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 (*cp4 epsps*, *cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis) (MON88017, OECD UI: MON-88Ø17-3) |
| Content of the Type 1 Use of Living Modified Organism | Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them. |
| Method of the Type 1 Use of Living Modified Organism | — |

**Outline of the Biological Diversity Risk Assessment Report**

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

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

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

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

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

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

(2) History and present state of Use

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

ii) At present, maize is used mainly for feed, but also for food and various food products including cooking oil and starch. Currently, it is the most widely cultivated grain in the world and can be grown in the area from a latitude of 58 degrees north to 40 degrees south mainly in the US, China, Brazil, Argentina, and European countries and others. Based on the statistical information of the Food and Agriculture Organization (FAO) of the United Nations, in 2002 the world's cultivated area of maize was about 140 million hectares. The seven top countries were the US (28 million hectares), China (25 million hectares), Brazil (12 million hectares), Mexico (7 million hectares), India (6 million hectares), Nigeria (4 million hectares), and South Africa (3 million hectares). Also, according to the same statistical information of the FAO, the cultivated area of maize in Japan in 2002 was about 30 thousand hectares.

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

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

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

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

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

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

ii) Mode of propagation or reproduction

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

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

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

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

d) Maize has a typical wind pollinated flower. A tassel of maize has 1,200-2,000 spikelets, and produces 16 million to 30 million pollen grains. The longevity of pollen is within 24 hours in the conditions of a field in high summer, but there is a range of longevity of from two hours to eight days according to the environmental conditions. Maize pollen is spherical and the diameter of the pollen is 90-100 μm. Maize mainly propagates in through cross-pollination by wind, but there is a 1-5% possibility of self-pollination in normal field conditions. The pollens dispersing from bloomed tassels attach to the silks extracted from ears and then they germinate. The fertilization of maize is completed within 24 hours. The dispersion distance of maize pollen differs by the presence of barriers such as woods or mountains, or the direction of the wind, but is considered to be approximately 300-500 m.

iii) 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, etc., 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

The composition of donor nucleic acid and the origins of component elements used for the development of the maize tolerant to glyphosate herbicide and resistant to Coleoptera (*cp4 epsps, cry 3Bb1, Zea mays* subsp. *mays* (L.) Iltis) (MON88017, OECD UI: MON-88Ø17-3) (hereinafter referred to as “this recombinant maize”) are shown in Table 1 (p 5-6). In this recombinant maize, *cry3Bb1* gene, which was modified from the wild-type *cry3Bb1* gene was inserted. Hereinafter the gene is referred to as “modified *cry3Bb1* gene” and the protein being expressed is referred to as “modified Cry3Bb1 protein”.

Table 1 Component elements of plasmid PV-ZMIR39 which were used for insertion, and their functions

| Component elements | Size (Kbp) | Origin and function |
|---|---|---|
| *cp4 epsps* gene cassette | | |
| P-ract | 0.93 | Promoter region of actin 1 gene derived from rice. It makes target genes expressed. |
| ract1 intron | 0.46 | Intron of rice actin gene. It makes target genes expressed by enhancing splicing. |
| CTP2 | 0.23 | N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast. |
| *cp4 epsps* | 1.37 | 5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4. Details of functions are shown on page 8. |
| NOS 3’ | 0.26 | 3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation. |
| Modified *cry3Bb1* gene cassette | | |
| P-e35S | 0.61 | A promoter from the cauliflower mosaic virus (CaMV). It has the function to express introduced genes in all tissues constantly. |
| wt CAB leader | 0.07 | 5’-terminal untranslated region of wheat chlorophyll a/b binding protein. Enhances the expression of target gene. |
| ract1 intron | 0.46 | Intron of rice actin gene. It makes target genes expressed by enhancing splicing. |
| Modified *cry3Bb1* | 1.96 | The gene which encodes modified Cry3Bb1 protein of *Bacillus thuringiensis.* Details of its functions are described on p 9. |
| tahsp 17 3’ | 0.23 | 3’-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation. |
| Components of T-DNA backbone | | |
| RB | 0.02 | 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. |
| *Aad* | 0.79 | The gene encoding the Tn7 adenyltransferase (AAD) derived from *Staphylococcus aureus.* Confers resistance to spectinomycin or streptomycin. |
| ori-322 | 0.63 | The replication origin isolated from pBR322. Permits autonomous replication of vectors in *E.coli*. |
| ROP | 0.19 | A coding sequence to repress primer protein to maintain the number of copies of plasmids in *E. coli*. |
| ori-V | 0.39 | The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens.* |
| LB | 0.02 | A DNA sequence of left border sequence derived from Ti plasmid pTiA6. Defines the terminal of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome. |

ii) Functions of component elements

Functions of component elements which were used for the development of this recombinant maize are shown in Table 1 (page 5).

