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

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Approved Type 1 Use Regulation

Name of the type of Living Modified Organism:	Maize resistant to Lepidoptera and Coleoptera (*cry1Ab*, *cry3Bb1*, *Zea mays* L.) (MON 810×MON 863, OECD UI: MON-ØØ81Ø-6×MON-ØØ863-5)
Content of the Type 1 Use of Living Modified Organism:	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.
Method of the Type 1 Use of Living Modified Organism:	-

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effects on Biological Diversity

1. Information concerning a recipient organism or the species to which the recipient organism belongs

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

The academic name for maize is *Zea mays* L. The origin is considered to be the area from the Southwest region of the United States to Mexico, Central America, and South America, but there is no conclusive theory. There is no report of natural distribution in Japan.

(2) History and present state of Use

The origin of maize is considered to be the area from the Southwest region of the United States to Mexico, Central America, and South America, but there is no conclusive theory. It is generally understood that the earliest cultivation could date back 9,000 years. The first introduction to Japan is said to be in 1579 to Nagasaki or Shikoku, and maize has long been cultivated since then. At present, it is used mainly for feed, but also for food and various food products including cooking oil and starch. Currently, it is the most widely cultivated grain in the world and can be grown in the area from 58 degrees north latitude to 40 degrees south latitude mainly in the US, China, Brazil, Argentina, and European countries.

Japan currently imports about 16 million metric tons of maize for feed and food.

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

The optimum germination temperature of maize seed is 32-36ºC and the minimum germinating and minimum growing temperatures are 6-10ºC. In practice, the optimum sowing season is considered to be the period when the temperature is 13-14ºC or over, and usually maize is sown in spring and harvested in autumn as an annual plant.

The dormancy of the seed is extremely low, and even when seeds fall to the ground, they do not germinate until the soil temperature reaches 10ºC. In most cases the seeds would decay and die before germinating.

ii) Mode of propagation or reproduction

Maize is a monoecious annual plant which propagates by seed, and can be self-pollinated but in most cases is cross-pollinated as a typical wind pollinated flower. It is not reported that maize seeds possess dormancy. The dispersion distance of maize pollen differs by the presence of barriers such as woods or mountains or the direction of the wind, but is considered to be approximately

300-500 m.

Related species of maize is teosinte of the same genus *Zea* as well as some other species classified into the genus *Tripsacum*. Maize can be hybridized only with teosinte in nature, and hybridization with any species of genus *Tripsacum* in nature is not known. In Japan, the growth of teosinte and wild species of the genus *Tripsacum* has not been reported.

iii) Production of harmful substances

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

iv) Other information

It has not been reported so far that maize seeds which were spilled during transportation on locations other than cultivation fields have grown.

2. Information concerning preparation of living modified organisms

This F1 hybrid variety of maize, (*cry1Ab, cry3Bb1, Zea mays* L.) (MON 810 X MON 863, OECD UI No. :MON-00810-6 X MON-00863-5) (hereinafter referred to as this stacked maize), was bred by a conventional F1 hybrid method, that is, by crossing 2 recombinant maize lines, maize resistant to Lepidoptera (*cry1Ab*, *Zea mays* L.) (MON 810, OECD UI No.: MON-00810-6) (hereinafter referred to as MON 810) and maize resistant to Coleoptera MON 863 (*cry3Bb1*, *Zea mays* L.) (MON 863, OECD UI No.: MON-00863-5) (hereinafter referred to as MON 863). This stacked maize has characteristics from both recombinant parent strains MON 810 and MON 863.

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

Composition of donor nucleic acid and origins of component elements used for the development of MON 810 are shown in Table 1

Composition of donor nucleic acid and origins of component elements used for the development of MON 863 are shown in Table 2.

ii) Functions of component elements

Functions of component elements which were used for the development of MON 810 are shown in Table 1. Functions of components which were used for the development of MON 863 are shown in Table 2.

