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 tolerant to glyphosate herbicide and resistant to Coleoptera and Lepidoptera (*cp4 epsps, cry3Bb1, cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (MON88017×MON810, OECD UI: MON-88Ø17-3×MON-ØØ81Ø-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 maize tolerant to glyphosate herbicide and resistant to Coleoptera and Lepidoptera (cp4 epsps, cry3Bb1, cry1Ab, Zea mays subsp. mays (L.) Iltis) (OECD UI: MON-88Ø17-3×MON-ØØ81Ø-6) (hereinafter referred to as "this stack maize") was from the crossing of the following two recombinant maize with the use of traditional crossbreeding method. The two recombinant maize are; i) Maize tolerant to glyphosate herbicide and resistant to Coleoptera (cp4 epsps, cry3Bb1, Zea mays subsp. mays (L.) Iltis) (MON88017, OECD UI: MON-88Ø17-3) (hereinafter referred to as "MON88017"), and ii) Maize resistant to Lepidoptera (cry1Ab, Zea mays subsp. mays (L.) Iltis) (MON810, OECD UI: MON-ØØ81Ø-6) (hereinafter referred to as "MON810"). Therefore, this stack maize possesses the both characteristics of these two parent recombinant maize lines, MON88017 and MON810. Then, the information concerning preparation of MON88017 and MON810 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 MON88017 are shown in Figure 1 and Table 1. In addition, the nucleotide sequence of component elements is included in Annex 1 of the Biological Diversity Risk Assessment Report for MON88017. In this recombinant maize, the *cp4 epsps* gene, which was the modified gene of the wild-type *cp4 epsps* gene, and the *cry3Bb1* gene, which was the modified gene of the wild-type *cry3Bb1* gene, were inserted, and hereinafter these genes are referred to as "modified *cp4 epsps* gene" and "modified *cry3Bb1* gene" and the proteins being expressed are referred to as "modified Cry3Bb1 protein" and “modified CP4 EPSPS protein”.

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

The nucleotide sequence of the *cry1Ab* gene is included in the Annex 1 of the Biological Diversity Risk Assessment Report for MON810.

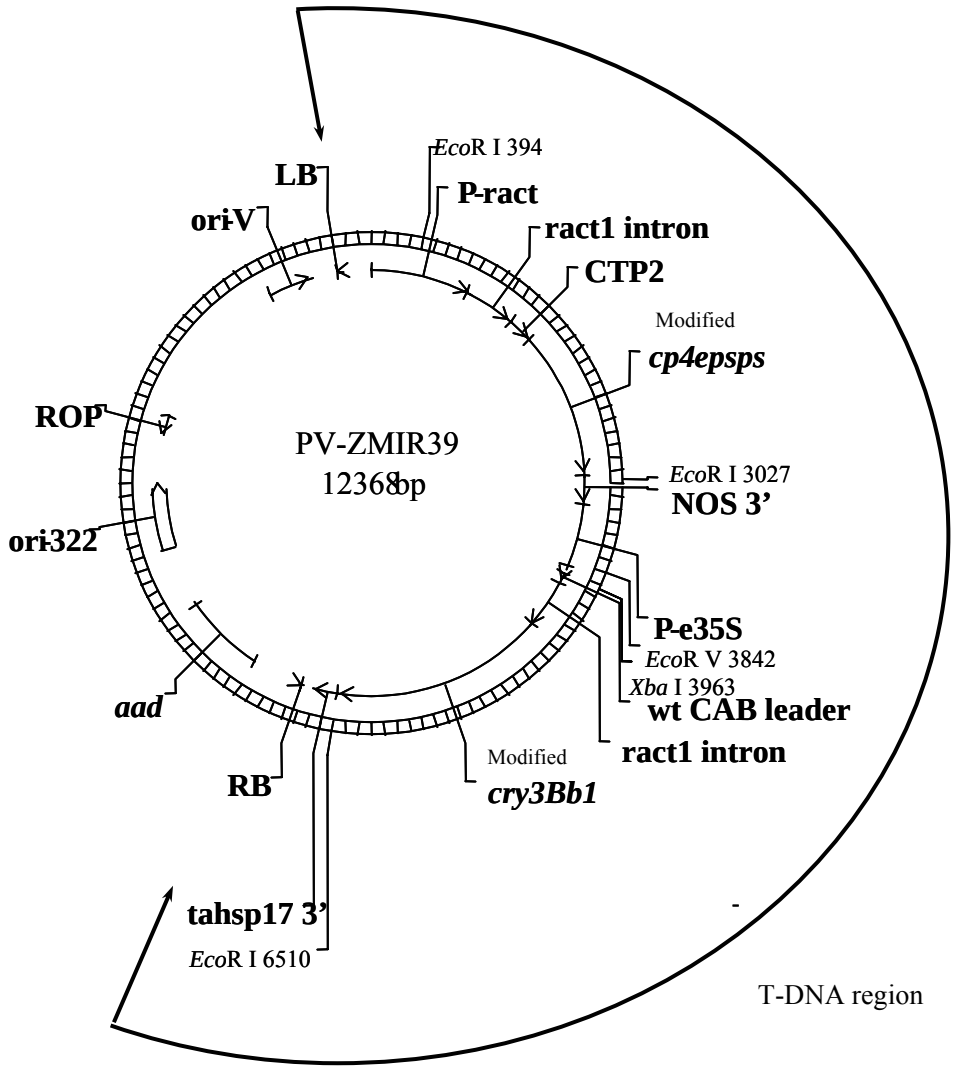

Figure 1 Plasmid PV-ZMIR39 used for developing MON88017[1]

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

Table 1 Component elements of the plasmid PV-ZMIR39, which were used for the production of MON88017, and their origins and functions[2]

