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 Coleoptera and Lepidoptera, and tolerant to glyphosate (*cry3Bb1, cry1Ab, cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (MON863×MON810×NK603, OECD UI: MON-ØØ863-5×MON-ØØ81Ø-6×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 a recipient organism or the species to which the recipient organism belongs

(1) Taxonomical position and state of distribution in natural environment

i) The general academic name for maize is *Zea mays* L. However, in recent years, since the annual teosinte, a related species of maize has been classified into *Z. mays*, maize has been classified into *Z. mays* subsp. *mays* (L.) Iltis as a subspecies of *Z. mays*.

ii) The recipient organism is *Zea mays*, which belongs to the genus *Zea* of the family *Gramineae*. The recipient organism belongs to the dent type.

iii) The origin is considered to be the area from the southwest region of the United States to Mexico, Central America, and South America, but there is no conclusive theory. There are two theories; one is that the independent origins are considered to be each of the aforementioned regions, and the other is that the exclusive origin is considered to be the south region of Mexico. There is no report of natural distribution in Japan.

(2) History and present state of Use

i) It is generally understood that the earliest cultivation could date back 9,000 years. It is considered that cultivation and breeding were later carried out by indigenous inhabitants, and in about 3000 BC to 1500 BC maize near to the modern cultivation type was cultivated in earnest, and was introduced to the various regions of the northern and southern Americas. It is understood that in the process of the introduction, various types such as dent, pop and sweet were differentiated. The first introduction to Japan is said to have been in 1579 to Nagasaki or Shikoku, and maize has long been cultivated in Japan since then.

ii) At present, maize is used mainly for feed, but also for food and various food products including cooking oil and starch. Currently, it is the most widely cultivated grain in the world and can be grown in the area from a latitude of 58 degrees north to 40 degrees south mainly in the US, China, Brazil, Argentina, and European countries and others. Based on the statistical information of the Food and Agriculture Organization (FAO) of the United Nations, in 2002 the world's cultivated area of maize was about 140 million hectares. The seven top countries were the US (28 million hectares), China (25 million

hectares), Brazil (12 million hectares), Mexico (7 million hectares), India (6 million hectares), Nigeria (4 million hectares), and South Africa (3 million hectares). Also, according to the same statistical information of the FAO, the cultivated area of maize in Japan in 2002 was about 30 thousand hectares.

Japan currently imports about 16 million tons of maize for feed and food. Maize imported for feed totals about 11 million tons and about 5 million tons is imported for food, and is mainly used for starch and isomerized sugar.

The practical cultivation method of maize for feed in Japan is as follows. The optimum sowing season in cold to mild-temperature regions is May, and April to June in some warmer regions. The optimum density is 6,000 to 8,000 plants per 10 arces. For weed control, herbicides are sprayed and intertillage and molding are applied two to three times at the early stages of growing maize. Aerial parts of maize are harvested in the yellow ripe stage, 35 to 45 days after ear emergence.

In addition, based on the lists of maize varieties of major seed and plant companies in Japan, currently almost all maize for cultivation available on the market is F1 hybrid, and it is not general to sow the harvested seeds for cultivation in the following year.

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

The optimum germination temperature of maize is 32-36℃ and the minimum germinating and minimum growing temperature is 6-10℃. In practice, the optimum sowing season is considered to be the period when the temperature is 13-14℃ or over. It varies somewhat by variety and place, but usually maize is sown in spring and harvested in autumn as an annual plant. In addition, maize is usually a short-day plant, and its photosensitivity is higher in the late variety and lower in the early-season variety. Other than temperature, the following environmental conditions affect the growth of maize. Regarding the absorption of water, 70% of seed weight for dent type and 90% of seed weight for sweet type allows the maize to germinate. Moreover, humid soil with a pH of 5.5-8.0 is suitable for maize cultivation.

Modern maize is a plant highly acclimatized for human cultivation, and it has lost the ability to reproduce and grow in natural conditions.

ii) Mode of propagation or reproduction

a) A fully-ripened seed is covered with the bract of the ear, and the seed does not have natural shedding habits. Maize has long been cultivated and it has lost the ability to survive as a wild plant. Maize requires the assistance of human beings to disperse its seeds. The dormancy of the seed is extremely low, and even when seeds fall to the ground, they do not germinate until the soil temperature reaches 10°C. In most cases the seeds would decay and die before germinating.

Moreover, even if seeds germinate, they cannot subsist under conditions of exposure to temperatures below 0℃ for more than 6-8 hours or over at the early stage of growth (5th-7th leaf stage)after the growing point reaches the above-ground level. The longevity of seeds is short if stored at room temperature, and the germination rate decreases from the second year.

b) Maize does not reproduce by vegetative propagation. It reproduces only by seed. There are no reports of maize having the budding property in the tissues or organs that can regenerate the plant body.

c) Maize is a monoecious annual plant, which propagates by seed mostly through cross-pollination, although can be self-pollinated due to the absence of self-incompatibility. Species related to maize are teosinte, of the same genus *Zea,* as well as some other species classified into the genus *Tripsacum*. Maize can be hybridized only with teosinte in nature, and natural crossing with any species of the *Tripsacum* is not known. Natural distribution of teosinte is only seen in Mexico and Guatemala. On the other hand, the distribution area of the genus *Tripsacum* is divided broadly into three areas including the south-eastern part of North America, the lowland area of the eastern Andes from Colombia to Bolivia, and the area of Mexico and Guatemala that is considered the center of this genus. In Japan, the growth of teosinte and wild species of the *Tripsacum* has not been reported.

d) Maize has a typical wind-pollinated flower. A tassel of maize has 1,200-2,000 spikelets, and produces 16 million to 30 million pollen grains. The longevity of pollen is within 24 hours in the conditions of a field in high summer, but there is a range of longevity of from two hours to eight days according to the environmental conditions. Maize pollen is spherical and the diameter of the pollen is 90-100 μm. Maize mainly propagates through cross-pollination by wind, but there is a 1-5% possibility of self-pollination in normal field conditions. The pollens dispersing from bloomed tassels attach to the silks extracted from ears and then they germinate. The fertilization of maize is completed within 24 hours. The dispersion distance of maize

pollen differs by the presence of barriers such as woods or mountains, or the direction of the wind, but is considered to be approximately 300-500 m.

iii) Productivity of harmful substances

Regarding maize, productivity of harmful substances that can affect the growth or habitat of other wild fauna and flora has not been reported.

iv) Other information

There are no reports that maize seeds which were spilled during transportation, etc., on locations other than cultivation fields have grown.