The *cry1Ab* gene, the target gene to confer Lepidoptera resistance, is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium that universally exists in soil. The Cry1Ab protein which is encoded by the *cry1Ab* gene has an insecticidal activity against corn borers (*Ostrinia nubilalis*), one of the major pest

insects of the order Lepidoptera in maize cultivation in the US. The part of the plant damaged by corn borers is the whole plant above the ground. B.t. crystal proteins which are produced by the bacterium B.t. including the Cry1Ab protein bind to specific receptors in the midgut epithelium of the target insects and form cation selective pores, leading to inhibition of the digestive process and resulting in the insecticidal activity.

The Cry1Ab protein exhibits insecticidal activity only against insects of the order Lepidoptera and not against insects of other orders. The Cry1Ab protein is also known to exhibit insecticidal activity against major US maize pest insects of the order Lepidoptera, including the 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*). *O. furnacalis,* which belongs to the same genus as *O. nubilalis* mentioned above, is known as the major maize pest insect of the order Lepidoptera in Japan.

To discover whether the Cry1Ab protein shares functionally important amino acid sequences with known contact allergens, comparisons were conducted using the database. As a result, the Cry1Ab protein did not share structurally related similar sequences with any of the known allergens examined.

The *cry3Bb1* gene, the target gene to confer Coleoptera resistance, is derived from *Bacillus thuringiensis* subsp. *kumamotoensis,* a gram-positive bacterium that universally exists in soil. The Cry3Bb1 protein which is encoded by the *cry3Bb1* gene possesses insecticidal activity against corn rootworms (*Diabrotica* sp.), which is one of the major maize pest insects of the order Coleoptera in the US. This insect damages the root system of maize.

The Cry3Bb1 protein has an extremely narrow insecticidal spectrum of activity that only targets against the Colorado potato beetle (*Leptinotarsa decimlineata*) and corn rootworm, respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, within the order Coleoptera. Closely-related species of the same genera with these two insect species do not inhabit Japan.

The *nptII* (neomycin phosphotransferase type II) gene, which is an antibiotic resistance marker gene introduced for the selection of transgenic plants, 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, transgenic cells can be selected by the addition of kanamycin to the medium.

To discover whether the Cry3Bbl protein and the NPTII protein shares functionally important amino acid sequences with known contact allergens, comparisons were conducted using publicly available databases. As a result, the Cry3Bb1 and NPTII proteins did not share structurally related sequences with known allergens.

Table 1 Component elements of plasmid PV-ZMBK-07 and PV-ZMGT10 which were used for the development of MON 810, and their origin and function

Component elements	Origin and function
cry1Ab gene cassette	
E35S	Contains 35S promoter (Odell *et al*., 1985) and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp 70 intron is used to enhance the expression of foreign genes in plants.
cry1Ab	The gene which encodes the Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain in the soil. The details of its function are described in p.2-3.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
CP4 EPSPS gene cassette（As a result of inserted gene analysis, it was not inserted into MON 810.）	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp 70 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.
CP4 EPSPS	A synthetic sequence generated based on 5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium.*
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
GOX gene cassette （As a result of inserted gene analysis, it was not inserted into MON 810.）	
E35S	Contains 35S promoter (Odell *et al*., 1985) and duplicated enhancer from cauliflower mosaic virus (CaMV).
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp 70 intron is used to enhance the expression of foreign genes in plants.
CTP 1	N-terminal chloroplast transit peptide of the small subunit 1A of rubisco gene from *A. thaliana*.
GOX	A synthetic sequence generated based on glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from *Agrobacterium tumefaciens.* Contains transcription terminator and polyadenylation signal for mRNA.
Backbone (common to PV-ZMBK07 and PV-ZMGT10)（As a result of inserted gene analysis, it was not inserted into MON 810.）	
LacZ	Partial coding sequence for β-D-galactosidase or LacZ protein.
Ori-pUC	A segment containing replication origin for *E.coli* plasmid pUC.
NptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II.