Component elements	Origin and function
Modified *cp4 epsps* gene cassette	
P-ract	Promoter region of actin 1 gene derived from rice. It makes target genes expressed (Reference 9).
ract1 intron	Intron of rice actin gene. It makes target genes expressed by enhancing splicing (Reference 12).
CTP2	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 13). Transfers the CP4 EPSPS protein to the chloroplast, where aromatic amino acids are synthesized.
Modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4 strain (Reference 14; Reference 15). A modification is given to the nucleotide sequence to enhance its expression in plants without changing the function of the CP4 EPSPS protein. Only a single modification is introduced to the amino acid sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine. Details of functions are shown on page 8-9.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation (Reference 16).
Modified *cry3Bb1* gene cassette	
P-e35S	A promoter from the cauliflower mosaic virus (CaMV) (Reference 17). It has the function to express introduced genes in all tissues constantly.
wt CAB leader	5'-terminal untranslated region of wheat chlorophyll a/b binding protein. Enhances the expression of target gene (Reference 18).
ract1 intron	Intron of rice actin gene. It makes target genes expressed by enhancing splicing (Reference 12).
Modified *cry3Bb1*	The gene which encodes modified Cry3Bb1 protein of *Bacillus thuringiensis* (Reference 19). It differs from the modified Cry3Bb1 protein in MON863 at one site in the amino acid sequence; the 166th amino acid sequence is aspartic acid in MON88017 whereas glycine in MON863. Details of its functions are described on page 9-10.
tahsp 17 3'	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (Reference 20).

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

Table 1 Component elements of the plasmid PV-ZMIR39, which were used for the production of MON88017, and their origins and functions (Continued)[3]

Component elements other than T-DNA range	
RB	A DNA sequence of right border sequence of nopaline type T-DNA derived from Ti plasmid pTiT37 . Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome (Reference 21).
aad	The gene encoding the Tn7 adenyltransferase (AAD) derived from *Staphylococcus aureus.* Confers resistance to spectinomycin or streptomycin (Reference 22).
ori-322	The replication origin isolated from pBR322. Permits autonomous replication of vectors in *E.coli* (Reference 23).
ROP	A coding sequence to repress primer protein to maintain the number of copies of plasmids in *E. coli* (Reference 24).
ori-V	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* (Reference 25).
LB	A DNA sequence of left border sequence derived from Ti plasmid pTi15955. Defines the terminal of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome (Reference 26).

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

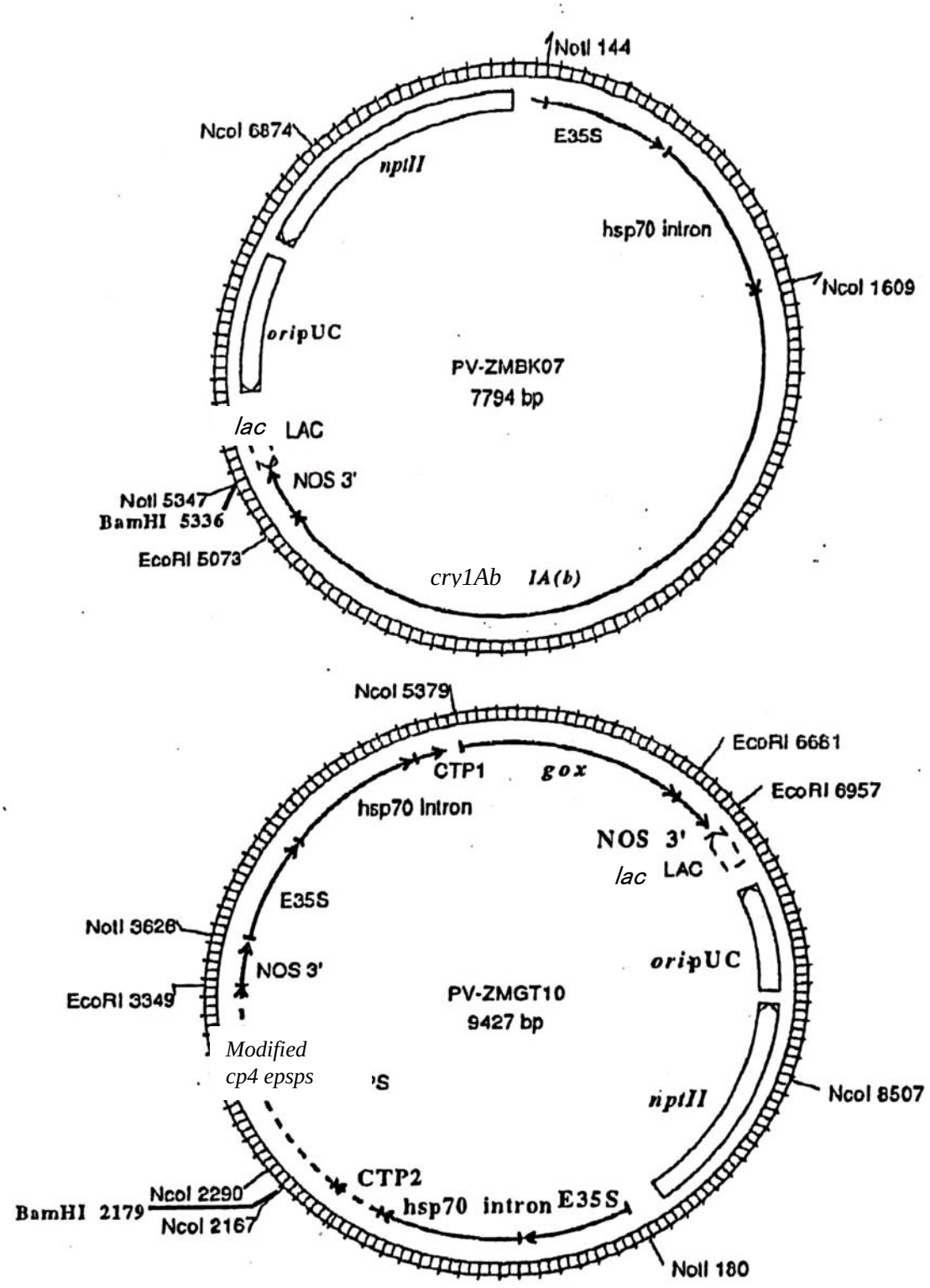

Figure 2 Plasmid PV-ZMBK07 and PV-ZMGT10 used for developing MON810 [4]