2. Information concerning preparation of living modified organisms

The selfed line (hereinafter referred to as "autogamy line of MON863×MON810") was produced by repeated autogamy after the hybridization of the following two recombinant maize with the use of traditional-breeding method. The two recombinant maize are, 1) Maize resistant to Coleoptera (*cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis) (MON863, OECD UI: MON-ØØ863-5) (hereinafter referred to as "MON863"), and 2) Maize resistant to Lepidoptera (*cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (MON810, OECD UI: MON-ØØ81Ø-6) (hereinafter referred to as "MON810"). Then, the hybridization posterity cultivar (*cry3Bb1, cry1Ab, cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (OECD UI No.: MON-ØØ863-5×MON-ØØ810-6×MON-ØØ6Ø3-6) (hereinafter referred to as "this stack maize") was produced by the hybridization of the following two recombinant maize with the use of traditional-breeding method. The two recombinant maize are, 1) autogamy line of MON863×MON810 on the above, and 2) Maize tolerant to glyphosate (*cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (NK603, OECD UI: MON-ØØ6Ø3-6) (hereinafter referred to as "NK603"). Therefore, this stack maize possesses the characteristics of the three recombinant maize including MON863, MON810 and NK603, which are the parent line of this stack maize. The information concerning preparation of MON863, MON810 and NK603 are explained individually in the followings.

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of MON863 are shown in Table 1. In this recombinant maize, *cry3Bb1*gene, which was modified from the wild- type *cry3Bb1* gene was inserted.

Hereinafter the gene is referred to as "modified *cry3Bb1* gene", and the protein being expressed is referred to as "modified Cry3Bb1 protein".

The composition of donor nucleic acid and the origins of component elements used for the development of MON810 are shown in Table 2.

The composition of donor nucleic acid and the origins of component elements used for the development of NK603 are shown in Table 3.

ii) Functions of component elements

The functions of component elements which were used for the development of MON863 are shown in Table 1. The functions of component elements which were used for the development of MON810 are shown in Table 2. The functions of component elements which were used for the development of NK603 are shown in Table 3.

【Modified *cry3Bb1* gene】

a) In MON863, the modified *cry3Bb1* gene, the target gene to confer Coleoptera resistance, is derived from *Bacillus thuringiensis* subsp. *Kumamotoensis,* a gram-positive bacterium, universally exists in soil. The modified Cry3Bb1protein which is encoded by the modified *cry3Bb1* gene possesses an insecticidal activity against corn rootworm (*Diabrotica* sp.) (hereinafter referred to as CRW), which is one of the major pest insects of order Coleoptera to maize cultivation in the US. This insect damages the roots of maize. *B.t.* proteins which are produced by the bacterium *B.t.* including modified Cry3Bb1 protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pores, which lead to the inhibition of the digestive process and result in the insecticide activity. *B.t.* protein does not possess enzyme activity and it functions independently of the metabolic system of recipient organism.

The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow, and the modified Cry3Bb1 protein shows the insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata*) (hereinafter referred to as CPB) and corn root worm (CRW), which are respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera. There was no report that related species of the same genera with these two insects have ever inhabited in Japan.

Compared with the wild-type Cry3Bb1 protein, the modified Cry3Bb1 protein has 98.9% homology. In practice, the analysis of the insecticidal spectrum is examined in with the use of this modified Cry3Bb1 protein.

b) In order to investigate whether the modified Cry3Bb1 protein shares functionally important amino acid sequences with known allergens, the modified Cry3Bb1 protein was compared with the contact allergens in the database. As a result, the modified Cry3Bb1 protein did not share structurally related sequences with known allergens.

【*nptII* gene】

a) In MON863, the *nptII* (neomycin phosphotransferase type II) gene, which is an antibiotic resistance marker gene introduced for the selection of transgenic plant, is derived from *Escherichia coli* transposon Tn5. The encoded NPTII protein shows resistance to aminoglycoside antibiotics (kanamycin and others) by inactivating these antibiotics through phosphorylation. As a result, trasgenic cells can be selected by the addition of kanamycin to the medium.

b) To discover whether the NPTII protein shares functionally important amino acid sequences with known allergens, comparison was conducted using the database. As a result, the NPTII protein did not share structurally related sequences with known allergens.

【*cry1Ab* gene】

a) In MON810, the *cry1Ab* gene, the target gene to confer Lepidoptera resistance, is derived from *Bacillus thuringiensis (B.t.)* subsp. *kurstaki*, a gram-positive bacterium, universally exists in soil. The Cry1Ab protein which is encoded by the *cry1Ab* gene possesses an insecticidal activity against corn borer (*Ostrinia nubilalis*), which is one of the major pest insects of order Lepidoptera to maize cultivation in the US. Corn borer (*Ostrinia nubilalis*) damages all part of maize above ground level. *B.t.* proteins which are produced by the bacterium *B.t.,* including Cry1Ab protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pores, which lead to the inhibition of the digestive process and result in the insecticidal activity. *B.t.* protein does not possess enzyme activity, and it functions independently of the metabolic system of recipient organism.

Cry1Ab protein shows insecticidal activity only against order Lepidoptera, and it does not possess insecticidal activity against the insects other than order Lepidoptera.