Table 2 Component elements of plasmid PV-ZMIR13L which was used for the development of MON 863, and their origin and function

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).
wt CAB	5'-terminal untranslated region of wheat chlorophyll a/b binding protein.
ract1 intron	Intron of rice actin gene.
cry3Bb1	The gene which encodes modified Cry3Bb1 protein of *Bacillus thuringiensis.* The detail of its function is described in p.3.
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 bacteria.
Ble	A part of bleomycin resistance gene isolated from Tn5. Encodes 50 amino acids in 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.

In the plant body of this stacked maize, Cry1Ab protein, Cry3Bb1 protein, and NptII protein are expressed as the products of genes that have been inserted into the parent strains, MON 810 and MON 863. Since Cry1Ab protein and Cry3Bb1 protein, both of which have no enzymatic activity, function independently, it is not considered that there would be interaction by the introduced genes or effect on the metabolic system of the recipient oraganism.

Growth chamber bioassays were carried out in this stacked maize for its resistance against insects of the orders of Lepidoptera and Coleoptera, using European corn borers and corn rootworms, target pest insects in the US, as the subjects. As a result, the level of resistance of this stacked maize against each of the pest insects was equivalent to the MON 810 F1 hybrids that express Lepidoptera resistance and to the MON 863 F1 hybrids that express Coleoptera resistance, and no statistical difference was found. Thus, it was shown that the expression level of these genes is free from mutual interaction due to hybridization.

It is known also from the findings of *B.t.* products that have been used as biological pesticides since 1960, that the Cry1A family to which the Cry1Ab protein belongs and the Cry3 family to which the Cry3Bb1 protein belongs exhibit insecticidal activity specifically against larvae of different orders, Lepidoptera and Coleoptera, respectively. *B.t.* products have a long history of use but there is no report that their insecticidal spectrum has changed during this process.

Additionally, it was confirmed that non-target insects that are insensitive to the Cry1Ac protein which belongs to the Cry1A family like Cry1Ab protein and to the Cry3Aa protein which belongs to the Cry3 family like Cry3Bb1 protein, still remain insensitive even if they were exposed to a mixture of these *B.t.* proteins that belonged to two different families, without inferred synergistic effect by simultaneous exposure to the Cry1Ac protein and Cry3A protein.

From the above, it is considered that there is no risk of mutual interaction of inserted genes affecting physiological and ecological traits of this hybrid maize.

(2) Information concerning vector

i) Name and origin

The vector used for the development of MON 810 and MON 863 is plasmid pUC119 from *Escherichia coli.*

ii) Properties

The vectors contain a kanamycin/neomycin-resistant gene (*nptII* gene) derived from *E.coli* transposon Tn5 as the selectable marker gene for the construction vector.

(3) Method of preparing living modified organisms

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

Refer to Tables 1 and 2.

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

For the development of MON 810, the mixture of plasmids PV-ZMBK07 and PV-ZMGT10 was introduced by particle gun bombardment to the F2 generation of a cross A188 X B73, between two inbred lines of dent type.

For the development of MON 863, normal chain DNA fragment, ZMIR13L was introduced by particle gun bombardment to inbred line A634.

iii) Processes of rearing of living modified organisms

For the development of MON 810, the callus to which plasmid DNA was 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. Pedigree selection was started in 1992, and field experiments were carried out from 1993 to 1995. Finally, MON 810 was selected as a commercial event. In isolated field tests carried out at 6 sites in the US in 1994, the morphological and growth characteristics of this event were investigated and also analysis of the expression of the Cry1Ab protein and inserted genes were implemented. Based on these

results, regulatory approval was obtained in the US and general commercial cultivation began in 1997.

For the development of MON 863, the callus to which ZMIR13L was 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 paromomycin-containing medium. From the selected callus, the regenerated plant was obtained and the expression of the Cry3Bb1 protein was analyzed. Pedigree selection was started in 1997, and field experiments were carried out from 1998 to 2002. Finally, MON 863 event was selected as a commercial event. In isolated field tests carried out at one site in Illinois in 1999, the morphological and growth characteristics of this event were investigated and also analysis of the expression of the Cry3Bb1 protein and inserted genes were implemented. Based on these results, regulatory approval was obtained in the US and general commercial cultivation began in 2003.

