaline synthase (*nos*) gene derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).
CS-*nptII*	A gene derived from *E. coli* transposon Tn5 (Reference 28). Encodes neomycin phosphotransferase type II (*NPT II*) enzyme protein and confers resistance to kanamycin. Used as marker to select the transgenic plant during the gene transfer (Reference 29).
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 18). Involved in the constant expression of the target gene in the entire tissue of plant body.
B-Left Border	A DNA fragment containing the left border sequence (25 bp) 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[Note 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-PBR322*	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).

[Note 1] B – border
[Note 2] P – promoter
[Note 3] L – leader
[Note 4] I – intron
[Note 5] CS – coding sequence
[Note 6] T – transcript termination sequence
[Note 7] TS – targeting sequence
[Note 8] OR – Origin of Replication

[3] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 2 Component elements of plasmid PV-ZMGT32L which were used for transferring to NK603, and their functions[4]

Component elements	Origin and function
Modified *cp4 epsps* gene cassette (1)	
P[Note 1]-*Ract1*	Promoter region of actin 1 gene derived from rice. It makes target genes expressed (Reference 20). Makes target genes expressed in all tissues constantly.
I[Note 2]-*Ract1*	Rice actin gene intron (Reference 34). Activates the expression of target gene.
TS[Note 3]-*CTP2*	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 35). Transfers target proteins from cytoplasm to chloroplast.
CS[Note 4]-modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 36; Reference 37). 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[Note 5]-*nos*	3' untranscribed region of nopaline synthase (*nos*) 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 gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants (Reference 38).
TS-*CTP2*	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 35). 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 36; Reference 37). 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*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 26).

Note 1 P – promoter
Note 2 I – intron
Note 3 TS – targeting sequence
Note 4 CS – coding sequence

[4] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

Note 5 T – transcript termination sequence

2) Functions of component elements

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

Functions of component elements of the donor nucleic acid that were used for the development of MON89034 and NK603 are shown in Table 1 and Table 2. Details of the target genes, *cry1A.105* gene, modified *cry2Ab2* gene, and modified *cp4 epsps* gene, are described below.

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 39; Reference 40; Reference 41). In addition, several studies have shown that the Bt protein is composed of several Domains and what functions individual Domains possess. It has been revealed that the Bt protein is composed of Domains I, II, and III and C-terminal Domain, and Domain I is involved in formation of cation-selective pores to inhibit the digestive process, Domain II is involved in recognition of specific receptors, Domain III is involved in binding to receptors, and C-terminal Domain is involved in the crystal structure of the Bt protein (Reference 42; Reference 43).

[*cry1A.105* gene]

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 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 (Annex 1).

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 species of insects including 5 insects of the order Lepidoptera. As a result, the Cry1A.105 protein exhibited the insecticidal activity against the larvae of Corn earworm (*Helicoverpa zea*), Black cutworm (BCW; *Agrotis ipsilon*), Fall armyworm (FAW; *Spodoptera frugiperda*), Southwestern corn borer (*Diatraea grandiosella*), and European corn borer (ECB; *Ostrinia nubilalis*), which are the major pest insects of order Lepidoptera for maize, though it did not exhibit any insecticidal activity against honeybee, ladybug and other beneficial insects except the insects of order Lepidoptera (Table 3).

Based on the above results, it was confirmed that the Cry1A.105 protein exhibits a selective insecticidal activity against only the insects of the order Lepidoptera similarly to the Cry1Ab protein, the Cry1F protein and the Cry1Ac

protein, which are the component elements for Cry1A.105, and it does not possess any insecticidal activity against the other species of insects.