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

Table 2 Component elements of the plasmids PV-ZMBK07 and PV-ZMGT10, which were used for the production of MON810, and their origins and functions[5]

Component elements	Origin and function
cry1Ab gene cassette	
E35S	Contains 35S promoter (Reference 17) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 27).
hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 28).
cry1Ab	The gene which encodes Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain in the soil (Reference 29). The detail of its function was described in p10-11.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 16).
Modified *cp4 epsps* gene cassette（As a result of inserted gene analysis, this was not inserted into MON810）	
E35S	Contains 35S promoter (Reference 17) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 27).
hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 28)
CTP2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene (Reference 13). Transfers target proteins from cytoplasm to chloroplast.
Modified *cp4 epsps*	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4 strain (Reference 14; Reference 15).
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 16).
Gox gene cassette （As a result of inserted gene analysis, this was not inserted into MON810）	
E35S	Contains 35S promoter (Reference 17) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 27).
hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 28).
CTP 1	N-terminal chloroplast transit peptide of the small subunit 1A of rubisco gene from *A. thaliana* (Reference 30). Transfers target protein from cytoplasm to chloroplast, where aromatic amino acids are synthesized. .
gox	A sequence that encodes the C-terminal of the variant v247 derived from glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA (Reference 31). GOX protein degrades glyphosate.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 16).
Component elements other than above (common to PV-ZMBK07 and PV-ZMGT10)（As a result of inserted gene analysis, this was not inserted into MON810）	
lac	Partial coding sequence for β-D-galactosidase or Lac protein (Reference 32). It degrades β-galactoside to form β-galactose.
ori-pUC	A segment containing replication origin for *E. coli* plasmid pUC (Reference 33). 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 bacteria (Reference 34).

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

(2) Functions of component elements

Functions of component elements of donor nucleic acid that was used for the development of MON88017 are shown in Table 1. Functions of component elements of donor nucleic acid that was used for the development of MON810 are shown in Table 2.

[Modified *cp4 epsps* gene]

1) 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 (Reference 35; Reference 36). As a result, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. The modified *cp4 epsps* gene expresses the modified CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The activity of the modified CP4 EPSPS protein that is produced by the modified *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus, the recombinant plants that express this protein have normal functions of shikimate synthesis and can grow.

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 (Reference 37). The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants (Reference 38; Reference 36). This pathway is regulated by 3-deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway, but 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) which is catalyzed by the EPSPS are inhibited or suppressed by metabolic intermediates or end products of this pathway (Reference 39; Reference 40). This suggests that EPSPS is not a rate-determining enzyme in this pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In 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 (Reference 41). 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 have been commercialized with the feature of tolerance 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 (Reference 42; Reference 43; Reference 44; Reference 45). These facts support the theory 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 phosphate (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 46), and is known to specifically react with these substrates (Reference 47). 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.

2) In order to investigate whether the modified CP4 EPSPS protein shares functionally important amino acid sequence with known contact allergens, the CP4 EPSPS protein was compared with contact allergens in the database (GenBank, EMBL, PIR, NRL3D, SwissProt). As a result, the CP4 EPSPS protein did not share structurally related homologous sequences with any of the known allergens examined.

[Modified *cry3Bb1* gene]

1) 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 USA. This insect damages the roots of maize. *B.t.* proteins which are produced by the bacterium *B.t.* including the modified Cry3Bb1 protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pores, which leads to the inhibition of the digestive process and results in the insecticidal activity (Reference 48; Reference 49; Reference 50).

The insecticidal spectrum of the Cry3Bb1 protein is extremely narrow, and the Cry3Bb1 protein shows the insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata*) (hereinafter referred to as "CPB") and CRW, which are respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). It was indicated by literature searching that there was no report that related species of the same genera with these two insects have ever inhabited in Japan (Annex 3 of Biological Diversity Risk Assessment Report for MON88017; Reference 51).

Compared with the wild-type Cry3Bb1 protein, the modified Cry3Bb1 protein has 98% or more homology (Annex 2 of Biological Diversity Risk Assessment Report for MON88017). The modified Cry3Bb1 protein in the Coleoptera-resistant maize MON863 and the modified Cry3Bb1 protein in MON88017 differ from each other in the amino acid sequence at one site; the 166th amino acid sequence is glycine in MON863 whereas aspartic acid in this recombinant maize. In practice, the insecticidal efficiency is examined in the field with the use of modified Cry3Bb1 protein of MON88017.

2) In order to investigate whether the modified Cry3Bb1 protein shares functionally important amino acid sequences with known contact allergens, the modified Cry3Bb1 protein was compared

with the contact allergens in the database (GenBank, EMBL, PIR, NRL3D, SwissProt). As a result, the modified Cry3Bb1 protein did not share structurally related sequences with known allergens.

[*cry1Ab* gene]

1) The *cry1Ab* gene, the target gene to confer Lepidoptera resistance to MON810, is derived from *Bacillus thuringiensis* 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 European corn borer (*Ostrinia nubilalis*), which is one of the major pest insects of order Lepidoptera to maize cultivation in the USA (Frankenhuzen, 1993). The European corn borer damages the entire plant body above ground. *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 leads to the inhibition of the digestive process and results in the insecticidal activity (Reference 48; Reference 50). *B.t.* proteins do not possess enzyme activity and function independently from the metabolic system of the recipient organism.

The Cry1Ab protein has an insecticidal activity only against Lepidoptera, and not against other insects than Lepidoptera. In addition, the Cry1Ab protein is known to show an insecticidal activity against the following major Lepidoptera pests that affect maize cultivation in the USA: 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*) (Reference 52; Reference 53; Reference 54; Reference 55; Reference 56). Among them, *O. furnacalis*, which belongs to the same family as *O. nubilalis*, is known to be major Lepidoptera pest that affects maize cultivation in Japan (Reference 57).

2) In order to investigate whether the Cry1Ab protein shares functionally important amino acid sequences with known allergens, the Cry1 Ab protein was compared with the allergens in the database (GenBank, EMBL, PIR, NRL3D, SwissProt). As a result, the Cry1 Ab protein did not share structurally related sequences with known allergens.