In addition, Cry1Ab protein shows insecticidal activity against European corn borer (*Ostrinia nubilalis*), southwestern corn borer (*Diatraea grandiosella*), southern cornstalk borer (*Diatraea crambidoides*), sugarcane cornstalk borer (*Diatraea saccharalis*), corn earworm (*Helicoverpa zea*), Fall armyworm (*Spodoptera frugiperda*), and stalk borer (*Papaipema nebris*), which are major pest insects to maize cultivation in the US. Among them, *O. furnacalis*, the same genus of *O. nubilalis*, is known as the major pest insect of order Lepidoptera to maize cultivation in Japan.

b) In order to investigate whether Cry1Ab protein shares functionally important amino acid sequences with known allergens, Cry1Ab protein was compared with the known allergens in the database. Results showed the Cry1Ab protein did not share structurally related sequences with known allergens.

【cp4 epsps gene】

a) Glyphosate is the active ingredient in Roundup, a nonselective herbicide, and inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis by specifically binding to the enzyme. As a result, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. A *cp4 epsps* gene, the inserted gene of NK603, expresses the CP4 EPSPS protein, which has high tolerance to the herbicide glyphosate. The activity of the CP4 EPSPS protein that is produced by *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 and grow normally.

EPSPS is one of the enzymes that catalyze the shikimate pathway for aromatic amino acid biosynthesis that is specific to plants and microorganisms, and is located in chloroplasts or plastids in plants. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It has been clarified to be extremely unlikely that the stages from DAHP to the synthesis of chorismic acid, through the production of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) catalyzed by EPSPS, are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, 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 practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids. In addition, Monsanto Co. examined amino acid contents in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the Roundup herbicides.Those results confirmed that there is no difference in the content of aromatic amino acids, which are the final product of shikimate pathway, between the original non-recombinant plants and recombinant plants. These facts support that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS is the enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), and is known to specifically react with these substrates. The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

b) In order to investigate whether the CP4 EPSPS protein shares functionally important amino acid sequences with known contact allergens, the CP4 EPSPS protein was compared with contact allergens in the database. As a result, the CP4 EPSPS protein did not share structually related homologous sequences with any of the known allergens examined.

【Modified *cry3Bb1* gene + *cry1Ab* gene】

It is known that each of the Cry1A family in which Cry1Ab protein belongs and Cry3 family in which modified Cry3Bb1 protein belongs show insecticidal activity specifically against larvae in different order including insect of order Lepidoptera and insect of order Coleoptera, from the information of *B.t.* preparation which has been used as biological pesticide since 1960.

In addition, both proteins on the above have same functions to inhibit the digestive process by binding to the specific receptors on the midgut epithelium of the target insect. However, the pH in midgut of order Lepidoptera is alkalinity (pH 10.5～pH 11.0), and that of order Coleoptera is neutrality (pH 6.5～pH 7.0), and both proteins show insecticidal activity in different chemical conditions.

Moreover, it is known that the non-target insect does not show the sensitivity against Cry1Ac protein which belongs to Cry1A family as same as Cry1Ab protein, and

Cry3Aa protein which belongs to Cry3 family as same as Cry3Bb1 protein. This non-target insect remained non-sensitive even if the mixture of *B.t.* protein which belongs to the different two families was given to this insect, and it was confirmed that this non-target insect does not receive synergistic effect by being exposed to Cry1Ac protein and Cry3A protein at the same time (Table 4). Consequently, both Cry1Ab protein and Cry3Bb1 protein which are derived form their parent lines are expressed in this recombinant maize, but it is considered that the possibility is extremely low that these proteins show the insecticidal activity synergistically against the non-target insects of each other.

Table 1 Component elements of PV-ZMIR13L, which were used for the production of MON863,and their origins and functions

Component elements	Origin and function
cry3Bb1 gene cassette	
4-AS1	A promoter that contains 4 copies of AS-1 element and a part of 35S promoter from cauliflower mosaic virus (CaMV). Has a function to make target genes expressed in all the tissues constantly.
wt CAB	5'-terminal untranslated region of wheat chlorophyll a/b binding protein. Activate the expression of target genes.
ract1 intron	Intron of rice actin gene. Activate the expression of target genes.
Modified *cry3Bb1*	The gene which encodes modified Cry3Bb1 protein of *Bacillus thuringiensis.* The detail of its function is described in p5-6.
LacZ	Partial coding sequence for β-d-galactosidase or lacZ protein.
tahsp 17 3'	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation.
nptII gene cassette	
35S	A promoter from cauliflower mosaic virus (CaMV).
nptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II. Utilized as a selectable marker for transformation since it confers resistance to kanamycin when being expressed in microorganism
Ble	A part of bleomycin resistance gene isolated from Tn5. It confers bleomycin resistance when being expressed in microorganism. It encodes 50 amino acids at the N-terminal region of the Ble protein in MON863, but does not confer bleomycin resistance.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* Terminates transcription and induces polyadenylation of mRNA.

Table 2 Component elements of PV-ZMBK07 and PV-ZMGT10, which were used for the production of MON810, and their origins and functions

Component elements	Origin and function
cry1Ab gene cassette	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
cry1Ab	The gene which encodes Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain in the soil. The detail of its function was described in p6-7.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
cp4 epsps gene cassette（As a result of inserted gene analysis, this was not inserted into MON810.）	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP2	N-terminal side of EPSPS protein chloroplast transit peptide sequence in *Arabidopsis epsps* gene. Transfers target gene from cytoplasm to chloroplast.
cp4 epsps	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
GOX gene cassette （As a result of inserted gene analysis, this was not inserted into MON810.）	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 1	N-terminal side of rubisco small subunit 1A chloroplast transit peptide sequence in small subunit 1A gene of rubisco derived from *Arabidopsis epsps* gene. Transfer target protein from cytoplasm to chloroplast.
GOX	Sequence encoding C-terminal of v247, a variant derived from glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein degrades glyphosate.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* Contains transcription terminator and polyadenylation signal for mRNA.
Backbone (common to PV-ZMBK07 and PV-ZMGT10)（As a result of inserted gene analysis, this was not inserted into MON810.）	
LacZ	Partial coding sequence for β-D-galactosidase or LacZ protein. It resolves β-galactoside and produces β-galactose.
Ori-pUC	A segment containing replication origin for *E. coli* plasmid pUC. Starts the replication of the plasmid.
nptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II. Utilized as a selectable marker for transformation since it confers resistance to kanamycin when being expressed in microorganism.