[Modified *cry2Ab2* gene]

The modified Cry2Ab2 protein, which is encoded by the modified *cry2Ab2* gene used for the development of MON89034, has the site broken by the restriction enzyme transferred during the cloning and then, a single aspartic acid is introduced 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 4 insects of the order Lepidoptera. As a result, the modified Cry2Ab2 protein exhibited the insecticidal activity against the larvae of Corn earworm, Fall armyworm, and European corn borer among the 4 species of major pest insects of the order Lepidoptera used in the investigation and not against Black cutworm (Table 3). Also, the modified Cry2Ab2 protein did not exhibit any insecticidal activity against honeybee, ladybug and other beneficial insects except the insects of the order Lepidoptera (Table 3); therefore, it was confirmed that the modified Cry2Ab2 protein offers specific insecticidal activity against only the insects of the order Lepidoptera and not against the other species of insects.

Table 3 Insecticidal spectrum of Cry1A.105 protein and modified Cry2Ab2 protein[5]

Order	Family	English name (Scientific name)	Insect Stage	Cry1A.105 LC_{50}[a]	Reference	Cry2Ab2 LC_{50}[a]	Reference
Lepidoptera	Noctuidae	Corn Earworm (*Helicoverpa zea*)	Larva	15	Reference 44	9.9	Reference 46
		Black Cutworm (*Agrotis ipsilon*)	Larva	33	Reference 45	>100[b]	Reference 54
		Fall Armyworm (*Spodoptera frugiperda*)	Larva	6.9	Reference 45	<50[c]	Reference 54
	Crambidae	Southwestern Corn Borer (*Diatraea grandiosella*)	Larva	37	Reference 45	—	—
		European Corn Borer (*Ostrinia nubilalis*)	Larva	0.43	Reference 46	1.5	Reference 46
Collembola	Isotomidae	Collembola (*Folsomia candida*)	Young adult	>80[d]	Reference 47	>70[d]	Reference 47
Coleoptera	Curculinoidae	Boll Weevil (*Anthonomus grandis grandis*)	Larva	>100	Reference 48	>100	Reference 54
	Chrysomelidae	Southern Corn Rootworm (*Diabrotica unecimpunctata howardi*)	Larva	>100	Reference 48	>100	Reference 54
	Coccinellidae	Spotted Lady Beetle (*Coleomegilla maculata*)	Larva	>240	Reference 49	>120	Reference 55
Hymenoptera	Ichneumonidae	Parasitic wasp (*Ichneumon promissorius*)	Adult	>240	Reference 50	>100	Reference 56
		Jewel wasp (*Nasonia vitripennis*)	Adult	—	—	>4500	Reference 57
	Apidae	European Honey Bee (*Apis mellifera*)	Adult	>550	Reference 51	>68	Reference 58
		European Honey Bee (*Apis mellifera*)	Larva	>11μg/cell	Reference 52	>0.6μg/cell	Reference 59
Hemiptera Sub-order: Homoptera	Aphididae	Green Peach Aphid (*Myzus persiscae*)	Adult/Young adult	>80	Reference 48	>80	Reference 54
Hemiptera Sub-order: Heteroptera	Miridae	Western Tarnished Plant Bug (*Lygus hesperus*)	Young adult	>80	Reference 48	>80	Reference 54
	Anthocoridae	Insidious Flower Bug (*Orius insidiosus*)	Young adult	>240	Reference 53	>100	Reference 60

[a] The unit used is μg/mL or g diet. The values with the sign "＞" refer to the highest density among the samples used in testing. The values with the sign "＜" refer to the lowest density among the samples used in testing.

[b] Mortality rate was 42% when the maximum dose of 100μg/mL was given.

[c] Mortality rate was 61% when the minimum dose of 50μg/mL was given.

[d] Testing was conducted using the freeze-dry leaves of MON89034.

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

[*cry1A.105* gene＋modified *cry2Ab2* gene]

MON89034 is given the resistance to the target insects of the order Lepidoptera with simultaneous expression of both Cry1A.105 protein and modified Cry2Ab2 protein. Actually, as a result of tests of 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 Argentine, 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 (Table 3). 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 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 the both Bt proteins (Table 1 in p.14 and Table 2 in p.15 of Annex 2).