[Modified *cry3Bb1* gene+*cry1Ab* gene]

It is known based on the findings about *B.t.*-based pesticides which have been used as biological agrochemicals since 1960 that the Cry3 family to which the modified Cry3Bb1 protein belongs and the Cry1A family to which the Cry1Ab protein belongs possess specific insecticidal activities against larvae of the insects classified into different order of insects Coleoptera and Lepidoptera, respectively.

In addition, the both proteins possess the similar mechanism of action to inhibit the digestive process of the target insects by binding to the specific receptors on the midgut epithelium. However, pH values in the midgut of the insects of order Lepidoptera and Coleoptera are found alkaline (pH

10.5 to pH 11.0) and neutral (pH 6.5 to pH 7.0), respectively. Therefore the both proteins exhibit the insecticidal activities under different chemical conditions (Reference 58).

Moreover, according to MacIntosh *et.al*, it is reported that non-target insects, which do not show sensitivity against Cry1Ac protein belonging to the same Cry1A family similarly as Cry1Ab protein, and Cry3Aa protein belonging to the same Cry3 family similarly as the modified Cry3Bb1 protein, remain unsusceptible even if treated with a mixture of the *B.t.* proteins belonging to the two different families, as demonstrated in Table 3, and thus free from any synergistic effects due to the simultaneous exposure to Cry1Ac protein and Cry3A protein (Reference 59). Consequently, in this stack maize, a cross progeny line developed from the crossing with the use of traditional crossbreeding method, Cry1Ab protein and modified Cry3Bb1 protein derived from the parent lines are expressed, though it was considered to be extremely low that they exhibit the insecticidal activities synergistically against the non-target insects.

Table 3 Investigational results of insecticidal activities of Cry1 and Cry3 proteins against different species of insects (based on the research paper by 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- Cry1Ab protein was given to the target insects in a controlled concentration of 50μg/mL in artificial feed.

2- Cry1Ac protein was given to the target insect in a controlled concentration of 50μg/mL in artificial feed.

3- Cry3A protein was given to the target insects in a controlled concentration of 500μg/mL in artificial feed.

4- Cry1Ac protein was given in a controlled concentration of 50μg/mL in artificial feed.
Cry3A protein was given to the target insects in a controlled concentration of 500μg/mL.

＋ The test fields showing a mortality of 25% or more of larvae tested

－ Fields showing a mortality of 25% or less of larvae tested

2. Information concerning vector

(1) Name and origin

The plasmid vector used for the production of MON88017 is assembled from plasmids including pBR322, which is a synthetic plasmid from *Escherichia coli* (Reference 23). The plasmid vector used for the production of MON810 is assembled from plasmids including pUC119, which is a synthetic plasmid from *Escherichia coli* (Reference 33).

(2) Properties

The total number of base pairs of PV-ZMIR39 used for the production of MON88017 is 12,368 bp. The vector PV-ZMIR39 possesses the *aad* gene to confer the resistance to spectinomycin or streptomycin as a selective marker for construction vector in *E.coli* (Reference 22). The infectivity of this vector is not known.

The total number of base pairs of PV-ZMBK07 and PV-ZMGT10 used for the production of MON810 is 7,794 bp and 9,427 bp, respectively. These vectors contain a kanamycin/neomycin-resistant gene (*nptII* gene) derived from *E.coli* transposon Tn5 as the selective marker gene for the construction vector. The infectivity of these vectors is not known.

3. Method of preparing living modified organisms

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

For the production of MON88017, based on pBR322 which contains the above-mentioned *aad* gene, the plasmid vector PV-ZMIR39 was constructed. T-DNA region in this vector transferred in the recipient organism is constructed with the modified *cp4 epsps* gene cassette [P-ract]-[ract1 intron]-[CTP2]-[*cp4 epsps*]-[NOS 3'] and modified *cry3Bb1* gene cassette [P-e35S]-[wt CAB leader]-[ract1 intron]-[*cry3Bb1*]-[tahsp17 3']. Map of the plasmid PV-ZMIR39 is shown in Figure 1 on Page 3.

For the production of MON810, two plasmids were constructed based on a basic vector derived from pUC119 containing the above-mentioned *nptII* gene: i) the plasmid PV-ZMBK07 to which the *cry1Ab* gene cassette ([E35S]-[hsp70 intron]-[*cry1Ab*]-[NOS3']) is connected, and ii) the plasmid PV-ZMGT10 to which the modified *cp4 epsps* gene cassette ([E35S]-[hsp70 intron]-[CTP2]-[*cp4 epsps*]-[NOS 3']) and *gox* gene cassette ([E35S]-[hsp70 intron]-[CTP1]-[*gox*]-[NOS 3']) are connected. These two plasmids were used as vectors to create MON810. Map of the plasmids PV-ZMBK07 and PV-ZMGT10 is shown in Figure 2.

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

In the production of MON88017, the T-DNA region of the plasmid vector PV-ZMIR39 was introduced by the *Agrobacterium* method to the variety A x HiII that is classified into dent type.

In the production of MON810, the mixture of plasmids PV-ZMBK07 and PV-ZMGT10 was introduced by particle gun bombardment to the F2 generation of the maize inbred line A188 X B73 that is classified into dent type.

(3) Processes of rearing of living modified organisms

[Process of rearing of MON88017]

1) The development of MON88017 was started in 1999. T-DNA region of plasmid vector PV-ZMIR39 was introduced by *Agrobacterium* method to A x HiII, and then the transformed callus was selected on a glyphosate-containing medium. From the selected callus, the regenerated plant was obtained and the expression of the modified Cry3Bb1 protein was analyzed by ELISA method, and the strain invested with tolerance to glyphosate herbicide and resistance to pest insect was selected.

2) In this process, the transformed callus was grown on a tissue culture media containing carbenicylin, and then the transformed callus was removed to the culture media for regenerating which does not contain these antibiotics, to confirm the absence of remaining Agrobacterium (Reference 60).