【cp4 epsps gene】

a) Glyphosate is the active ingredient in Roundup, a nonselective herbicide, and inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis by specifically binding to the enzyme. As a result, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. A *cp4 epsps* gene expresses the CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The activity of the CP4 EPSPS protein that is produced by *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus, the recombinant plants that express this protein have normal functions of shikimate synthesis and 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. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway, but it has been clarified to be extremely unlikely that the stages from DAHP to the synthesis of chorismic acid are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids. In addition, Monsanto Co. examined amino acid contents in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the Roundup herbicides, and confirmed that there is no difference in the aromatic amino acid content between the original non-recombinant plants and recombinant plants. These facts support that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS is the enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), and is known to specifically react with these substrates. The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

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

【Modified *cry3Bb1* gene】

a) The modified *cry3Bb1* gene, the target gene to confer Coleoptera resistance, is derived from *Bacillus thuringiensis* subsp. *kumamotoensis,* a gram-positive bacterium, universally exists in soil. The modified Cry3Bb1protein which is encoded by the modified *cry3Bb1* gene possesses an insecticidal activity against corn rootworm (*Diabrotica* sp.) (hereinafter referred to as CRW), which is one of the major pest insects of order Coleoptera to maize cultivation in the US. This insect damages the roots of maize. *B.t.* proteins which are produced by the bacterium *B.t.* including modified Cry3Bb1 protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pores, which leads to the inhibition of the digestive process and results in the insecticide activity.

   The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow, and the modified Cry3Bb1 protein shows the insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata*) (hereinafter referred to as CPB) and CRW, which are respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera. 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.

   Compared with the wild-type Cry3Bb1 protein, the alternation-type modified Cry3Bb1 protein has 98% or more homology. In practice, the insecticidal efficiency is examined in the field with the use of modified Cry3Bb1 protein.

b) 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. As a result, the modified Cry3Bb1 protein did not share structurally related sequences with known allergens.

(2) Information concerning vector

i) Name and origin

The vector used for the production of this recombinant maze is plasmid pBR322 from *Escherichia coli.*

ii) Properties

The total number of base pairs in the vectors are 12,368 bp. These vectors contain a spectinomycin/streptomycin-resistant gene (*aad* gene) derived from *E. coli* as the selectable marker gene for the construction vector. The infectivity of this vector is not known.

(3) Method of preparing living modified organisms

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

Based on the above mentioned vector derived from pBR322, the plasmid PV-ZMIR39 to be used for the production of this recombinant maize was constructed.

T-DNA region in this vector transferred in the recipient organism is constructed with *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’].

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

T-DNA region in plasmid vector PV-ZMIR39 was introduced by Agrobacterium method to the hybrid A x F1 (HiII) that is classified into dent type.

iii) Processes of rearing of living modified organisms

a) The development of this recombinant maize was started in 1999. T-DNA region in plasmid vector PV-ZMIR39 was introduced by Agrobacterium method to the hybrid A x F1 (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, and the strain invested with torelance to glyphosate herbicide and resistance to pest was selected.

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

c) Field experiments were carried out at 169 field sites from 2000 to 2001. The strain for the final commercial cultivation was selected, and its environmental safety was evaluated.

In Japan, in April 2003, based on the “Guideline for the use of recombinant in agriculture, forestry and fisheries”, import to Japan (to be used for processing and feed) and cultivation were approved by the Ministry of Agriculture, Forestry and Fisheries.

(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 T-DNA region was inserted at a site in the genome of this recombinant maize. Also, the integrity of *cp4 epsps* gene expression cassette in T-DNA region and modified *cry3Bb1* gene expression cassette was examined by analyzing the component elements of each of the gene expression cassettes as the probe. In the *cp4 epsps* gene expression cassette, each of the component elements of P-ract + ract1 intron, CTP2 + *cp4 epsps* and NOS 3’ were analyzed as the probe. While in modified *cry3Bb1* gene expression cassette, each of the component elements of P-e35S, wt CAB leader + ract1 intron, modified *Cry3Bb1 and* tahsp17 3’ were analyzed as the probe. As a result, each of the component elements of *cp4 epsps* gene expression cassette and modified *cry3Bb1* gene expression cassette were inserted in integrity condition. The backbone region other than T-DNA region which includes *aad* gene and others was not inserted. In addition, as a result of Southern blotting analyses on multiple generations, it was indicated that inserted genes were inherited stably in offspring. Also, glyphosate-tolerance test and ELISA with the use of antibody of Cry3Bb1 protein in the process of selection indicated that the torelance to glyphosate herbicide and resistance to Coleoptera were inherited stably in offspring.