[Modified *cp4 epsps* gene]

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 modified *cp4 epsps* gene has the nucleotide sequence in the wild-type *cp4 epsps* gene modified to enhance the expression level in plants without changing the functional activity of the wild-type CP4 EPSPS protein, with only a single modification 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 the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19) is inhibited and then the synthesis of aromatic amino acids essential for synthesis of proteins fails. The modified *cp4 epsps* gene, the target gene of NK603, produces the modified CP4 EPSPS protein which has high tolerance to the glyphosate herbicide. The activity of the modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus the recombinant plants that express this protein

have normal functions of shikimate synthesis pathway and can grow.

(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 (excluding allergenicity as food)

In order to investigate whether the Cry1A.105 protein and the modified Cry2Ab2 protein expressed in MON89034, and the modified CP4 EPSPS protein expressed in NK603 share any functionally important amino acid sequences with known contact allergens, they were compared with the allergens in the database (including GenBank, EMBL, PIR, and SwissProt). As a result, they did not share structurally related sequences with known allergens.

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

The Cry1A.105 protein and the modified Cry2Ab2 protein are crystalline insecticidal proteins called the Bt proteins derived from *B. thuringiensis*. For the mechanism of the insecticidal activity by the Bt proteins, many studies have been made (Reference 61), 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 enzyme activity and they would affect the metabolic system of recipient organism.

The EPSPS, identical functionally 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 an rate-determining enzyme in the pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In addition, EPSPS is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P)(Reference 62), and the only shikimate that is known to react with EPSPS other than these offers the reactivity of only one-two millionth that of S3P; therefore, it is considered unlikely to react as the substrate of EPSPS in any living organisms. Consequently, it is considered not to affect the metabolic system of recipient organism.

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

(2) Information concerning vector

1) Name and origin

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

MON89034: PV-ZMIR245 assembled from plasmids including the vector pBR322 derived from E. coli

NK603: PV-ZMGT32 assembled from plasmids including the vector 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 production of parent lines are as follows.

MON89034: PV-ZMIR245; 17,600bp

NK603: PV-ZMGT32; 9,308bp

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

The PV-ZMIR245 used for the production of MON89034 contains the *aadA* gene derived from *E. coli* transposon Tn7, which confers resistance to spectinomycin and streptomycin, as a selectable marker gene for construction vectors in *E. coli*, outside of the T-DNA region.

The PV-ZMGT32 used for the production of NK603 contains the kanamycin/neomycin resistant gene (*nptII* gene) derived form transposon Tn5, as a selectable marker gene (Reference 28).

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

The infectivity of PV-ZMIR245 and PV-ZMGT32 is not known.

(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 transferred in the recipient organism for development of MON89034 and NK603 are listed in Table 1 and Table 2. In addition, the location and section broken by restriction enzyme of the component elements of the donor nucleic acid in the vector are shown in Figure 1 and Figure 2.

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

NK603: Particle gun method

3) Processes of rearing of living modified organisms

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

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

MON89034: Paromomycin

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 63). 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.

Presence or absence of remaining *Agrobacterium* was not confirmed for NK603, since a DNA fragment was transferred by the particle gun method.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line with 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

In the development of MON89034, those individuals were selected based on the PCR method that contain only T-DNA I region.

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 climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters were examined in outdoor field tests. The MON89034 was selected upon the comprehensive evaluation of these results.

In the development of NK603, 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 excelent line was selected.

The status of application for approval of MON89034 in Japan is the following.

October, 2007: The safety of use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques" was certified by the Ministry of Agriculture, Forestry and Fisheries.

November, 2007: The safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" was certified by the Ministry of Health, Labour and Welfare.

January, 2008: Approval was obtained from the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) under the provisions of the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms."

The status of approval of NK603 in Japan is the following.