3) Field experiments were carried out at 169 field sites in total from 2000 to 2001. The strain for the final commercial cultivation was selected, and its environmental safety was evaluated (Process of rearing of the strain used in the field tests is shown in Figure 3).

The situation of approval of MON88017 in Japan is the following.

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.

August, 2004: Assessment by the Ministry of Agriculture, Forestry and Fisheries and Ministry of Environment for conformity with the Type 1 Use Regulations in the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Law) (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) was completed.

[Process of rearing of MON810]

1) The callus to which PV-ZMBK07 and PV-ZMGT10 were introduced was grown on a tissue culture media 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. In practice, it was confirmed as a result of Southern blotting analysis of the genes introduced into MON810 that there exist no expression cassettes of *nptII* gene, modified *cp4 epsps* gene and *gox* gene (Annex 2 of Biological Diversity Risk Assessment Report for MON810). The achieved selection by glyphosate even without introduction of modified *cp4 epsps* gene to MON810 was considered due to the segregation of the introduced genes in the subsequent generation (BC0F1) to the regenerated individuals (R0). However, definite reasons could not be identified because MON810 was selected as pest-resistant maize and glyphosate assay and Southern blotting analysis were not carried out in the subsequent generation of regenerated individuals.

2) A DNA fragment was introduced to MON810 by particle gun bombardment, so, confirmation of remaining Agrobacterium was not carried out.

3) 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 tests conducted at 6 sites in the USA in 1994, the morphological and growing characteristics of this line were investigated and also analysis of the expression of the Cry1Ab protein and inserted genes were implemented (The line used in the tests is shown in Figure 4). Based on these results, necessary approval was obtained in the USA and general commercial cultivation began in 1997.

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

Oct., 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 (Ministry of Health and Welfare, at the 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.

June 2004: Approval of Type 1 Use under the provisions of the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Law) (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) was received by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment.

[Process of rearing of MON88017×MON810]

This stack maize was developed by the crossing of the two inbred lines MON88017 and MON810 with the use of traditional crossbreeding method (Figure 5).

Not made available or disclosed to unauthorized person

Figure 3　Process of rearing of the glyphosate-tolerant and Coleoptera-resistant maize MON88017

Not made available or disclosed to unauthorized person

Figure 4　Process of rearing of the recombinant maize MON810

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Figure 5　Process of rearing of the stack line maize MON88017×MON810

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 MON88017 and stability of expression of traits caused by the nucleic acid]

As a result of Southern blotting analysis of the inserted gene in MON88017, it was confirmed that one copy of T-DNA region is inserted into the genome of this recombinant maize at one site (Annex 2 of Biological Diversity Risk Assessment Report for MON88017). The schematic diagram of the inserted gene, which became clear from the results of the analysis and the analytical result of base sequence, is shown in Figure 6. As a result of Southern blotting analysis in multiple generations, it was also proved that the inserted gene is descended stably to the progeny (for the generations analyzed, refer to the lines identified with the asterisk * in Figure 3) (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). In addition, it was also confirmed as a result of glyphosate-spraying test and ELISA analysis using the antibody to Cry3Bb1 protein that the glyphosate herbicide tolerance and Coleoptera resistance are stably expressed in multiple generations.

[State of existence of nucleic acid transferred in MON810 and stability of expression of traits caused by the nucleic acid]

As a result of Southern blotting analysis of the inserted gene in MON810, it was confirmed that one copy of DNA fragment derived from PV-ZMBK07 which is necessary for the expression of *cry1Ab* gene is inserted into the genome of MON810 at one site (Annex 2 of Biological Diversity Risk Assessment Report for MON810). The schematic diagram of the inserted gene, which became clear from the results of the analysis and the analytical result of base sequence, is shown in Figure 7. As a result of Southern blotting analysis in multiple generations (the generations identified with the superscript [d] in Figure 4), it was also proved that the inserted gene is descended stably to the progeny (Annex 2 and Annex 3 of Biological Diversity Risk Assessment Report for MON810). In addition, it was also confirmed as a result of bioassay that the resistance to the insects of order Lepidoptera is stably expressed in multiple generations.

As a result of Southern blotting analysis of the inserted gene in MON810, it was confirmed that the gene insertion in the genome of maize is limited to the region necessary for the expression of the *cry1Ab* gene derived from PV-ZMBK07 and that there exist no expression cassettes of the modified *cp4 epsps* gene and *gox* gene derived from *nptII* gene and PV-ZMGT10, respectively (Annex 2 and Annex 5 of Biological Diversity Risk Assessment Report for MON810).

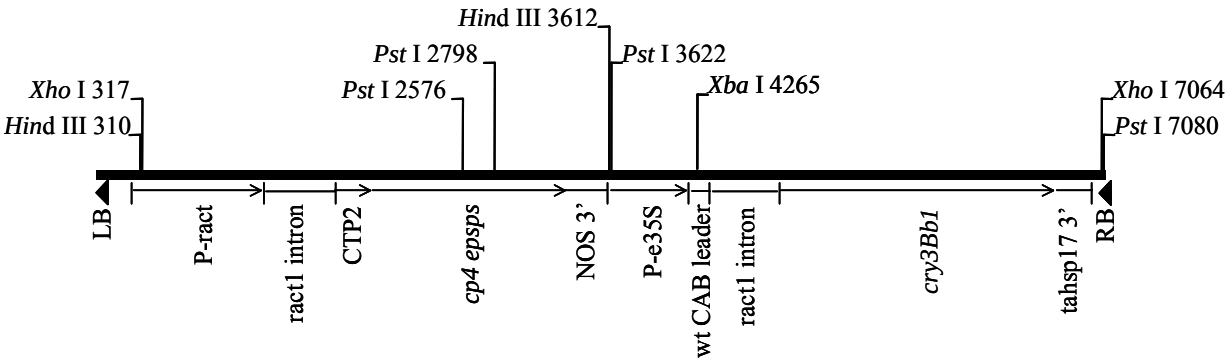

Figure 6　Map of the inserted gene in MON88017 [6]

