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:	Lepidoptera resistant maize (*cry1Ab, Zea mays* L.) (MON810, OECD UI: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 Report

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

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

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

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 and others.

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

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

The optimum germination temperatures of maize seed are 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 temperatures are 13-14ºC or more than those, 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 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 others, or the direction of the wind or others, 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 and others on locations other than cultivation fields have grown.

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

Composition of donor nucleic acids and origins of component elements used for the development of the Lepidoptera resistant maize (*cry1Ab, Zea mays* L.*)*(MON810, OECD UI No.: MON-ØØ81Ø-6) (hereinafter referred to as this recombinant maize) is shown in Table 1.

ii) Functions of component elements

Functions of component elements which were used for the development of this recombinant maize is shown in Table 1.

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

The Cry1Ab protein exhibits insecticidal activity only against insects of the order Lepidoptera and not against insects of other orders. Also, this Cry1Ab protein is known to exhibit insecticidal activity against the major pest insects of the order Lepidoptera for maize growing in the US, including 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 pest insect of the order Lepidoptera for maize growing in Japan.

In order to investigate whether the Cry1Ab protein shares functionally important amino acid sequences with known contact allergens, the Cry1Ab protein was compared with contact allergens in the database. As a result, the Cry1Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

(2) Information concerning vector

i) Name and origin

The vector used for the production of this recombinant maze is plasmid pUC119 from *Escherichia coli.*

ii) Properties

The total numbers of base pairs of the vectors are 7,794 bp for PV-ZMBK07 and 9,427 bp for PV-ZMGT10. These 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

For the production of this recombinant maize, two plasmids, PV-ZMBK07 and PV-ZMGT10 were used as vectors. PV-ZMBK07 was constructed by connecting the *cry1Ab* gene cassette ([E35S]-[hsp70 intron]-[*cry1A*b]-[NOS3']), and PV-ZMGT10 was constructed by connecting the*CP4 EPSPS* gene cassette ([E35S]-[hsp70 intron]-[CTP2]-[*CP4 EPSP*S]-[NOS 3']) and *GOX gene cassette* ([E35S]-[hsp70 intron]-[CTP1]-[*GO*X]-[NOS 3']), respectively to a basic vector derived from pUC119 containing the above-mentioned *nptII* gene.

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

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 that are classified into dent type.

iii) Processes of rearing of living modified organisms

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 an excellent event. In field experiments 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, necessary approval was obtained in the US and general commercial cultivation began in 1997.

Table 1 Component elements of plasmids PV-ZMBK07 and PV-ZMGT10 which were used for insertion, and their origins and functions

Component elements	Origin and function
cry1Ab gene cassette	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
cry1Ab	The gene which encodes Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain in the soil. The detail of its function was described in p.2.
NOS 3’	3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
CP4 EPSPS gene cassette（As a result of inserted gene analysis, this was not inserted into this recombinant maize.）	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis EPSPS* gene. Transfers target proteins from cytoplasm to chloroplast.
CP4 EPSPS	A synthetic sequence generated based on 5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium.* Expresses CP4 EPSPS protein, which has high levels of glyphosate tolerance.
NOS 3’	3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
GOX gene cassette （As a result of inserted gene analysis, this was not inserted into this recombinant maize.）	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV) Makes target genes expressed in all the tissues constantly.
Hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 1	N-terminal chloroplast transit peptide of the small subunit 1A of rubisco gene from *A. thaliana*. Transfers target protein from cytoplasm to chloroplast.
GOX	A synthetic sequence generated based on glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein degrades glyphosate.
NOS 3’	3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* Contains transcription terminator and polyadenylation signal for mRNA.
Backbone (common to PV-ZMBK07 and PV-ZMGT10)（As a result of inserted gene analysis, this was not inserted into this recombinant maize.）	
LacZ	Partial coding sequence for β-D-galactosidase or LacZ protein. Used as a selectable marker in cloning experiments in *E.coli,* since blue color appears as the substrate Xgal is degraded by β-D-galactosidase.
Ori-pUC	A segment containing replication origin for *E. coli* plasmid pUC. Starts the replication of the plasmid.
NptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II. Utilized as a selectable marker for transformation since it confers resistance to kanamycin when being expressed in bacteria.