This stacked maize is an F1 hybrid produced by hybridizing 2 kinds of recombinant maizes, MON 810 and MON 863, using a conventional hybridizing method.

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

Based on Southern blot analyses, it was confirmed that 1 copy of the DNA fragment from PV-ZMBK07 which is necessary for the expression of the *cry1Ab* gene is inserted into the genome of MON 810 at one site. Also, Southern blot analyses of multiple generations of the plant indicated that the inserted gene is stably inherited in offspring. It was also confirmed that resistance to Lepidoptera pest insects is stably expressed in multiple generations.

In addition, as a result of Southern blot analyses, it was confirmed that only the region from PV-ZMBK07 which is required for the expression of *cry1Ab* gene was inserted into the genome of MON 810, but neither the *nptII* gene nor expression cassettes of the *CP4 EPSPS* gene and *GOX* gene from PV-ZMGT10.

While, as a result of Southern blot analyses, it was confirmed that 1 copy of the DNA fragment from ZMIR13L which is necessary for the expression of the *cry3Bb1* gene and *nptII* gene is inserted into the genome of MON 863 at one site. Also, southern blot analyses and Western blot analyses of multiple generations of the plant indicated that the inserted *cry3Bb1* gene and *nptII* gene of the DNA fragment are stably inherited in offspring. It was also confirmed that resistance to Coleoptera pest insects is stably expressed in multiple generations.

Therefore, in the genome of this stacked maize, 1 copy of DNA fragment from PV-ZMBK07 which is necessary for the expression of the *cry1Ab* gene from MON 810 exists at 1 site, and 1 copy of DNA fragment from ZMIR13L which is necessary for the expression of the *cry3Bb1* gene from MON 863 exists at 1 site.

In addition, as a result of growth chamber bioassays on the resistance of this stacked maze against insects of the order Lepidoptera and Coleoptera using the European

cornborer and corn rootworm, target pest insects in the US, as test subjects, it has been shown that the resistance of this stacked maize against both insects were equivalent to that of the MON 810 F1 hybrids that express Lepidoptera resistance and to the MON 863 F1 hybrids that express Coleoptera resistance, and that there is no statistically significant difference.

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

As was stated in I 2-(1), it is not considered that the transfer of the introduced genes affects the metabolic system of the recipient oraganism in this stacked maize.

This stacked maize is an F1 hybrid which was produced by the hybridization of MON 810 and MON 863, and possesses the traits of both parent strains. Heterosis is expected to occur by hybridizing the inbred lines of MON 810 and MON 863, the parent strains. However, since the *cry1Ab* gene and *cry3Bb1* gene function independently, and their products do not have enzymatic activities, it is not considered that they affect the metabolic system of the recipient oraganism or that they interact with each other. As such, it is considered that these introduced genes do not affect heterosis by hybridization, and that the change in the various traits of this stacked maize due to heterosis is seen to be within the range of changes that occur due to heterosis that are observed in the conventional F1 hybrids which have been developed between non-recombinant maizes.

From the above, the difference between this stacked maize and maize which is a taxionomic species to which the recipient organism belongs is to be discussed based on the results of individual investigation of various traits of MON 810 and MON 863.

i) MON 810 is conferred with resistance to insect damage by major insects of the order Lepidoptera found in maize cultivation in the US through the expression of the Cry1Ab protein which is encoded by the *cry1Ab* gene, and a reduction in insect damage by the European cornborer was confirmed. The Cry1Ab protein is constantly expressed in all the tissues of the plant body.

MON 863 is conferred with resistance to insect damage by major insects of the order Coleoptera found in maize cultivation in the US through the expression of the Cry3Bb1 protein which is encoded by the *cry3Bb1* gene, and a reduction in insect damage by corn rootworms was confirmed. The Cry3Bb1 protein is constantly expressed in all the tissues of the plant body. Also, NPTII protein is constantly expressed in all the tissues of the body.