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

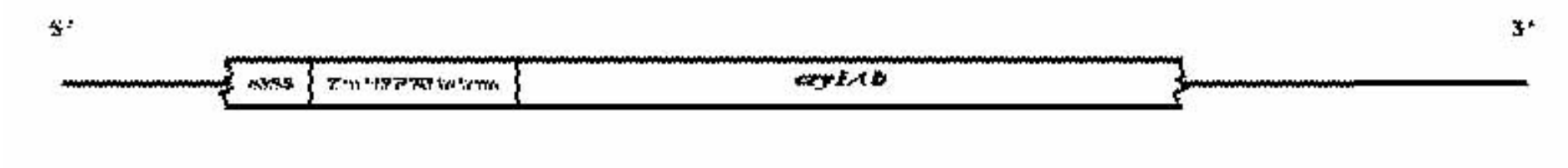

Figure 7　Map of inserted gene in MON810[7]

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

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

Specific method for detection and identification of MON88017 is available by using the DNA sequence of the plant genome of the inserted gene and its surroundings as primers (Annex 4 of Biological Diversity Risk Assessment Report for MON88017).

For detection and identification of MON810, a standard analysis method is disclosed at http://www.maff.go.jp/sogo_shokuryo/jas/manual00.htm (Annex 4 of Biological Diversity Risk Assessment Report for MON810).

For detection and identification of this stack maize, the above-mentioned two methods must be applied to each grain of maize seeds.

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

It has been indirectly demonstrated that the modified CP4 EPSPS protein, modified Cry3Bb1 protein and Cry1Ab protein are expressed in the plant body of this stack maize by the function of genes which were inserted in parent lines MON88017 and MON810. It is suggested that EPSPS protein, which possesses the same functions as the modified CP4 EPSPS protein, is not a rate-determining enzyme in the shikimate pathway. In addition, Monsanto Co. examined aromatic 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 the metabolic pathway of recipient organism. Furthermore, the modified CP4 EPSPS protein has high substrate specificity. In addition, as mentioned in I-1-(2), modified Cry3Bb1 protein and Cry1Ab protein do not possess enzyme activity and function independently of the metabolic system of recipient organism, and it is considered to be extremely low that the proteins in the Cry1 family and Cry3 family interact with each other. Based on the above understanding, there is no reason to suspect that these three proteins would affect each other.

To confirm the above understanding in practice, regarding tolerance to glyphosate herbicide of this stack maize, Roundup-spraying tests were carried out in the USA, and regarding resistance to Coleoptera and Lepidoptera of this stack maize, the bioassay to take western corn rootworm (*Diabrotica vergifera virgifera*) and European corn borer (*Ostrinia nubilalis*) as the objects of pot tests was carried out. The process of rearing of this stack maize used in the spraying tests and bioassay is shown in Figure 8. As a result, it was confirmed that under the test conditions applied, this stack maize expresses the tolerance to glyphosate herbicide (Table 4). Then, resistance of this stack maize to western corn rootworm (*Diabrotica vergifera virgifera*) was same as that of MON88017 which expresses the modified Cry3Bb1 protein (Table 5). In addition, resistance of this

stack line to European corn borer was almost same as that of MON810 which expresses Cry1Ab protein alone (Table 6).

Based on the above results, it was considered that the individual proteins expressed in this stack maize function independently from each other. Then, for the information used in the Item-by-item assessment of Adverse Effects on Biological Diversity in II about the difference between this stack maize and the taxonomic maize species to which the recipient organism belongs, the results of individual examinations for the various characteristics of MON88017 and MON810 were attached as follows.

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Figure 8　Process of rearing of MON88017×MON810 used in the bioassay

Table 4　Result of bioassay by spraying of glyphosate herbicide (Product name: Roundup Weathermax) to cross progeny line of MON88017×MON810[8]

Cross progeny line	Rate of growth inhibition (%)
MON88017×MON810	0
MON88017	0
Non-recombinant plant	100

Ten (10) plant bodies for each cross progeny line were cultivated in pots, and on the 13th day after cultivation, glyphosate herbicide (Product name: Roundup Weathermax) was sprayed in a concentration of 1.125 lb a.e./ac [equivalent to 350 mL spraying per 10a (70% of the normal dose)]. On the 10th day after spraying of glyphosate, the rate of inhibited growth (the degree of overall growth inhibition in 10 individuals) was evaluated by visual inspection. The rate of growth inhibition was determined based on the comparison to overall growth inhibition in 5 plant bodies which were not sprayed with glyphosate.

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

Table 5 Investigational result of the severity of damage by western corn rootworm (*Diabrotica vergifera virgifera*), order Coleoptera, based on bioassay of cross progeny line of MON88017×MON810[9]

Cross progeny line	Nodal injury score (NIS)
MON88017×MON810	0.19
MON88017	0.17
MON810	2.42
Non-recombinant plant	2.68

Table 6 Investigational result of the severity of damage by European corn borer, order Lepidoptera, based on bioassay of cross progeny line of MON88017×MON810[10]

Cross progeny line	Leaf damage rate (LDR)
MON88017×MON810	1.10
MON88017	5.10
MON810	1.11
Non-recombinant plant	5.80

[Notes on Tables 5 and 6]

Ten (10) plant bodies for each cross progeny line were cultivated in pots, and eggs of western corn rootworm (*Diabrotica vergifera virgifera*) were inoculated at the 2nd leaf stage (1200 eggs/pot). At the 4th leaf stage, the first instar larvae of European corn borer were additionally inoculated (45 larvae/pot). On the 21st day after inoculation of European corn borer, the severity of insect damage by European corn borer was determined based on the leaf damaging rate (LDR), a typical method for evaluating insect damage severity on a scale from 0 (no damage) to 9 (serious damage: a greater part of leaf is damaged) (Reference 61) (Annex 1).