Table 3 Component elements of PV-ZMGT32L, which were used for insertion, and their origins and functions

Component elements	Origin and function
cp4 epsps gene cassette (1)	
P-ract1	Promoter region of actin 1 gene derived from rice. It makes target genes expressed.
ract1 intron	Intron of rice actin gene. It makes target genes expressed by enhancing splicing.
CTP 2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
cp4 epsps	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4. Details of functions are shown on p7-8.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
cp4 epsps gene cassette (2)	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 Intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
cp4 epsps	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4. Details of functions are shown on p7-8.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.

Table 4 Result of the examination of insecticidal activity of Cry1 protein and Cry3 protein against the different order insects (Based on the treatises of MacIntosh et al.)

	Cry1Ab (1	**Cry1Ac** (2	**Cry3A** (3	**Cry1Ac + Cry3A** (4
Cockroach (*Blatella germanica*)	-	-	-	-
Alfalfa weevil (*Hypera postica*)	-	-	-	-
Cotton boll weevil (*Antonomus grandes*)	-	-	-	-
Horseradish flea beetle (*Phyllotreta armoraciae*)	-	-	-	-
Southern corn rootworm (*Diabrotica undecimpunctata howardii*)	-	-	-	-
Japanese beetle (*Popilla japonica*)	-	-	-	-
Colorado potato beetle (*Leptinotarsa decemlineata*)	-	-	+	+
Mosquito (*Aedes aegypti*)	-	-	-	-
Green peach aphid (*Myzus persicae*)	-	-	-	-
Termite (*Reticulitermes flavipes*)	-	-	-	-
Beet armyworm (*Spodoptera exigua*)	+	+	-	+
Black cutworm (*Agrotis ipsilon*)	+	+	-	+
Cabbage looper (*Trichoplusia ni*)	+	+	-	+
Corn earworm (*Heliothis zea*)	+	+	-	+
European corn borer (*Ostrinia nubilialis*)	+	+	-	+
Tobacco budworm (*Heliothis virescens*)	+	+	-	+
Tobacco hornworm (*Manduca sexta*)	+	+	-	+
Spider mite (*Tetranychus urticae*)	-	-	-	-

1- Set the concentration of Cry1Ab protein as 50μg/ml in artificial feed, and gave to the insect order.
2- Set the concentration of Cry1Ac protein as 50μg/ml in artificial feed, and gave to the insect order.
3- Set the concentration of Cry3A protein as 500μg/ml in artificial feed, and gave to the insect order.
4- Set the concentration of Cry1Ac protein as 50μg/ml、and that of Cry3A protein as 500μg/ml in artificial feed, and gave them to the insect order.

＋ Examination Unit with the death rate of examined larvae of 25% and over

— Examination Unit with the death rate of examined larvae of 25% and below

(2) Information concerning vector

i) Name and origin

The vectors used for the production of MON863, MON810, and NK603 are respectively assembled from plasmids including pUC 119 from *Escherichia coli*.

ii) Properties

The vectors for the production of MON863, MON810, and NK603 contain kanamycin/neomycin-resistant gene (*nptII* gene) derived from *E.coli* transposon Tn5 as the selectable marker gene for the constructive vector. The infection of this vector is not known.

(3) Method of preparing living modified organisms

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

Table 1, Table 2, and Table 3 show the structure of the entire nucleic acid transferred in the recipient organism.

ii) Method of transferring nucleic acid transferred in the recipient organism

For the production of MON863, PV-ZMIR13L, a linear DNA fragment, was introduced by particle gun bombardment to the maize inbred line A634 that is classified into dent type.

For the production of MON810, the mixture of plasmid PV-ZMBK07 and PV-ZMGT10 was introduced by particle gun bombardment to the F2 generation from a cross between 2 maize inbred lines A188×B73 that is classified into dent type.

For the production of NK603, PV-ZMGT32L, a linear DNA fragment
, was introduced by particle gun bombardment to the maize variety AW×CW that is classified into dent type.

iii) Processes of rearing of living modified organisms

【Process of rearing of MON863】

a) The callus to which PV-ZMIR13L was introduced was grown on a tissue culture medium containing 2,4-D for a certain period of time, and then the recombinant plant was selected on a kanamycin-containing medium. From the selected callus, the regenerated plant was obtained and the expression of the Cry3Bb1 protein was analyzed.

b) A plasmid was introduced in MON863 by particle gun bombardment, so confirmation of remaining Agrobacterium was not carried out.

c) Pedigree selection was started in 1997, and field experiments were carried out from 1998 to 1999. Finally, an excellent line was selected. In the field experiments at one site in Illinois in 1999, the morphological and growth characteristics of this line were investigated and also analysis of the expression of the Cry3Bb1 protein and inserted genes were implemented. Based on these results, necessary approval was obtained in the US and general commercial cultivation began in 2003.

The situation of approval of MON863 in Japan is the following.

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.

February, 2002: Based on the "Procedure for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare.
Based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

March, 2003: Based on the "Procedure to confirm the safety of feed and additives derived from
recombinant-DNA plants", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

April, 2003: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being cultivated in Japan was certified by the Ministry of Agriculture, Forestry and Fisheries.

【Process of rearing MON810】

a) The callus to which PV-ZMBK07 and PV-ZMGT10 were introduced was grown on a tissue culture medium containing 2,4-D for a certain period of time, and then the recombinant plant was selected on a glyphosate-containing medium. From the selected callus, the regenerated plant was obtained and the expression of the Cry1Ab protein was analyzed. As a result of Southern blotting analysis of inserted gene of MON810, it was confirmed that the expression cassettes of *nptII* gene, *cp4 epsps* gene, and *GOX* gene do not exist. The reason of MON810 to be selected in glyphosate even though *cp4 epsps* gene was not inserted in MON810 might be that the segregation would have happened for inserted gene in the following generation of re-differentiated plant. However, the reason was not specified, because MON810 was selected as maize resistant to pest insect, and glyphosate examination and Southern blotting analysis were not performed in the following generation of re-differentiated plant.

b) MON810 was introduced to the recombinant plant by particle gun bombardment, so confirmation of remaining Agrobacterium was not carried out.

c) Pedigree selection was started in 1992, and field experiments were carried out from 1993 to 1995. Finally, an excellent line was selected. In the field experiments at 6 fields in the US in 1994, the morphological and growth characteristics of this line were investigated and also analysis of the expression of the Cry1Ab protein and inserted genes were implemented. Based on these results, necessary approval was obtained in the US and general commercial cultivation began in 1997.