Thus, Cry1Ab protein, Cry3Bb1 protein, and NPTII protein is constantly expressed in all the tissues of the plant body of this stacked maize.

ii) Isolated field tests were carried out using MON 810AX and MON 810BX which belong to the MON 810 strain, and their control MON 810AC and MON 810BC as the test sample. In addition, isolated field tests were carried out using MON 863 AX, MON 863 BX and MON 863CX which belong to the MON 863 strain, and their control MON 863AC, MON 863BC and MON 863CC as the test sample.

(a) Morphological and growth characteristics

Evaluation between MON 810 and the non-recombinant control maize was conducted in relation to germination rate, uniformity of germination, time of tassel exsertion, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, tiller number, total number of ears, number of productive ears, culm length, height of ear production, grain color, grain shape, and fresh weight after harvesting. Statistically significant differences were not observed between MON 810 and the non-recombinant control maize lines in all of the examined characteristics except for culm length. Statistically significant difference was found for culm length between recombinant maize MON 810BX and non-recombinant control maize MON 810BC, with an average length of 248.1 cm and 229.3 cm, respectively. No statistically significant difference was found between recombinant maize MON 810AX and non-recombinant control maize MON 810AC.

Evaluations were conducted between MON 863 and the non-recombinant control maize in relation to germination rate, uniformity of germination, time of tassel exsertion, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, tiller number, total number of ears, number of productive ears, culm length, height of ear production, grain color, grain shape, and fresh weight after harvesting, in the same manner as for MON 810. Statistically significant difference was not observed between MON 863 and the non-recombinant control maize lines in all of the characteristics examined.

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

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

Chilling sensitivity (highest temperature 12-14 ºC, lowest temperature 2 ºC) of the seedlings of MON 810 and the non-recombinant control maize was evaluated. All of the opened leaves presented withered conditions, on the 21st day, and no difference was observed between MON 810 and the non-recombinant control maize in chilling sensitivity tests.

Chilling sensitivity (4 ºC) of the seedlings of MON 863 and the non-recombinant control maize was evaluated. All of the plants died on the 14th day, and no difference was observed between MON 863 and the non-recombinant control maize in chilling sensitivity.

Thus, it is considered that there is no difference in chilling sensitivity between this stacked maize and maize which is the taxonomic species to which the recipient oraganism belongs.

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

Maize is a summer annual plant, and after ripening it usually dies down to the

ground naturally in winter. Overwintering test for the matured plant of MON 810 and MON 863 was not carried out, since it does not regrow and propagate vegetatively, norproduce seeds.

(d) Fertility and size of the pollen

To examine the fertility and size of the pollen of MON 810 and the non-recombinant control maize, pollen grains were stained with 0.1% neutral red solution and potassium iodine solution and observed under a microscope. As a result, no difference was observed between MON 810 and the nonrecombinant control maize.

To examine the fertility and size of the pollens of MON 863 and the nonrecombinant control maize, pollen grains were stained with potassium iodine solution and observed under a microscope. As a result, no difference was observed between MON 863 and the non-recombinant control maize.

Thus, it is considered that there is no difference in fertility and diameter of the pollen between this stacked maize and maize which is the taxonomic species to which the recipient oraganism belongs.

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

Ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, of the ears harvested after sib-mating, and the germination rate of the harvested seeds were examined. As a result, no difference was observed between of MON 810 and MON 863 and non-recombinant control maize in all of the characteristics examined. Also, no difference was observed between MON 810 or MON 863 and the non-recombinant control maize in terms of dormancy and shedding habit.

Thus, it is considered that there is no difference in ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, and germination rate, dormancy, and shedding habit of the harvested seeds, between this stacked maize and maize which is the taxonomic species to which the recipient oraganism belongs.

(f) Hybridization

Hybridization test was not performed since there are no wild relatives that can be hybridized in Japan.

(g) Production of harmful substances

Plow-in test, succeeding crop test and soil microflora tests were performed between MON 810 and the non-recombinant control maize. Statistically significant difference were not observed in all items. Plow-in, succeeding crop and soil microflora tests were also performed between MON 863 and the non-recombinant control maize. Statistically significant difference was not observed in any of the items between MON 863 and the non-recombinant control

maize.