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

Based on Southern blotting 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 this recombinant maize at 1 site. Also, Southern blotting analyses of multiple generations of the plant indicated that the inserted gene is stably inherited in offspring. It was also confirmed that resistance to Lepidopteran pest insects is stably expressed in multiple generations.

In addition, as a result of Southern blotting analyses, it was confirmed that only the region from PV-ZHMBK07 which is required for the expression of Cry1Ab protein was inserted into the genome of the maize, but neither the *nptII* gene nor expression cassettes of the CP4 EPSPS gene and *GOX* gene from PV-ZMGT10 was inserted.

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

i) With the expression of the Cry1Ab protein, which is encoded by this *cry1Ab* gene, in various regions of the plant, resistance to corn borers (*Ostrinia nubilalis*), which is the major pest insect of the order Lepidoptera, was conferred to this recombinant maize.

ii) Isolated field tests were carried out using MON810AX and MON810BX which belong to the event of this recombinant maize, as well as MON810AC and MON810BC as the control lines.

(a) Morphological and growth characteristics

Providing this recombinant maize and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tassel exsertion, time of silking, time of flower initiation, time of flower completion, flowering period, time of maturation, plant type, tiller number, total number of ears, number of productive ears, culm length, height of ear, grain color, grain shape, and fresh weight after harvesting. Statistically significant difference was not observed between recombinant and non-recombinant control maize lines in all of the characteristics except for culm length. In culm length, statistically significant difference was found between recombinant maize MON810BX and non-recombinant control maize MON810BC, with the average length of 248.1 cm and 229.3 cm, respectively. While, no statistically significant difference was found between recombinant maize MON810AX and non-recombinant control maize MON810AC.

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

Sensitivity to low temperatures (between highest temperatures of 12-14ºC, and lowest temperature of 2ºC) of the seedlings of this recombinant maize and non-recombinant control maize was evaluated. All of the opened leaves presented withered conditions, on the 21st day, and no difference was observed between this recombinant maize and non-recombinant control maize.

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

Maize is an annual plant, and after ripening it usually dies down to the ground naturally in winter. Overwintering test for the matured plant of this recombinant maize was not carried out, since it does not regrow and propagate vegetatively, or produce seeds.

(d) Fertility and size of the pollen

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

(e) Production, germination rate, dormancy, and shedding habit 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 statistically significant difference was observed between this recombinant maize and non-recombinant control maize in all of the characteristics examined.

(f) Hybridization

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

(g) Production of harmful substances

Plow-in tests, succeeding crop tests, and soil microflora tests were performed providing this recombinant maize and a non-recombinant control maize. Statistically significant difference was not observed in all the items except in the succeeding crop test between this recombinant maize and the non-recombinant control maize. Only in the succeeding crop test, statistically significant difference was observed in the growth of the test plant (lettuce) between recombinant maize MON810AX and non-recombinant maize MON810AC. Since this was considered to be attributable to the nonuniformity of germination and growth of the lettuce, the following changes were made and the test was carried out again: paper mulch was utilized after sowing; water was supplied from the bottom of the pot to prevent crust formation on the soil surface due to surface affusion; and temperature was set at the optimum level during the test period, in order to ensure uniformity of growth. As a result, the lettuce germinated and grew uniformly. Retest under these test conditions revealed no statistically significant difference in the growth of lettuce between MON810AX and MON810AC.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

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

(2) Emergency measures which should be taken to prevent Adverse Effect on Biological Diversity in case Adverse Effect on Biological Diversity could arise

Refer to the attached Plans of Emergency Measures.

(3) Information obtained from Use abroad

From 1993 to 1995, field tests were conducted at 98 sites in the US to examine morphological and growing characteristics including germination rate, plant vigor, plant height, flowering time, and time of silking, as well as yield, pest sensitivity except for pest sensitivity to Lepidoptera, and wintering ability. As a result, no difference has been reported between the recombinant and the non-recombinant maize.

This recombinant maize has been commercially cultivated overseas including the United States, Canada, Argentina, South Africa, Philippine, Uruguay, and Spain.

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

1. Dominance in competition

As a result of comparative examination of various traits of the recombinant maize and non-recombinant control maize relating to dominance in competition, statistically significant difference was observed in the culm length between them. This recombinant maize possesses Lepidoptera resistance, which may raise the survival rate of the recombinant plant temporarily. However, since there was no significant difference in the traits relating to dominance in competition other than culm length and Lepidoptera resistance between the recombinant maize and the non-recombinant control maize, it cannot be considered that dominance in competition is raised with just these traits.

