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

Monsanto Japan Limited

Ginza Sanno Bldg. 8F
4-10-10, Ginza, Chuo-ku, Tokyo

Approved Type 1 Use Regulation

Name of the type of Living Modified Organism	Maize tolerant to glyphosate herbicide and resistant to Coleoptera (*cry3Bb1, cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (MON863 ×NK603, OECD UI: MON-ØØ863-5×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

I. Information concerning preparation of living modified organisms

The cross progeny line (*cry3Bb1, cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (OECD UI: MON-ØØ863-5×MON-ØØ6Ø3-6) (hereinafter referred to as "this stack maize") was from the crossing of the following two recombinant maize with the use of traditional cross-breeding method. The two recombinant maize are, i) Maize resistant to Coleoptera (*cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis) (MON863, OECD UI: MON-ØØ863-5) (hereinafter referred to as "MON863"), and ii) Maize tolerant to glyphosate herbicide (*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 both characteristics of these two recombinant maize lines, MON863 and NK603. The information concerning preparation of MON863 and NK603 are explained individually in the following sections.

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of MON863 are shown in Table 1 (p6). In MON863, *cry3Bb1* gene which was the modified gene of the wild-type *cry3Bb1* gene was inserted, and 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 NK603 are shown in Table 2 (p8).

(2) Functions of component elements

Functions of component elements which were used for the development of MON863 are shown in Table 1 (p6).

Functions of component elements which were used for the development of NK603 are shown in Table 2 (p8).

【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 Cry3Bb1 protein 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*) and corn root worm, 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 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. Results showed 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.

【*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 5-enol-pyruvylshikimate-3-phosphate (EPSP) 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, canola, cotton and maize) that are tolerant to the Roundup herbicides, and 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 allergens, the CP4 EPSPS protein was compared with allergens in the database. As a result, the CP4 EPSPS protein did not share structurally related homologous sequences with any of the known allergens examined.

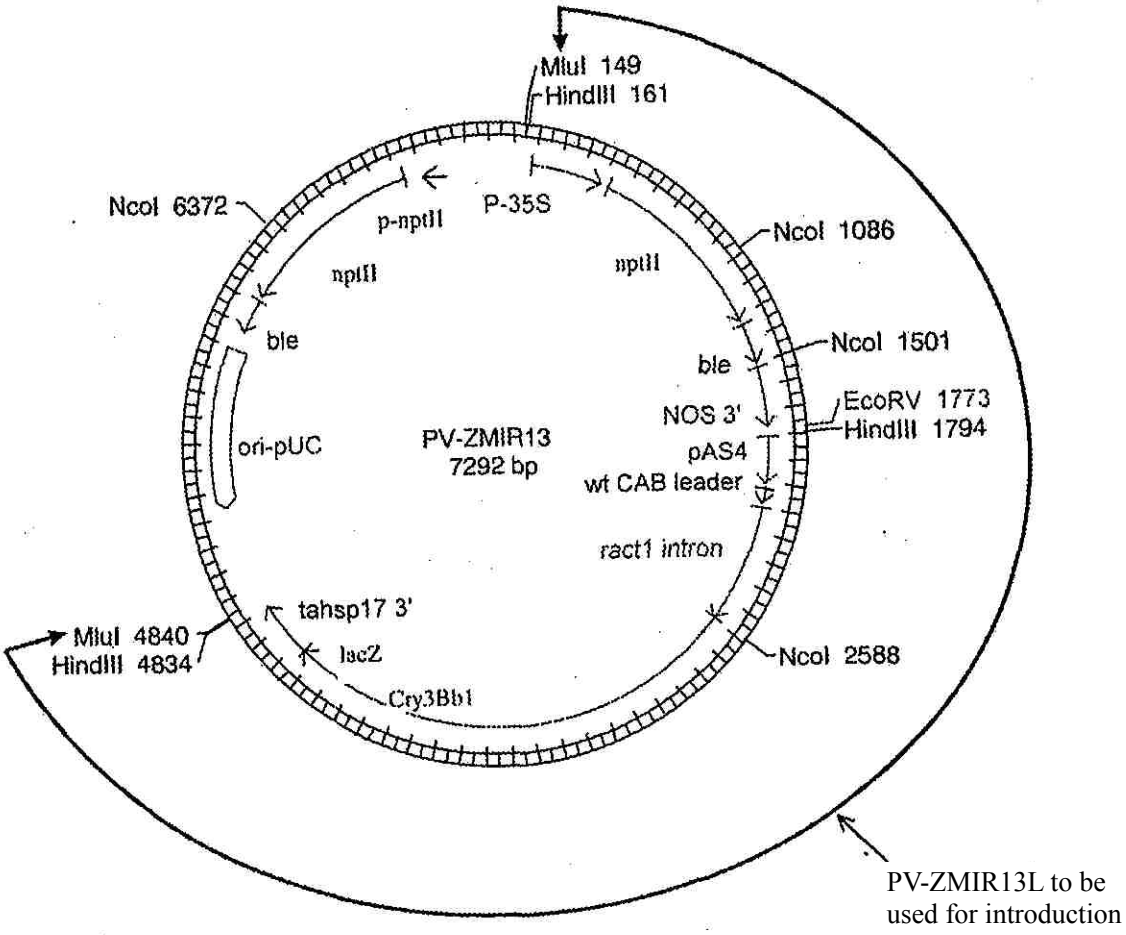

Figure 1 Plasmid PV-ZMIR13 used for developing MON863

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)that 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 p2.
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 microorganisms. It encodes 50 amino acids at the N-terminal region of the Ble protein, 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.