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

3. Information concerning the Use of living modified organisms

(1) Content of the Use

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

(2) Information obtained from Use abroad

As a way to set a refuge at the time of cultivation of this stacked maize, the US EPA confirmed in October 2003 that it would be sufficient to grow maize cultivars which do not produce B.t. proteins in the 20% of the field area where this stacked maize is cultivated, which is the same to set a refuge for MON 810 and MON 863, the parent strains.

Commercial cultivation of this stacked maize overseas is scheduled to commence in the US and Canada in April 2004.

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

This stacked maize is an F1 hybrid which was produced by the hybridization of MON 810 and MON 863, and therefore possesses the traits of both MON 810 and MON 863. As was stated in I 2-(1), it is not considered that the introduced genes affect the metabolic system of the recipient oraganism or interact with each other in this hybrid maize. Thus, the evaluation of the adverse effect on the biological diversity of this stacked maize was carried out based on the results of individual investigation of various traits of MON 810 and MON 863.

1. Dominance in competition

This stacked maize has resistance to insects of the order Lepidoptera as well as to insects of the order Coleoptera. Also, various traits concerning the dominance in competition (morphological and growth characteristics, chilling tolerance at the early stage of growth, fertility and size of the pollen, and productivity, germination rate, dormancy and shedding habit of the seeds) were examined between MON 810 or MON 863, the parent strains of this stacked maize, and the non-recombinant control maize. As a result, statistically significant difference was observed in the culm length of MON 810. However, it was not considered that such a difference in the trait raises dominance in competition.

Introduced genes do not affect the traits concerning dominance in competition of each of the parent strain, and they function independently. Thus, it is not considered that introduced genes affect heterosis by hybridization. Therefore, the change in the various traits concerning dominance in competition of this stacked maize due to heterosis is seen to be within the range of changes that occur due to heterosis that are observed in conventional F1 hybrids.

In Japan, maize that grows as self-seeding in nature has not been reported since the introduction of maize into the country.

Based on the above, it was judged that there is no risk of adverse effect on the biological diversity attributable to dominance in competition.

2. Production of harmful substances

As a result of comparative examination of the production of harmful substances in the plow-in test, succeeding crop test, and soil microflora test, no difference was observed in comparison with the non-recombinant control maize.

This stacked maize expresses the Cry1Ab protein and Cry3Bb1 protein. In addition, it is also known from the findings of B.t. products that have been used as biological pesticides since 1960, that the Cry1A family to which the Cry1Ab protein belongs and the Cry3 family to which the Cry3Bb1 protein belongs exhibit insecticidal activity specifically against larvae of different orders, Lepidoptera and Coleoptera, respectively. B.t. products have a long history of use but there is no report that their insecticidal spectrum has changed during this process. Thus, as the species that may possibly be affected in some way by the dispersion of the pollen of this stacked maize, a total of 14 species (including 2 varieties) including 11 species of insect of the order Lepidoptera (including 2 varieties) that were specified for MON 810 and 3 species of the order Coleoptera that were specified for MON 863 were identified.

As a result of growth chamber bioassays, no difference was observed in the activity against target insects of this stacked maize from those of the parent strains MON 810 and MON 863. Thus, the specifics of the adverse effect of the pollen of this stacked maize on non-target insects were evaluated by the results of bioassays using the pollen of MON 810 and MON 863. The distance within which pollen dispersion exerts an effect was calculated by assigning 2,000-4,000 particles/cm^2, which is the pollen density that exerted effect on the survival of *Zizeeria maha argia* and Colorado potato beetles, to the model equation, which describes the relationship between the distance from the field and the number of dropped maize pollen (maximum number of deposited pollen). As a result, it was estimated that the maximum ranges within which pollen deposit at the density of 4,000 particles/cm^2 and 2,000 particles/cm^2 are 10 m and 20 m, respectively.