The situation of approval of MON810 in Japan is the following.

October, 1996: 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.

May, 1997: Based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare (the Ministry of Health and Welfare, at that time).

June, 1997: Based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

March, 2001: Based on the "Procedure for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare.

March, 2003: Based on the "Procedure to confirm the safety of feed and additives derived from
recombinant-DNA plants", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

April, 2003: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being cultivated in Japan was certified by the Ministry of Agriculture, Forestry and Fisheries.

【Process of rearing of NK603】

a) The callus to which PV-ZMGT32L was introduced was grown on a tissue culture medium containing 2,4-D for a certain period of time, and then the recombinant plant was selected on a glyphosate-containing medium. From the selected callus, the regenerated plant was obtained and the expression of the CP4 EPSPS protein was analyzed.

b) A plasmid was introduced to NK603 by particle gun bombardment, so confirmation of remaining Agrobacterium was not carried out.

c) Pedigree selection was started in 1997, and field experiments were carried out in a total number of 103 fields from 1997 to 1999. Finally, an excellent line was selected. In these field experiments, the morphological and growth characteristics of this line were investigated and also analysis of the expression of the CP4 EPSPS protein and inserted genes were implemented. Based on these results, necessary approval was obtained in the US and general commercial cultivation began in 2001.

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

March, 2001: Based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare.

March, 2001: Based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)", safety of use for feed was approved 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) and being cultivated was certified by the Ministry of Agriculture, Forestry and Fisheries.

March, 2002: Additional information regarding insertion genes was submitted for committees regulating the safety for food, feed and environment, which was approved to give no effect for the judgment of safety as stated above.

March, 2003: Based on the "Procedure to confirm the safety of feed and additives derived from recombinant-DNA plants", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

【Process of rearing of MON863×MON810×NK603】

This stack maize was produced from the hybridization of following two recombinant maize, with the use of traditional cross-breeding method. The two recombinant maize on the above are, 1) the inbred line produced from the repeated selfing after hybridization of two recombinant maize, MON863 and MON810 with the use of traditional cross-breeding method (hereinafter referred to as "the inbred line of MON863×MON810"), and 2) the inbred line of NK603.

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

【State of existence of nucleic acid transferred in MON863 and stability of expression of traits】

As a result of the analyses of inserted gene by Southern blotting analysis of MON863, it was confirmed that one copy of the DNA fragment derived from PV-ZMIR13L which is essential for the expression of modified *cry3Bb1*gene and *nptII* gene is inserted into the genome of MON863 at one site. Also, it was shown that modified *cry3Bb1* gene and *nptII* gene on the inserted DNA fragment were stably descended to the progeny by Southern blotting analysis and Western blotting analysis conducted in multiple generations. In the process of selection, it was also confirmed that resistant to Coleoptera was stably expressed in multiple generations by biological examination.

【State of existence of nucleic acid transferred in MON810 and stability of expression of traits】

As a result of the analyses of inserted gene by Southern blotting analysis of MON810, it was confirmed that one copy of the DNA fragment derived from PV-ZMBK07 which is essential for the expression of *cry1Ab* gene is inserted into the genome of MON810 at one site. Also, it was shown that inserted gene was stably descended to the progeny by Southern blotting analysis conducted in multiple generations. In the process of selection, it was also confirmed that resistance to Lepidoptera was stably expressed in multiple generations by biological examination.

As a result of the analyses of inserted gene by Southern blotting analysis of MON810, only the essential region for expression of *cry1Ab* gene which was derived from PV-ZMBK07 was inserted into the genome of the maize. Also it was confirmed that the *nptII* gene and the expression cassettes of *cp4 epsps* gene and *GOX* gene derived from PV-ZMGT10 did not exist.

【State of existence of nucleic acid transferred in NK603 and stability of expression of traits】

Regarding NK603, state of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid, including additional information regarding inserted genes which were already approved for safety in March 2002, is the following.

As a result of the analyses of inserted gene by Southern blotting analysis of NK603 and the analyses of base sequence of 3'-terminal, it was confirmed that one copy of the DNA fragment which was derived from PV-ZMGT32 including two *cp4 epsps* gene cassettes was inserted into the genome of NK603 at one site. Also it was proved that 217bp fragment which is P-ract1 promoter exists in the reverse direction near the 3'-terminal of the inserted gene. It was also proved that the inserted gene is descended stably to the progeny in multiple generations. In addition, as a result of the glyphosate-spraying test, it was confirmed that CP4 EPSPS protein is stably expressed in multiple generations.

Also, as a result of strand-specific RT-PCR to confirm whether transcription product is produced in this region regarding 217bp fragment which is P-ract1 promoter near the 3'-terminal of this inserted gene, transcription product was found which was considered to start from either P-ract1 promoter of the inserted gene or E35S promoter and to read through NOS 3' terminator. However, as a result of Western blotting analysis that used a polyclonal antibody of CP4 EPSPS protein in NK603, only CP4 EPSPS protein which was approximately 46kDa was detected, and no larger protein was detected. It was reported that the reading through of terminator commonly takes place in plants, and single protein is transferred from transcription product because of the function of static codon. Consequently, it was confirmed that the reason only CP4 EPSPS protein was found in NK603 is owing to the function of static codon preserved in the upstream of the transcription termination signal in the transcription product to read through the terminator of the insertion gene of NK603. It was concluded that this reading through does not affect the safety evaluation.