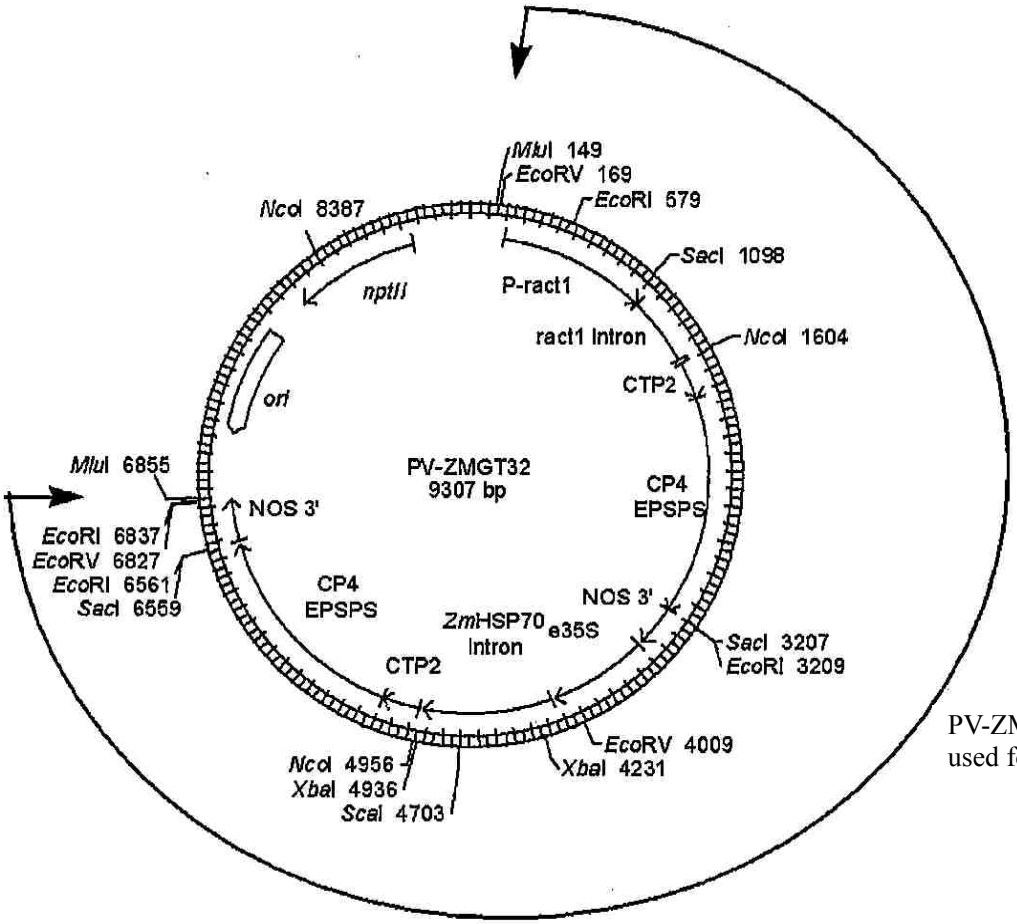

Figure 2 Plasmid PV-ZMGT32L used for developing NK603

Table 2 Component elements of PV-ZMGT32L, which were used for the production of NK603, 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 p3-4.
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 region from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 Intron	Intron of heat shock protein gene from maize. ZmHsp70 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-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4. Details of functions are shown on p3-4.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.

2. Information concerning vector

(1) Name and origin

The vectors used for the production of MON863 and NK603 are plasmid pUC119 from *Escherichia coli*.

(2) Properties

The vectors for the production of MON863 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 infectivity of this vector is not known.

3. Method of preparing living modified organisms

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

Refer to Table 1 (p6) and Table 2 (p8).

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

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

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

(3) 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 linear DNA fragment 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, Labor and Welfare.

February, 2002: 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 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, Labor 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×NK603】

This stack maize was developed from the crossing of inbred lines derived from MON863 and NK603, with the use of traditional cross-breeding method.

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 cells of 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 resistance to Coleoptera was stably expressed in multiple generations by biological examination.

【State of existence of nucleic acid transferred in cells of 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, are 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 in 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 by Southern blotting analyses 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 that 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 a static codon preserved in the upstream of the transcription termination signal in the transcription product to read through the terminator of the inserted gene in 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 induced by 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 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 has been indirectly demonstrated that the 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 and NK603. As mentioned in (2)-1-I, modified Cry3Bb1 protein does not possess enzyme activity as mentioned above, and functions 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 the shikimate pathway. 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, canola, 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 does not possess enzyme activity, and CP4 EPSPS protein has high substrate specificity. Based on the above understanding, there is no reason to suspect that these two proteins would affect each other.