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

2. Production of harmful substances

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

As a result of expression of the Cry1Ab protein, the recombinant maize is conferred with resistance to corn borer (*Ostrinia nubilalis)*, one of the major maize pests. This creates the possibility that Lepidopteran insects which populate Japan will become affected if they consume pollen dispersed from the recombinant maize together with their feed plants when they are larvae.

Thus, insect species in the order Lepidoptera classified as endangered or sub-endangered species, and for whom the possibility of suffering the adverse effect resulting from the cultivation of the recombinant maize could not be denied, were first selected from the "Ministry of Environment's Red-list (revised edition 2000)". The list was narrowed down following consideration of 1) relationship between the active season (feeding season) of the larvae and flowering season of this recombinant maize, and 2) the possibility that the feed plant of the larvae is in contact with the pollen. Eleven species (including 2 subspecies) were identified.

The specifics of the adverse effect were then evaluated using *Zizeeria maha argia,* which is selected by the National Institute for Agro-Environmental Sciences as the insect for bioassays. This insect was selected because it meets the following criteria. 1) high sensitivity to the pollen of Bt maize, 2) ease of collective maintenance, 3) ease of sourcing and successive raising, and 4) sensitivity to various types of Bt toxin against Lepidopteran pests.

As a result of comparison of the survival rate of the 1st instar larvae of *Zizeeria maha argia* fed with pollen from the recombinant maize and the non-recombinant control maize, statistically significant difference was observed within a pollen density range of 2,000-4,000 particles/cm^2, and the LC_{50} on the 5th day from the start of pollen feeding was 2,300 particles/cm^2.

As a result of comparison of the quantity and size of pollen between the recombinant maize and the non-recombinant control maize, no statistically significant difference was observed.

The distance within which pollen dispersion of the recombinant maize exerts the adverse effect was calculated by assigning the pollen density of 2,000-4,000 particles/cm^2 to the model equation of Kawashima et al., which indicates the relationship between the distance from the corn field and the number of dropped maize pollen. This model equation represents the maximum number of deposited pollen that cannot be exceeded under normal climatic conditions. As a result, it was estimated that the maximum ranges on which the pollen of recombinant maize deposit at the density of 4,000 particles/cm^2 and 2,000 particles/cm^2 are 10 m and 20 m, respectively.

Also, according to literature information, the plants consumed by larvae of the 11 species (including 2 subspecies), which were identified as the Lepidopteran insects possible to be affected by the recombinant maize, inhabit broad areas including open fields and mountains. The cultivation fields of the maize surrounding area of the fields are not the main habitat for these plants.

From these results, it was concluded that the possibility where the dispersion of pollen would disturb the maintenance of species or individual populations is extremely low, based on the assessment of the range within which the dispersion of the pollen of the recombinant maize is expected to exert adverse effect and the judgment regarding the habitat of plants fed by the larvae of the 11 species of Lepidopteran insects (including 2 subspecies).

Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity attributable to the 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 understanding, it was concluded that there is no risk of adverse effect on biological diversity attributable to hybridization.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

Maize, the species to which the recipient organism belongs, has long been used in Japan. Also, there was no difference in the various traits relating to dominance in competition between the recombinant maize and the non-recombinant maize. Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity attributable to dominance in competition.

Various traits related to the production of harmful substances were evaluated in the plow-in test, succeeding crop test, and soil microflora test, and no difference was found between the recombinant maize and the non-recombinant maize. In addition, the adverse effect on 11 species (including 2 subspecies) of insects of genus Lepidoptera which were regarded to be susceptible to the adverse effect of pollen dispersion of this recombinant maize in Japan were examined. It was concluded that the possibility that they are affected by pollen at the individual population level is extremely low because the range within which the pollen of this recombinant maize exert adverse effect is estimated within 10-20 m around the field and also because the nontargeted Lepidopteran insects which originally inhabit the natural ecosystem do not inhabit mainly around the maize cultivating field. Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity attributable to the production of harmful substances.

Since there is no wild plant which can be hybridized with maize, it was considered that there is no risk of adverse effect on biological diversity attributable to hybridization

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