March, 2001: The safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" was certified by the Ministry of Health, Labour and Welfare.

March, 2001: The safety of use of the cultivar for feed was approved in accordance with "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)" by the Ministry of Agriculture, Forestry and Fisheries.

May, 2001: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was certified by the Ministry of Agriculture, Forestry and Fisheries.

March, 2002: Additional materials of transferred genes submitted for the safety of food, feed and environmemt were confirmed that they do not affect to the safety criteria on the above.

March, 2003: The safety of use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques" was certified by the Ministry of Agriculture, Forestry and Fisheries.

November, 2004: Approval was obtained from the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) under the provisions of the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms."

[Process of rearing of MON89034×NK603]

This stack line maize is an F1 hybrid developed from the inbred lines of MON89034 and NK603 as both parents (Figure 3).

Figure 3 Process of rearing of MON89034×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

As a result of examination for presence and segregation pattern of transferred genes in MON89034 and NK603 based on the chi-square (χ^2) test, no statistically significant difference was observed. Consequently, it was confirmed that the transferred genes in MON89034 and NK603 exist on the chromosome.

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

As a result of Southern blotting analysis of the transferred genes, it was confirmed that one copy of T-DNA I region and T-DNA region are transferred into the genomic DNA of MON89034 and NK603 at one site. In addition, it was also confirmed as a result of Southern blotting analysis on multiple generations that the transferred genes are inherited stably in offspring.

In addition, as a result of analysis on the nucleotide sequence of transferred genes of MON89034, it is found that 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 due to the homologous recombination (Annex 3). However, it was concluded that this homologous recombination could not cause formation of any new open reading frame.

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, and it was considered owing to the reason that static codon is preserved in the upstream of the transcription termination signal in the transcription product to read through the terminator of the transferred gene of NK603. Then it was concluded that this reading through does not affect the safety evaluation.

In addition, in the transferred gene of NK603, each of 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 proved 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.

The change of the base was confirmed in multiple generations, and stably descended to the progeny.

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

This item is not applicable because of one copy for both MON89034 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 proteins by Western blotting analysis

NK603: Confirming the expression by herbicide glyphosate spraying test

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

Regarding the plasmid PV-ZMIR245 used for the production of 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.

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

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

For detection and identification of this stack line maize, the above-mentioned methods

must be applied to each grain of maize seeds.

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

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

This stack line maize is given the traits as described below.

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

NK603: Tolerance to herbicide glyphosate due to the modified CP4 EPSPS protein which is derived from the transferred genes

The Cry1A.105 protein and the modified Cry2Ab2 protein expressed in MON89034 are crystalline insecticidal proteins 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 destructed midgut epithelium cells and inhibited digestive process in the sensitive species of insects, leading to death of the insects (Reference 39; Reference 41). For the mechanism in which Bt proteins develop the insecticidal activity, a number of studies have been made (Reference 61), and there is no report published to date that Bt proteins possess any other functions. Based on the above understanding, Bt proteins are considered not to offer any enzyme activity and thus they are judged not to affect the metabolic pathway of plants.

Similarly, the modified CP4 EPSPS protein expressed in NK603 is also considered not to affect the metabolic pathway of plants based on the facts that it 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.

In addition, it was confirmed that the Cry1A.105 protein and the modified Cry2Ab2 protein which exhibit the insecticidal activity against the insects of order Lepidoptera do not offer any synergistic insecticidal activity against the target insects of the order Lepidoptera which show sensitivity to the both Bt proteins (Table 1 in p.14 and Table 2 in p.15 of Annex 2).

In addition, it has been suggested that little consideration is required for possible interaction between proteins except for the proteins which are likely to cause interaction, namely such proteins that can act when two proteins are combined in a pair otherwise bind to the same receptors (Reference 64). Consequently, the Bt proteins and the modified CP4 EPSPS protein expressed in this stack line maize are considered not to fall under the proteins referred to in Reference 64 as requiring examinations on possible interaction.