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

a) With the expression of the CP4 EPSPS protein, which is encoded by the *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.

With the expression of the modified Cry3Bbl protein, which is encoded by modified *cry3Bb1* gene, resistance to corn rootworms (CRW), which is the major pest insect of the order Coleoptera, was conferred to this recombinant maize. Also, a decrease in feeding damage by CRW was confirmed. Roots of maize are damaged by CRW, but the modified Cry3Bb1 proteinprotects the leaves, pollens and roots of this recombinant maize.

b) The tests were carried out in the isolated field using MON88017-A and MON88017-B (hereinafter referred to as 017-A and 017-B) lines which belong to this recombinant maize, as well as Cont-A and Cont-B as the control lines. 017-A and 017-B are the F1 hybrids derived from the different rearing process from the first generation (RO) of this recombinant maize. While, Cont-A and Cont-B are F1 hybrids of non-recombinant control maize hybridized to have the same hereditary background with 017-A and 017-B.

(a) Morphological and growth characteristics

For this recombinant maize and the non-recombinant control maize, evaluation was conducted regarding uniformity of germination, germination rate, time of tassel exsertion, 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. Regarding culm length, statistically significant difference was found between the recombinant maize, 017-B and the non-recombinant maize, Cont-B, and the average value of culm length was 226.9 cm for 017-B and 233.4 cm for Cont-B. Meanwhile no statistically significant difference was observed between the recombinant maize, 017-A and the non-recombinant control maize, Cont-A.

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

Sensitivity to low temperature (temperature of 5℃) of the seeding of this recombinant maize 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.

(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 both this recombinant maize and 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, pollens were stained with 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

Regarding the production of the seed, 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 this recombinant maize and non-recombinant control maize in any of the characteristics examined except in ear diameter. 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. Meanwhile no statistically significant difference was observed between the recombinant maize, 017-A and the non-recombinant control maize, Cont-A. Regarding shedding habit of the seed, shedding habit was not observed in the natural condition, since the ears of both recombinant maize and non-recombinant control maize were covered with bracts at the time of harvesting. Regarding germination rate on the 10th day of sowing of harvested seeds, no difference was observed between the recombinant maize and non-recombinant control maize, and no dormancy of the seeds was examined.

(f) Hybridization

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

(g) Production of harmful substances

Soil microflora tests, succeeding crop tests, and plow-in tests were performed for this recombinant maize and non-recombinant control maize. Statistically significant differnce 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. Regarding the fresh weight of *Raphanus sativus* var. *radicula*, which was the assay plant of the plow-in test, statistically significant differnce was found between the recombinant maize, 017-A and the non-recombinant control maize, Cont-A, and the average value was 7.17g for 017-A and 8.38g for Cont-A.

## 3. Information concerning the Use of living modified organisms

(1) Content of the Use

Use, cultivation, processing, storage, transportation, and disposal of the LMO to be served as food or feed, and other acts incidental to them.

(2) Information obtained from Use abroad

Field tests were implemented in the US in 2001 to observe the characteristics including morphological and growth characteristics, sensitivity to pests, and characteristics of propagation between this recombinant maize and non-recombinant control maize. As a result, no statistically significant difference was observed between the recombinant maize, MON88017 and the non-recombinant control maize in any of the characteristics examined except seedling establishment and the number of mature seedlings in morphological and growth characteristics. Regarding seedling establishment and the number of mature seedlings in which statistically significant difference was observed, the difference of the mean value was small (within 10%) between the recombinant maize, MON88017 and the non-recombinant control maize.

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

### 1. Competitiveness

Regarding the maize (*Zea mays* subsp. *mays*（L.）Iltis.), to which the recipient organism belongs, the Type 1 Use has been conducted in Japan, but there is no report that it has become self-seedling so far.