To confirm the above understanding in practice, regarding resistance to Coleoptera of this stack maize, the biological examination to take corn rootworm 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 corn rootworm of this stack maize was same as that of MON863 which expresses Cry3Bb1 protein alone. Also, tolerance to glyphosate herbicides of this stack maize was almost same as that of NK603 which expresses CP4 EPSPS protein alone. Based on the above results, it is suggested that the degree of expression of these proteins does not affect each other by crossing.

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 determined with the use of the results of individual examinations for the various characteristics of MON863 and NK603.

Table 3 Result of damage degree examination to corn rootworm, order Coleoptera by biological examination of hybrid progeny line of MON863×NK603

Hybrid progeny line	Nodal injury score (NIS)
MON863×NK603	0.18
MON863	0.14
Non-recombinant plant	2.80

Ten plant bodies for each hybrid progeny line were cultivated in pots, and eggs of corn rootworm were inoculated at the 2nd leaf stage. On the 21st day of cultivation, the plant bodies were taken out from the pots and soils on the plants were carefully removed, then the

degree of insect damage by corn rootworm was examined 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 feed on crown roots from the lower node (usually 5th node), then feed on crown 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 such as 0.00 -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.

Table 4 Result of biological examination by spraying of glyphosate herbicide (Product name: Roundup) to hybrid progeny line of MON863×NK603

Hybrid progeny line	Rate of chlorosis (%)	Rate of malformation (%)	Rate of growth retardation (%)
MON863×NK603	5	0	0
NK603	7	3	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 chlorosis (the degree of all yellowing part in 10 plant bodies), the rate of malformation (the degree of all malformed leaves including shrinking leaves in total leaf-area of 10 plant bodies), and the rate of growth retardation (the degree of growth retardation in 10 plant bodies) were visually observed and evaluated.

（1）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 corn rootworm was confirmed. Root systems of maize are damaged by corn rootworm, but modified Cry3Bb1 protein constantly expresses in various regions of plant body in MON863.

In addition, it was confirmed that *nptII* gene exists in MON863, and NPTII protein also expresses constantly in various parts in plant bodies. Regarding *nptII* gene, there are many examples of use, and still there is no report that the use of *nptII* gene causes any effects.

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 and CP4 EPSPS protein express in various regions of this stack maize.

(2) 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 F1 hybrid lines to have the same genetic background which are derived from the different rearing processes starting from the first generation (R0) of the MON863. It was confirmed that the recombinant maize being tested was MON863 line by Southern blotting analysis. MON863AC, MON863BC and MON863CC are F1 hybrid lines of the non-recombinant control maize crossed in a specific way to attain the same genetic background with MON863AX, MON863BX and MON863CX.

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 F1 hybrid lines derived from the different rearing processes from the first generation (R0) of the NK603. While, Cont-A and Cont-B are F1 hybrid lines of the non-recombinant control maize crossed 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, and 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 differences were not observed between MON863 and the non-recombinant control maize lines in any of the characteristics. In addition, statistically significant differences were not observed between NK603 and the non-recombinant control maize lines in any of the characteristics.

Thus, it is considered that there is no difference in 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 (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 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 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 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-recombinant control maize, Cont-A.

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

Regarding germination rate on the 10th day after sowing of harvested seeds of MON863,

and that on the 10th day after sowing of harvested seeds of NK603, 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 and NK603, since no wild relatives that can be crossed 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, 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, 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.

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

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

This stack maize was produced by crossing of Maize resistant to Coleoptera (MON-00863-5) and Maize tolerant to glyphosate herbicide (MON-00603-6). Regarding the parent lines on the above, it was already judged there is no risk that the use of each of this recombinant maize, in accordance with Type I Use Regulation in the same way as this stack maize is presumed, causes Adverse Effect on Biological Diversity.

It is suggested that Cry3Bb1 protein which is encoded by modified *cry3Bb1* gene (gene resistant to Coleoptera) derived from MON-00863-5 does not have enzyme activity, and CP4 EPSPS protein, which is encoded by *cp4 epsps* gene (gene tolerant to glyphosate herbicide) derived from MON-00603-6 has high substrate specificity. Thus, it is considered that these two proteins have no interaction.

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

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 lines.

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

(1) Competitiveness

This stack maize has resistance to Coleoptera derived form MON-00863-5, 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 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 competitiveness. Thus, this stack maize is not considered to become dominant in competition compared with its parent lines. 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 productivity of Cry3Bb1 protein derived from MON-00863-5, and the productivity of CP4 EPSPS protein derived from MON-00603-6. Cry3Bb1 protein possesses insecticidal activity against order Coleoptera, but it is considered that CP4 EPSPS protein possesses the same functions as EPSPS which is originally in plant body except the traits not to receive activity inhibition by glyphosate, and 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 crossed with maize in natural environment has not been reported.

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

2. Conclusion

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stack 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 valid.