Based on the above understanding, also in this stack line maize developed by cross-breeding between MON89034 and NK603, it is considered unlikely that the proteins expressed exhibit interaction with each other and have a new effect on the metabolic pathway of plants.

To confirm the above understanding in practice, regarding resistance of this stack line maize to the insects of order Lepidoptera, the bioassay to take Fall armyworm as the object of pot tests was carried out in the U.S. As a result, it was confirmed that there was no statistically significant difference between this stack line maize and MON89034 in which only the Cry1A.105 protein and the modified Cry2Ab2 protein are expressed, regarding the resistance to Lepidopteran pest insects (Table 4). In addition, regarding tolerance to herbicide glyphosate, the bioassay based on the glyphosate (Product name: Roundup Weathermax) spraying tests was carried out in the U.S. As a result, it was confirmed that there was no statistically significant difference between this stack line maize and NK603 in which only the modified CP4 EPSPS protein is expressed, regarding tolerance to herbicide glyphosate (Table 5).

Based on the above results, it was considered that the individual proteins expressed in this stack line maize function independently from each other. Then, for the information used in the Item-by-item assessment of Adverse Effects on Biological Diversity in II about the difference between this stack line maize and the taxonomic maize species to which the recipient organism belongs, the results of individual examinations for the various characteristics of MON89034 and NK603 were attached as follows.

Table 4 Investigational result of the severity of damage by Fall armyworm, order Lepidoptera, based on bioassay of this stack line maize (mean value ± 95% confidence interval) [6]

F1 hybrid	Leaf damage rate (LDR)±95% confidence interval
MON89034×NK603	2.75±0.42 a[1]
MON89034	2.63±0.68 a
NK603	9.00±0.00 b
Non-transgenic plant	9.00±0.00 b

[1] Tukey's multiple comparison test was conducted. As a result, for each evaluation item, different alphabetical letters indicate that a significant difference was observed between the relevant mean values (significance level: 5%).

Twelve (12) individuals each of this stack line maize MON89034×NK603, MON89034, NK603, and the non-transgenic plant were cultivated in pots (3 individuals/repeat × 4 repeats), and at the 6th leaf stage, the first instar larvae of Fall armyworm were inoculated (54 individuals/pot). On the 14th day after inoculation of Fall armyworm, the severity of insect damage by Fall armyworm was determined based on the leaf damage rate (LDR) in accordance with the evaluation method proposed by Guthrie et al., a typical method for evaluating insect damage severity on 10-step scales from 0 (no damage) to 9 (serious damage: a greater part of leaf is damaged) (Reference 65).

[6] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 5 Investigational result of the severity of injury by spraying of herbicide glyphosate (Product name: Roundup Weathermax) to this stack line maize (mean value ± 95% confidence interval)[7]

Test sample	Concentration							
	0.0 kg a.e./ha		0.84 kg a.e./ha		13.44kg a.e./ha1		26.88kg a.e./ha1	
	7th day[1]	14th day[1]	7th day[2]	14th day[1]	7th day[2]	14th day[1]	7th day[2]	14th day[1]
MON89034 ×NK603	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00 a	0.0 ± 0.00	1.0 ± 0.00 a	1.0 ± 0.00	1.9 ± 0.56 a	1.0 ± 0.00
NK603	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00 a	0.0 ± 0.00	1.0 ± 0.00 a	1.0 ± 0.00	2.0 ± 0.00 a	1.0 ± 0.00
MON89034	0.0 ± 0.00	0.0 ± 0.00	7.8 ± 0.44 b	10.0 ± 0.00	8.6 ± 0.41 a	10.0 ± 0.00	7.9 ± 0.32 b	10.0 ± 0.00
Non-transgenic plant	0.0 ± 0.00	0.0 ± 0.00	8.0 ± 0.45 b	10.0 ± 0.00	8.4 ± 0.46 a	10.0 ± 0.00	8.0 ± 0.00 b	10.0 ± 0.00

[1] Statistical treatment was not carried out since no variation was observed in the data.