Later, the plant bodies were taken out from the pots and soils on the plants were carefully removed, then the severity of insect damage by western corn rootworm (*Diabrotica vergifera virgifera*) was evaluated by using the nodal injury score (NIS) (Reference 62) (Annex 2). This method is popularly used by various research institutions in the USA to evaluate the insect damage by corn rootworm. The corn rootworm feeds on crown roots sequentially, starting from those growing from the lower node (usually 5th node) to those growing from the upper nodes (usually 6th node, then 7th node), so the severity of insect damage is represented in the successive values ranging from 0.00 to 3.00 in this method. For example, the score of 2.80 indicates that the 5th and 6th nodes are completely damaged, and 80% of the 7th node is damaged.

[9-10] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

(1) Regarding MON88017, with the expression of the modified CP4 EPSPS protein, which is encoded by the modified cp4 epsps gene in various regions of the plant, tolerance to glyphosate herbicide was conferred to this recombinant maize. In practice, the non-recombinant control maize died due to the influence of glyphosate herbicide, while the recombinant maize grew normally (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). In addition, with the expression of the modified Cry3Bb1 protein, which is encoded by the modified cry3Bb1 gene, resistance to CRW, which is the major pest insect of the order Coleoptera in the maize cultivation in the USA, is conferred, and insect damage by CRW is decreased. CRW feeds on and damages the root systems of maize, though the modified Cry3Bb1 protein constantly expresses in various regions of plant body in MON88017 (Annex 2 of Biological Diversity Risk Assessment Report for MON88017).

Regarding MON810, with the expression of the Cry1Ab protein, which is encoded by the *cry1Ab* gene, resistance to insect damage by corn borers (*Ostrinia nubilalis*), which is the major pest insect of the order Lepidoptera in the maize cultivation in the USA, was conferred, and a decrease of insect damage by corn borers (*Ostrinia nubilalis*) was confirmed (Annex 2 of Biological Diversity Risk Assessment Report for MON810). *Ostrinia nubilalis* feeds on and damages the entire above-ground part of maize, though the Cry1Ab protein constantly expresses in various regions of plant body in MON810 (Annex 2 of Biological Diversity Risk Assessment Report for MON810). In addition, it was found as a result of Southern blotting analysis that, regarding MON810, *nptII* gene, modified *cp4 epsps* gene and *gox* gene are not present in MON810 and the expression of traits derived from these genes is not observed (Figure 5 and Table 3 in Annex 2 of Biological Diversity Risk Assessment Report for MON810).

Consequently, it is considered that modified CP4 EPSPS protein, modified Cry3Bb1 protein and Cry1Ab protein constantly express in various regions of plant body in this stack maize.

(2) The isolated field tests were carried out in Kawachi Research Farm (KRF) of Monsanto Japan Limited in 2002, using MON88017-A and MON88017-B (hereinafter referred to as "017-A" and "017-B", respectively), which belong to the line of [11]MON88017, as well as Cont-A and Cont-B as the control lines. 017-A and 017-B are the cross progeny lines which are derived from the different rearing processes starting from the first generation (R0) of the MON88017 as indicated by the pedigree in Figure 3. The control lines Cont-A and Cont-B are the cross progeny lines of the non-recombinant control maize crossed in a specific way to attain the same genetic background with 017-A and 017-B.

The isolated field tests were carried out in the National Institute for Agro-Environmental Science in 1996 and from 2001 to 2002, using MON810AX and MON810BX, which belong to the line of MON810, as well as MON810AC and MON810BC as the control lines. MON810AX and

[11] All the rights pertinent to the information in the paragraphs (a) through (g) following this section and the responsibility for the contents rest upon Monsanto Japan Limited.

MON810BX are the cross progeny lines derived from the different rearing processes from the first generation (R0) of the MON810 as indicated by the pedigree in Figure 4. The control lines MON810AC and MON810BC are the cross progeny lines of the non-recombinant control maize crossed in a specific way to attain the same genetic background with MON810AX and MON810BX.

(a) Morphological and growth characteristics

For MON88017 and the non-recombinant control maize, evaluation was conducted regarding uniformity of germination, germination rate, time of tasseling, time of silking, culm length, plant shape or plant type, tiller number, height of ear, maturation time, number of ears, and plant 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 (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). Regarding culm length, statistically significant difference was found between the recombinant maize 017-B and the non-recombinant control maize Cont-B, and the average value of culm length was 226.9 cm for 017-B and 233.4 cm for Cont-B (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). Meanwhile, no statistically significant difference was observed between the recombinant maize 017-A and the non-recombinant control maize Cont-A (Annex 3 of Biological Diversity Risk Assessment Report for MON88017).

For MON810 and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tasseling, 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 (Annex 2 and Annex 3 of Biological Diversity Risk Assessment Report for MON810). Regarding culm length, statistically significant difference was found between the recombinant maize MON810BX and the non-recombinant control maize MON810BC, and the average value of culm length was 248.1 cm for MON810BX and 229.3 cm for MON810BC (Annex 3 of Biological Diversity Risk Assessment Report for MON810). Meanwhile, no statistically significant difference was observed between the recombinant maize MON810AX and the non-recombinant control maize MON810AC (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

Sensitivity to low temperature (temperature of 5℃) of the seedlings of MON88017 and non-recombinant control maize was evaluated. Almost all plants died after 24 days, and no difference was observed between this recombinant maize and non-recombinant control maize in the cold-tolerance (Annex 3 of Biological Diversity Risk Assessment Report for MON88017).

Sensitivity to low temperature (maximum temperature of 12 to 14℃, minimum temperature of 2℃) of the seedlings of MON810 and non-recombinant control maize was evaluated. All the fully

developed leaves had wilted on the 21st day after the start of cold treatment. No difference between MON810 and the non-recombinant control maize was observed on cold-tolerance (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

(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 isolated field experiments for the parent lines MON88017 and MON810 and their relevant non-recombinant control maize in practice. Based on the above, 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 MON88017 and the non-recombinant control maize, pollens were stained with potassium iodide solution and observed under a microscope. As a result, no difference was observed between MON88017 and non-recombinant control maize (Annex 3 of Biological Diversity Risk Assessment Report for MON88017).