In addition, in the inserted gene of NK603 each of the 456th base and the 641st base from 5'-terminal of coding region in *cp4 epsps* gene 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 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) Seven amino acids essential for activating the EPSPS protein family are also preserved in L214P, and proline which is the 214th amino acid from N-terminal is not included in these seven amino acid residues; 2) This change of the amino acid does not affect the active site of the EPSPS protein and three-dimensional structure; and 3) As the traits of enzyme activity and immune response of L214P protein and CP4 EPSPS protein are substantially equal, the structure and function of L214P protein and CP4 EPSPS protein are substantially equal.

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.

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

It is guessed that modified Cry3Bb1 protein, NptII protein, Cry1Ab protein and CP4 EPSPS protein are expressed in the plant body of this stack maize by the function of genes which were inserted in parent lines, MON863, MON810 and NK603. As mentioned in (ii)-(1)-2-I, modified Cry3Bb1 protein and Cry1Ab protein do not possess enzyme activity as mentioned above, and function independently of the metabolic system of recipient organism. Also, it is suggested that EPSPS protein, which possesses the same functions as CP4 EPSPS protein, is not a rate-determining enzyme. In addition, Monsanto Co. examined amino acid content in the seeds of the recombinant crops in the

process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the Roundup herbicides, and confirmed that there is no difference in the aromatic amino acid content between the original non-recombinant plants and recombinant plants, thus it is considered not to affect to the metabolic pathway of recipient organism. Furthermore, Cry3Bb1 protein and Cry1Ab protein does not possess enzyme activity, and CP4 EPSPS protein has high substrate specificity. Based on the above understanding, it is not easy to consider that these three proteins would affect each other.

To confirm the above understanding in practice, regarding resistance to Coleoptera and Lepidoptera of this stack maize, the biological examination to take western corn rootworm (*Diabrotica virgifera virgifera*) and corn borer (*Ostrinia nubilalis*) as the object of pot tests was carried out, and regarding tolerance to glyphosate herbicide (Product name: Roundup-ultra) of this stack maize, Roundup-spraying tests were carried out in the US. As a result, resistance to western corn rootworm (*Diabrotica virgifera virgifera*) of this stack maize was same as that of MON863 which expresses Cry3Bb1 protein alone, and no statistically significant difference was observed (Table 5). In addition, resistance to corn borer (*Ostrinia nubilalis*) of this stack maize was same as that of MON810 which expresses Cry1Ab protein alone, and no statistically significant difference was observed (Table 6). Also, tolerance to glyphosate herbicides of this stack maize was almost same as that of NK603 which expresses CP4 EPSPS protein alone (Table 7). Based on the above results, it is suggested that the degree of expression of these proteins does not affect each other by hybridization.

Based on the above understanding, the difference between this stack maize and maize which is the taxonomic species to which the recipient organism belongs was guessed with the use of the results of individual examinations for the various characteristics of MON863, MON810 and NK603.

Table 5 Result of damage examination to western corn rootworm (*Diabrotica virgifera virgifera*), order Coleoptera by biological examination of hybrid progeny line of MON863×MON810×NK603

Hybrid progeny line	Nodal injury score (NIS)	Dispersion of NIS among sample plants
MON863×MON810×NK603	0.16	0.04
MON863	0.14	0.02
Non-recombinant plant	2.80	0.07

Table 6 Result of damage examination to corn borer (*Ostrinia nubilalis*), order Lepidoptera by biological examination of hybrid progeny line of MON863×MON810×NK603

Hybrid progeny line	Leaf damaging rate (LDR)	Dispersion of LDR among sample plants
MON863×MON810×NK603	0.20	0.13
MON810	0.11	0.11
Non-recombinant plant	5.30	0.37

【Footnote forTable 5 and Table 6】

10 plant bodies for each hybrid progeny line were cultivated in pots, and eggs of western corn rootworm (*Diabrotica virgifera virgifera*) were inoculated at the 2nd leaf stage, and 1st age larvae of corn borer (*Ostrinia nubilalis*) were inoculated at the 4th leaf stage. On the 21st day after inoculating corn borer (*Ostrinia nubilalis*), the degree of insect damage by corn borer (*Ostrinia nubilalis*) was examined in 10 grades, such as 0 (no insect damage)-9 (great insect damage: almost all parts of leaves are damaged) according to the "leaf damaging rate (LDR)". Regarding the values of the item of "Dispersion of LDR among sample plants", the values of LDR were calculated with the use of the formula to calculate standard error, to examine the dispersion of LDR in this examination.

Then, the plant bodies were taken out from the pots and soils on the plants were removed, and the insect damage by western corn rootworm (*Diabrotica virgifera virgifera*) was evaluated by using nodal injury score (NIS). This method is popularly used by various research institutions in the US to evaluate the insect damage by corn rootworm.

At first, corn rootworm eats roots which are from the lower node (usually 5th node), then eats roots which are from the upper nodes (usually 6th node, then 7th node), so the degree of insect damage is shown in the successive values between 0.00 and 3.00 in this method. For example, the score of 2.80 shows that the 5th and 6th nodes are completely damaged, and 80% of the 7th node is damaged. Regarding the values in the item of "Dispersion of NIS between each plant body among sample plants", the values of NIS were calculated with the use of the formula to calculate standard error, to examine the dispersion of NIS in this examination.

Table 7 Result of biological examination by spraying of glyphosate herbicide (Product name: Roundup-ultra) to hybrid progeny line of MON863×MON810×NK603

hybrid progeny line	Rate of growth inhibition (%)
MON863×MON810×NK603	5
NK603	5
Non-recombinant plant	100

10 plant bodies for each hybrid progeny line were cultivated in pots, and on the 13th day after cultivation, glyphosate herbicide (Product name: Roundup-ultra) was sprayed. On the 10th day after spraying glyphosate, the rate of growth inhibition (the degree of growth inhibition in 10 plant bodies) was visually observed and evaluated.

i) Regarding MON863, with the expression of the Cry3Bb1 protein, which is encoded by modified *cry3Bb1* gene in various regions of the plant, resistance to corn rootworm, which is the major pest insect of the order Coleoptera in the maize cultivation in the US was conferred, and a decrease of insect damage by western corn rootworm was confirmed. Root of maize are damaged by corn rootworm, but modified Cry3Bb1 protein constantly expresses in various regions of plant body in MON863.