The likelihood of dispersion of pollens of this stacked maize causing effect was evaluated. The plants consumed by the larvae of the 11 species (including 2 varieties) of the order of Lepidoptera and 3 species of the order Coleoptera, which were identified as the insects subject to the effect of MON 810 and MON 863, inhabit wide areas including open field and mountains. Therefore, the cultivation field of maize and its surroundings are not the main habitat for these larvae. Also, it has not been reported that maize seeds which were spilled during transportation on locales other than cultivation fields have grown. Even if they germinated, the number of plants would be extremely fewer than the number of maize cultivated in the field, and the effect of dispersion of their pollen on the non-target insects of the orders Lepidoptera and Coleoptera can be ignored. Also, the main habitat for the aforementioned 11 species (including 2 varieties) of insects of the order Lepidoptera and 3 species of insects of the order Coleoptera do not fall within the livestock barns or roads where spillage may occur.

From these results, it was concluded that the possibility that dispersion of the pollen disturbs

the maintenance of species or individual populations is extremely low.

Based on the above, it was considered that there is no risk of the effect on the biological diversity attributable to production of harmful substances.

3. Hybridization

Related species of maize are teosinte of the same genus *Zea* and some other species of genus *Tripsacum*, but teosinte is the only species that can be hybridized with maize in nature. In Japan, the growth of teosinte and wild species in the genus *Tripsacum* has not been reported.

Based on the above, it was judged that there is no risk of effect on the biological diversity attributable tohybridization.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

This stacked maize is an F1 hybrid which was produced by the hybridization of MON 810 and MON 863, and possesses the traits of both MON 810 and MON 863. As was stated in I 2-(1), it is not considered that the introduced genes affect the metabolic system of the recipient oraganism or interact with each other in this hybrid maize. Thus, the effect on the biological diversity of this stacked maize was assessed based on the results of individual investigation of various traits of MON 810 and MON 863.

Maize, the species to which the recipient organism belongs, has long been used in Japan. Also, there was no difference in the various traits concerning dominance in competition between MON 810 or MON 863 and the non-recombinant control maize. Therefore, it was considered that there is no risk of adverse effect on biological diversity attributable to dominance in competition. In addition, since the introduced genes of parent strains function independently and the products do not interact each other, it was considered that the heterosis by the introduced genes in this stacked maize will not be beyond the range of those observed in the conventional F1. From the above, it was considered that there is no risk of adverse effect on biological diversity attributable to dominance in competition.

Various traits of MON 810 and MON 863 related to the production of harmful substances were evaluated in plow-in test, succeeding crop test, and soil microflora tests. As a result, no difference was observed. Also, adverse effect to the 11 species (varieties) of insects of the order Lepidoptera and the 3 species of insects of the order Coleoptera, which were identified as the wild organism whose inhabitation or growth is subjected to the effect of dispersion of pollens of MON 810 and MON 863, were examined. From the conclusion that the range within which the pollens of MON 810 and MON 863 exert effect is estimated to be 10-20 m surrounding the maize cultivation field, and that non-target insects of the order Lepidptera and those of the order Coleoptera that originally inhabit in nature do not mainly inhabit near maize cultivation fields, it was concluded that the possibility that they are effected by the pollen at the level of individual populations are extremely low. From the above, it was concluded that there is no risk that MON 810 and MON 863 causes adverse effect on biological diversity attributable to production of harmful substances. Further, since in the bioassay with pollen, tests were carried out using larvae at their most susceptible, during their growth stage, and also using the maximum conceivable distance of pollen dispersion under normal climate conditions, it was concluded that the effect of MON 810 and MON 863 will

not exceed the assumed effect even if there is some fluctuation in the period of pollen dispersion or the amount of dispersion due to differences in genetic background or heterosis. From the above, it was judged that there is no risk that this stacked maize causes adverse effect on biological diversity attributable to production of harmful substances, similarly to MON 810 and MON 863, the parent strains

Wild plants that can be hybridized with maize do not exist in Japan. Thus, it was considered that there is no risk of adverse effect on biological diversity attributable tohybridization.

Consequently, it was judged that there is no risk of adverse effect on biological diversity in Japan attributable to the use of this stacked maize for provision as food, for provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.