[2] Tukey's multiple comparison test was conducted. As a result, for each evaluation item, different alphabetical letters indicate that a significant difference was observed between the relevant mean values (significance level: 5%).

Eight (8) individuals each of this stack line maize MON89034×NK603, NK603, MON89034, and the non-transgenic plant were cultivated in pots, and at the 4th leaf stage, herbicide glyphosate (Product name: Roundup Weathermax) was sprayed. On the 7th and 14th days after spraying 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 of glyphosate sprayed of 0.84 kg acid equivalent (a.e.)/ha refers to normal dosage, and 13.44 kg a.e./ha and 26.88 kg a.e./ha refer to 16-times higher and 32-times higher dosages than the normal dosage, respectively.

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

2) Differences between the recombinant plant and the taxonomic species to which the recipient organism belongs[8]

Regarding the difference between this stack line maize and the taxonomic species of maize to which the recipient organism belongs, evaluation was conducted on the parent lines MON89034 and NK603 based on the isolated field tests conducted in Japan.

For NK603, two different hybrid varieties NK603-A and NK603-B were subjected to the tests based on the "Concept of safety assessment of sibling lines" announced from the Agriculture, Forestry and Fisheries Research Council, the Ministry of Agriculture, Forestry and Fisheries, in March 29, 2001. For MON89034, only one variety was tested since the isolated field tests were conducted after the "Concept of safety assessment of sibling lines" was revised.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between MON89034 and NK603 and their non-transgenic control plants regarding the germination rate, uniformity of germination, time of tasseling, time of silking, culm length, plant shape, tiller number, height of ear, maturation time, number of ears, number of productive ears, row number per ear, grain number per row, 100-kernel weight, grain shape, and weight of above-ground parts at the harvest time. In addition, for MON89034, examination was conducted regarding the number of germinated seedlings, flowering time, culm diameter, ear length, ear diameter, grain color, grain shape, and grain number per ear. For NK603, examination was conducted regarding the ear length and ear diameter.

As a result, a statistically significant difference was observed between MON89034 and the non-transgenic control maize in ear diameter and grain number per ear, and between NK603-B for NK603 and the non-transgenic control maize in 100-kernel weight. However, these items were found to fall within the variable ranges for conventional maize, otherwise observed statistically significant difference was limited to only one of the two hybrid varieties tested (Table 2 in p.8 of Annex 4, Table 3-2 in p.17 and photos in pp.18-19 of Annex 5).

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

MON89034 and NK603 both withered and died due to the low temperature treatment at the early stage of growth similarly to their non-transgenic control plants, and no difference was observed in the withering and death (Figure 6-2 in p.14 of Annex 4, Table 3-4 in p.24 of Annex 5).

[8] All the rights pertinent to the information in (a) to (g) of this item and the responsibility for the content rest upon Monsanto Japan Limited.

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not re-grow and propagate vegetatively, or produce seeds. Actually, at the end of isolated field tests, start of withering and death after ripening was observed in MON89034 and NK603 (Figure 7 in p.15 of Annex 4, Table 3-4 in p.24 of Annex 5).

(d) Fertility and size of the pollen

MON89034 and NK603 both exhibited high fertility of the pollen similarly to their non-transgenic control plants, and no difference was observed also regarding the shape and size of pollen (Figures 8-1 and 8-2 in p.19 of Annex 4, Table 3-3 in p.21 and photo in pp.22 of Annex 5).

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

Regarding seed production, comparison was conducted between MON89034 and NK603 and their non-transgenic control plants for the characteristics listed in I-2-(6)-2)-(a). As a result, a statistically significant difference was observed between MON89034 and the non-transgenic control maize regarding ear diameter and grain number per ear, and between NK603-B of NK603 and the non-transgenic control maize regarding 100-kernel weight. However, these items were found to fall within the variable ranges for conventional maize, otherwise observed statistically significant difference was limited to only one of the two hybrid varieties tested (Table 6 in p.20 of Annex 4, Table 3-2 in p.17 and Table 3-4 in p.24 of Annex 5).