Regarding MON810, with the expression of the Cry1Ab protein, which is encoded by this *cry1Ab* gene, resistance to corn borer (*Ostrinia nubilalis*), which is the major pest insect of the order Lepidoptera was conferred, and a decrease of insect damage by corn borer (*Ostrinia nubilalis*) was confirmed. All parts of maize above ground are damaged by corn borer (*Ostrinia nubilalis*), but Cry1Ab protein constantly expresses in various regions of MON810. As a result of Southern blotting analysis, *nptII* gene and *cp4 epsps* gene did not exist in MON810, and the expression of characteristics derived from these genes was not confirmed.

Regarding NK603, with the constant expression of the CP4 EPSPS protein, which is encoded by this *cp4 epsps* gene, in various regions of the plant, tolerance to glyphosate herbicide is conferred to this recombinant maize. In practice, the non-recombinant control maize died due to the influence of glyphosate herbicide, while NK603 grew normally.

Consequently, it was considered that modified Cry3Bb1 protein, NPTII protein, Cry1Ab protein and CP4 EPSPS protein express in various regions of this stack maize.

ii) The isolated field tests were carried out in Kawachi Research Farm (KRF), Monsanto Japan Limited in 2000, and in the National Institute for Agro-Environmental Science in 2002, using MON863AX, MON863BX and MON863CX, which belong to the line of MON863, as well as MON863AC, MON863BC and MON863CC as the control lines. MON863AX, MON863BX and MON863CX are the hybrid progeny lines derived from the R0 plant by different rearing processes. They keep the same genetic background because they have started from the same cross combination. It was confirmed that the recombinant maize being tested was MON863 line by Southern blotting analysis. MON863AC, MON863BC and MON863CC are hybrid progeny lines of the non-recombinant control maize hybridized in a way to attain the same genetic background with MON863AX, MON863BX and MON863CX.

The isolated field tests were carried out in the National Institute for Agro-Environmental Science in 1996 and 2001-2002 using MON810AX and MON810BX, which belong to the line of MON810, as well as MON810AC and MON810BC as the control lines. MON810AX and MON810BX are the hybrid progeny lines derived from the different rearing process from the first generation (R0) of the same plant. While, MON810AC and MON810BC are hybrid progeny lines of the non-recombinant control maize hybridized in a way to attain the same genetic background with MON810AX and MON810BX.

The isolated field tests were carried out in Kawachi Research Farm (KRF), Monsanto Japan Limited in 2000 using NK603-A and NK603-B, which belong to the line of NK603, as well as Cont-A and Cont-B as the control lines. NK603-A and NK603-B are the hybrid progeny lines derived from the different rearing process from the first generation (R0) of the same plant. While, Cont-A and Cont-B are hybrid progeny lines of the non-recombinant control maize hybridized in a way to attain the same genetic background with NK603-A and NK603-B.

(a) Morphological and growth characteristics

For MON863 and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tassel exertion, time of silking, maturation time, plant type, tiller number, total number of ears, number of effective ears, culm length, height of ear and fresh weight at harvesting time. Statistically significant difference was not observed between MON863 and the non-recombinant control maize lines in any of the characteristics.

For MON810 and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tassel exertion, time of silking, maturation time, plant type, tiller number, total number of ears, number of effective ears, culm length, height of ear and fresh weight at harvesting time. Statistically significant difference was not observed between recombinant and non-recombinant control maize lines in any of the characteristics except in culm length. Regarding culm length, statistically significant difference was found between the recombinant maize MON810BX, and the non-recombinant maize MON810BC, and the average value of culm length was 248.1 cm for MON810BX and 229.3 cm for MON810BC.

For NK603 and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tassel exertion, time of silking, maturation time, plant type, tiller number, total number of ears, number of effective ears, culm length, height of ear and fresh weight at harvesting time. Statistically significant difference was not observed between NK603 and the non-recombinant control maize lines in any of the characteristics.

Thus, there is a possibility that statistically significant difference in culm length is observed between this stack maize and the maize which is the taxonomic species to which the recipient organism belongs. However, it is considered that there is no difference in other morphological and growth characteristics between this stack maize and maize which is the taxonomic species to which the recipient organism belongs.

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

Sensitivity to low temperature (temperature of 4°C) of the seedlings of MON863 and the non-recombinant control maize was evaluated. Almost all died on the 14th day after exposure to low temperature, and no difference was observed between MON863 and the non-recombinant control maize.

Sensitivity to low temperature (maximum temperature of 12-14℃, and minimum temperature of 2℃) of the seedling of MON810 and the non-recombinant control maize was evaluated. All leaves withered on the 21st day after exposure to low temperature, and no difference was observed between MON810 and the non-recombinant control maize.

Sensitivity to low temperature (temperature of 4°C) of the seedlings of NK603 and the non-recombinant control maize was evaluated. Almost all died on the 14th day after exposure to low temperature, and no difference was observed between NK603 and the non-recombinant control maize.

Consequently, regarding chilling-tolerance, it is considered that there is no difference between this stack maize and maize which is the taxonomic species to which the recipient organism belongs.

(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 regrow and propagate vegetatively, or produce seeds. It was observed that dying started after ripening at the end of the tests carried out in the isolated field for parent lines, MON863, MON810 and NK603 in practice. Based on the above, an overwintering test for the matured plant of this recombinant maize was not carried out.

(d) Fertility and size of the pollen

To examine the fertility (maturity) and size of the pollens of MON863 and the non-recombinant control maize, pollens were stained with potassium iodine solution and observed under a microscope. As a result, no difference was observed between MON863 and the non-recombinant control maize.

To examine the fertility (maturity) and size of the pollens of MON810 and the non-recombinant control maize, pollens were stained with 0.1% neutral red solution and potassium iodine solution, and observed under a microscope. As a result, no difference was observed between MON810 and the non-recombinant control maize.

To examine the fertility (maturity) and size of the pollens of NK603 and the non-recombinant control maize, pollens were stained with potassium iodine solution and observed under a microscope. As a result, no difference was observed between NK603 and the non-recombinant control maize.