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 iodide solution and observed under a microscope. As a result, no difference was observed between MON810 and non-recombinant control maize (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

Regarding the production of the seed of MON88017, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, and grain shape after sib-mating were examined. As a result, no statistically significant difference was observed between MON88017 and non-recombinant control maize in any of the characteristics examined except in ear diameter (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). Regarding ear diameter, statistically significant difference was found between the recombinant maize 017-B and the non-recombinant control maize Cont-B, and the average value of ear diameter was 44.0 mm for 017-B and 45.7 mm for Cont-B (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). Meanwhile, no statistically significant difference was observed between the recombinant maize 017-A and the non-recombinant control maize Cont-A (Annex 3 of Biological Diversity Risk Assessment Report for MON88017).

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 non-recombinant control maize (Annex 2 and Annex 3 of Biological Diversity Risk Assessment Report for MON810).

Regarding shedding habit of the seed, shedding habit was not observed in natural conditions, since the ears of both MON88017 and MON810 and their non-recombinant control plants were covered with bracts at the time of harvesting.

Regarding germination rate on the 10th day of sowing of harvested seeds of MON88017 and germination rate on the 4th day of sowing of harvested seeds of MON810, no statistically significant difference was observed between the recombinant maize and the non-recombinant control maize, and no dormancy of the seed was examined (Annex 3 of Biological Diversity Risk Assessment Report for MON88017 and Annex 3 of Biological Diversity Risk Assessment Report for MON810).

(f) Crossability

Crossability test was not performed for the parent lines MON88017 and MON810, since no wild relatives that can be crossed grow in Japan.

(g) Production of harmful substances

Soil microflora tests, succeeding crop tests, and plow-in tests were performed for MON88017 and non-recombinant control maize. Statistically significant difference was not observed in any of the items except the fresh weight of *Raphanus sativus* var. *radicula*, which was the assay plant of the plow-in test between MON88017 and non-recombinant control maize (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). Regarding the fresh weight of *Raphanus sativus* var. *radicula*, which was the assay plant of the plow-in test, statistically significant difference was found between the recombinant maize 017-A and the non-recombinant control maize Cont-A, and the average value of the fresh weight was 7.17 g for 017-A and 8.38 g for Cont-A (Annex 3 of Biological Diversity Risk Assessment Report for MON88017). However, for the recombinant maize 001-A and 012-A, which were subjected to the isolated field tests concurrently with 017-A, no statistically significant difference was found in the fresh weight of *Raphanus sativus* var. *radicula* in the plow-in tests. In addition, regarding germination rate of *Raphanus sativus* var. *radicula* in plow-in tests and regarding the succeeding crop tests and soil microflora tests, no statistically significant difference was observed between 017-A and Cont-A (Annex 3 of Biological Diversity Risk Assessment Report for MON88017).

Plow-in tests, succeeding crop tests, and soil microflora tests were performed for MON810 and non-recombinant control maize. Statistically significant difference was not observed in any of the items (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

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

This stack maize was developed by traditional crossbreeding method from the maize tolerant to glyphosate herbicide and resistant to Coleoptera (MON-88017-3) and the maize resistant to Lepidoptera (MON-00810-6), and the parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied for the Type 1 Use same as the stack maize.

It is reported that the modified CP4 EPSPS protein which is encoded by modified *cp4 epsps* (gene torelant to glyphosate) derived from MON-88017-3 is the enzyme that possesses high substrate specificity, and that the modified Cry3Bb1 protein which is encoded by modified *cry3Bb1* (gene resistant to Coleoptera) derived from MON-88017-3 and the Cry1Ab protein which is encoded by *cry1Ab* (gene resistant to Lepidoptera) derived from MON-00810-6 do not possess enzyme activity. In addition, it is known that Cry3 family to which Cry3Bb1 protein belongs and Cry1A family to which Cry1Ab protein belongs show specific insecticidal activities against larvae of the insects classified into different order of insects, Coleoptera and Lepidoptera, respectively and possess highly specific insecticidal activities. Consequently, it is considered to be extremely low that the characteristics conferred by modified *cp4 epsps* and those conferred by *cry3Bb1* and *cry1Ab* could interact with each other.

It has been confirmed based on the herbicide-spraying tests that the tolerance to glyphosate herbicide is expressed in this stack maize. In addition, based on the bioassay using the western corn rootworm (*Diabrotica virgifera virgifera*) and European corn borer (*Ostrinia nubilalis*), it is also confirmed that the resistance to Coleoptera and resistance to Lepidoptera are expressed in this stack maize, respectively.

Based on the above understanding, it is considered that there is no specific change in the characteristics in this stack maize except it possesses the same characteristics as the parent lines do.

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

(1) Competitiveness

This stack maize possesses the glyphosate herbicide tolerance and Coleoptera resistance derived from MON-88017-3 and the Lepidoptera resistance derived from MON-00810-6. However, it is generally considered unlikely that the glyphosate exerts pressure for selection under the natural environment. In addition, it is also considered that the insect damage by Coleoptera and Lepidoptera is not the major cause to make the maize difficult to grow in the natural environment in Japan. Consequently, it is considered that these characteristics do not increase the competitiveness and thus this stack maize is not predominant over the parent lines in the competitiveness. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack maize possesses the productivity of CP4 EPSPS protein and Cry3Bb1 protein derived from MON-88017-3 and the productivity of Cry1Ab protein derived from MON-00810-6. It is confirmed that the Cry3Bb1 protein possesses the insecticidal activity against insects of the order Coleoptera and the Cry1Ab protein possesses the insecticidal activity against insects of the order Lepidoptera, though their insecticidal activities are highly specific, while the CP4 EPSPS protein is not a harmful substance to animals and plants. Thus, it is considered that the productivity of harmful substances of this stack maize would not become higher than that of parent lines even though this stack maize contains all the three proteins. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is valid.

(3) Crossability

In Japan, no wild species that can be crossed with maize is growing in natural environment.

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 Biological Diversity Risk Assessment Report

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

[Bibliography]

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[Annex List]

Annex 1: Method for evaluating the severity of insect damage by Lepidoptera

Annex 2: Method for evaluating the severity of insect damage by Coleoptera