Regarding shedding habit of the seed, shedding habits of the seed were not observed in natural environment, since the ears of both MON89034 and NK603 and their non-transgenic control plants were covered with bracts at the time of harvesting.

Regarding germination rate in order to identify the dormancy of harvested seeds, germination test was carried out for the seeds harvested from MON89034 and NK603 and their non-transgenic control plants. As a result, no statistically significant difference was observed and then, no dormancy of the seed was identified (Table 3 and Table 4 in p.16 of Annex 4, Table 3-4 in p.24 of Annex 5).

(f) Crossability

A crossability test of the parent lines MON89034 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 and NK603 and their non-transgenic control plants, a statistically significant difference was not observed betweeen them. (Table 7-9 in p.23 of Annex 4, Table 3-5 in p.26, Table 3-6 in p.27, and Table 3-7 in p.28 of Annex 5).

[Bibliography]

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

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

The Cry1A.105 protein and the modified Cry2Ab2 protein encoded by the *cry1A.105* gene and the modified *cry2Ab2* gene (both are Lepidoptera resistant genes) derived from MON89034 possess the insecticidal activity against the specific insects of the order Lepidoptera but these proteins are considered not to have any enzyme activity. On the other hand, the modified CP4 EPSPS protein (5-enol-pyruvylshikimate-3-phosphate synthase) encoded by the modified *cp4 epsps* gene (glyphosate tolerant gene) derived from NK603 is the enzyme that possesses high substrate specificity.

Consequently, it is considered unlikely that traits conferred by the *cry1A.105* gene, the modified *cry2Ab2* gene, and the modified *cp4 epsps* gene would interact with each other.

It has been confirmed that this stack line maize expresses resistance to Lepidoptera and tolerance to glyphosate herbicide in the similar levels as offered by the parent lines, by Fall armyworm resistance tests and herbicide spraying tests, respectively.

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

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

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis) has been long used in Japan, though there is no report that it has become self-seeding in a natural environment in Japan.

This stack line maize is given the traits to be resistant to the insects of the order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein encoded by the *cry1A.105* gene and the modified *cry2Ab2* gene derived from MON89034, and to be tolerant to glyphosate due to the modified CP4 EPSPS protein encoded by the modified *cp4 epsps* gene derived from NK603. However, it is not generally considered that the insect damage by Lepidopteran insects is the major factor to inhibit the growth of maize under the natural environment in Japan, and that the herbicide glyphosate is sprayed and exerts pressure for selection in the natural environment in Japan.

Therefore, it is unlikely that these characteristics enhance the competitiveness of this recombinant maize and therefore this stack line maize would be more competitive than its parent lines.

Based on the above understanding, it was judged that the conclusion made by the

applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack line maize has both the Cry1A.105 protein and the modified Cry2Ab2 protein productivity derived from MON89034 and the modified CP4 EPSPS protein productivity derived form NK603. The Cry1A.105 protein, and the modified Cry2Ab2 protein possess the insecticidal action against Lepidopteran insects. However, the modified CP4 EPSPS protein confers tolerance to glyphosate herbicide, though it is confirmed not to be harmful substance to animals and plants. In addition, it is considered unlikely that the Cry1A.105 protein, the modified Cry2Ab2 protein, and the modified CP4 EPSPS protein would interact with each other.

As a result, even though this stack line maize contains these proteins in conjunction, it is unlikely that the productivity of harmful substances will be greater in this stack line maize than its parent lines.

Therefore, the conclusion that the use of this stack line maize poses no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances, which was made by the applicant, is valid.

(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 recombinant maize 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 reasonable.