Consequently, regarding fertility and size of pollen, it is considered that there is no difference between this stack maize and maize which is the taxonomic species to which the recipient organism belongs.

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

Regarding the production of the seed of MON863, ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight after sib-mating were examined. As a result, no statistically significant difference was observed between MON863 and the non-recombinant control maize in any of the characteristics examined.

Regarding the production of the seed of MON810, ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight after sib-mating were examined. As a result, no statistically significant difference was observed between MON810 and the non-recombinant control maize in any of the characteristics examined.

Regarding the production of the seed of NK603, ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight after sib-mating were examined. As a result, no statistically significant difference was observed between NK603 and the non-recombinant control maize in any of the characteristics examined except in 100-kernel weight. Regarding 100-kernel weight, statistically significant difference was found between NK603-B and the non-recombinant control maize, Cont-B, and the average value of 100-kernel weight was 33.6g for NK603-B and 35.1g for Cont-B. Meanwhile, no statistically significant difference was observed between NK603-A and the non-recombiant control maize, Cont-A.

Regarding shedding habit of the seed, shedding habit was not observed in the natural condition, since the ears of MON863, MON810 and NK603, and the non-recombinant control maize were covered with bracts at the time of harvesting.

Regarding germination rate on the 10th day of sowing of harvested seeds of MON863, and that on the 4th day of sowing of harvested seeds of MON810, and that on the 10th day of sowing of harvested seeds of NK603, germination rate of the recombinant maize and the non-recombinant control maize for each group was high, and no difference was observed between them, and no dormancy of the seeds was examined.

Consequently, regarding production, shedding habit and dormancy of the seed, it is considered that there is no difference between this stack maize and maize which is the taxonomic species to which the recipient organism belongs, except in 100-kernel weight.

(f) Crossability

Crossability test was not performed for parent lines, MON863, MON810 and NK603, since no wild relatives that can be hybridized grow in Japan.

(g) Productivity of harmful substances

Plow-in test, succeeding crop test and soil microflora tests were performed between MON863 and the non-recombinant control maize, MON810 and the non-recombinant control maize, and NK603 and the non-recombinant control

maize. Statistically significant difference was not observed in any of the items between MON863 and the non-recombinant control maize, MON810 and the non-recombinant control maize, and NK603 and the non-recombinant control maize.

Thus, it is considered that there is no difference in productivity of harmful substances between this stack maize and maize which is the taxonomic species to which the recipient organism belongs.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.

(2) Information obtained from Use abroad

In the US, to prevent the incidence of pest insects which show the resistance to *B.t.* protein, buffer zone is set up at the time of cultivation of the recombinant maize which expresses *B.t.* protein. For the buffer zone of this stack maize cultivation, the maize which does not produce *B.t.* protein would be cultivated in the 20% of total area of this stack maize cultivation, as well as maize resistant to Lepidoptera and Coleoptera (MON810×MON863).

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

This stack maize was produced by hybridization using the traditional hybridization breeding method of maize resistant to Coleoptera (MON-00863-5), maize resistant to Lepidoptera (MON-00810-6), and maize tolerant to glyphosate herbicide (MON-00603-6). The parent lines abovewere already judged to cause no risk of Adverse Effect on Biological Diversity when they are used in accordance with Type I Use Regulation, which is the same as this stack maize.

It is suggested that Cry3Bb1 protein which is encoded by the modified *cry3Bb1* gene derived from MON-00863-5, and Cry1Ab protein, which is encoded by the *cry1Ab* gene derived from MON-00810-6 do not have enzyme activity, and CP4 EPSPS protein, which is encoded by *cp4 epsps* gene derived from MON-00603-6 has high substrate specificity.

In addition, resistance to Coleoptera and Lepidoptera of this stack maize was examined by biological examination with the use of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and European corn borer (*Ostrinia nubilalis*), and tolerance to glyphosate herbicide was examined by a glyphosate-spraying test. As a result, significant difference was not confirmed between this stack maize and the parent line.

Based on the above understanding, regarding this stack maize, it is considered that there is no change of significant characteristics except having the characteristics of parent line.

1. Competitiveness

This stack maize has resistance to Coleoptera derived form MON-00863-5, resistance to Lepidoptera derived from MON-00810-6, and tolerance to glyphosate herbicide derived from MON-00603-6. However, it is not considered that the glyphosate exerts pressure for selection under a natural environment, and also the insect damage by Coleoptera and Lepidoptera is not the main factor to inhibit the growth of maize under a natural environment in Japan. Thus, it is considered that these characteristics are not the characteristics to raise dominance in competition. Also, it was suggested that even if Cry3Aa protein and Cry1Ac protein are given at the same time, insecticidal activity would change neither for target insects nor for non-target insects. Thus, this stack maize is not considered a risk to become dominance in competition as compared with parent line. Based on the above understanding, there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness.

2. Productivity of harmful substances

This stack maize has the characteristics to produce Cry3Bb1 protein derived from MON-00863-5, to produce Cry1Ab protein derived from MON-00810-6, and to produce CP4 EPSPS protein derived from MON-00603-6. However, 1) Cry3Bb1 protein and Cry1Ab protein have different insecticidal spectrum, 2) it was suggested that even if target insects and non-target insects are given Cry3Aa protein and Cry1Ac protein at the same time, insecticidal activity for both groups of insects would not interfere, and 3) it is considered that CP4 EPSPS protein possesses the same functions as plant EPSPS, except that the activity of CP4 EPSPS is not inhibited by glyphosate. It is considered not to possess the characteristic to raise productivity of harmful substances. Thus, it is considered that the productivity of harmful substances of this stack maize would not become higher than that of parent line. Based on the above understanding, it was concluded that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances.

3. Crossability

In Japan, the growth of wild species that can be hybridized with maize in natural environment has not been reported.

Based on the above understanding, it was concluded that no wild species can be specified as having some effects, and there is no risk of Adverse Effect on Biological Diversity attributable to crossability.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

Consequently, it was judged that there is no risk of Adverse Effect on Biological Diversity in Japan attributable to the use of this stack maize for provision as food, for provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.