This recombinant maize is given a trait to be strongly tolerant to glyphosate herbicide and to be resistant to Coleoptera by transferred *cp4 epsps* and modified *cry3Bb1 genes*. But as a result of the examination of traits concerning dominance in competitiveness in isolated fields in Japan, statistically significant difference was found in culm length and ear diameter between this recombinant maize and the non-recombinant control maize. However, it is not generally considered that tolerance to glyphosate becomes the pressure for selection under the natural environment, and also the difference in culm length and ear diameter between this recombinant maize and the non-recombinant control maize was small. Based on the above understanding, it is unlikely that this recombinant maize will be more competitive than the non-recombinant control maize in competition with wild plants. Also it is difficult to consider that this recombinant maize comes to dominate the other wild plants, even if this recombinant maize is given resistance to Coleoptera and is not damaged by Coleopteran insects.

Consequently, no wild animals and wild plants were specified to be possibly affected, and it was judged that the conclusion by the applicant that the use of the recombinant maize posed no significant risk of Adverse Effect on Biological Diversity attributable to dominance in competition is reasonable.

### 2. Productivity of harmful substances

Regarding the maize (*Zea mays* subp. *mays* (L.) Iltis.), to which the recipient organism belongs, there is no report that it produces harmful substances to affect wild animals and wild plants.

The recombinant maize possesses a trait to produce CP4 EPSPS protein that has a strong tolerance to glyphosate herbicide, but this protein is not reported as a harmful substance. EPSPS protein is one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis, but it is proved that EPSPS is not the rate-determining enzyme. It was also confirmed that the content of aromatic amino acid in the other recombinant maize in which *ep4 epsps* is transferred has not changed. It is thus concluded that the recombinant maize does not produce an excess of aromatic amino acid. In addition, since EPSPS protein is an enzyme that reacts specifically with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), CP4 EPSPS protein is considered not to produce different substances to catalyze the other substances.

In Japan, the productivity of harmful substances (including secretion from roots to affect the other plants, secretion from roots to affect microorganisms in soil, and substances in the plant body to affect the other plants) was investigated in isolated fields, but there is no significant difference between recombinant maize and non-recombinant maize.

This recombinant maize produces modified Cry3Bb1 protein which is one of the *B.t.* proteins and exhibits the insecticidal activity against Coleoptera. At present, modified Cry3Bb1 protein shows the insecticidal activity only against the Colorado potato beetle（*Leptinotarsa decimlineata*） and Corn rootworm（*Diabrotica* spp.）, which are classified respectively into two genera of the family *Chrysomelidae*, among the order Coleoptera. As a result of literature searching, it was confirmed that these Coleopteran insects mentioned above do not inhabit in Japan. However, it cannot be denied that this recombinant maize shows the insecticidal activity against uninvestigated Coleoptera, so the following investigation was carried out. Also, it was confirmed that only 1st and 2nd instar larvae were sensitve to *B.t.* protein.

a) Identification of wildlife likely to be affected

As the exposure pathway of modified Cry3Bb1 protein to the 1st and 2nd instar larvae of Coleoptera, the eating habit of Coleopteran insects of the plant bodies or pollens of this recombinant maize which was cultivated in the fields or spilled during transportation is identified. Taking into account the fact that maize-eating Coleopteran insects have been listed as an agricultural pest, and also taking into account the habitat, behavior and area of distribution of Coleopteran insects, the following Coleopteran insects are designated as wildlife likely to be affected: the 1st and 2nd instar larvae of *Chrysolina angsticollis*, *Catapionus virdimetallicus*, and *Epilachna niponica* LEWIS and others.

b) Evaluation of concrete details of adverse effect

Mortality was compared between the larvae of Colorado Potato Beetle (within 24H after hatching) fed by pollen of this recombinant maize and those fed by pollen of non-recombinant maize. It has been generally known that Colorado Potato Beetle has the highest sensitivity to the modified CryBb1 protein. As a result, significant differences in the mortality were observed at the density of 2,000 particles/$cm^2$ and more.

c) Evaluation of likelihood of adverse effect

In the case of cultivation of this recombinant maize in fields, the maximum distance of pollen dispersal at the density of 2,000 particles/$cm^2$ has been estimated as 20m based on a model equation. Therefore, if the Coleopteran insects, which is mentioned in a) as to be possibly affected by the CryBb1 protein, have a similar degree of sensitivity to that of the Colorado potato beetle, it is considered that the Coleopteran insects being within 20 m of the fields may be affected in some way. However, the main habitat of these Coleopteran insects is not in the cultivation fields of maize or the surroundings. Further, it is thought that even if these Coleopteran insects incidentally moved into an area within 20 m of the field, it would only be a small portion of these Coleopteran species or a group of individuals. In addition, even if the recombinant maize is spilled during transportation on locations other than the agricultural fields and grow, it is considered that this recombinant maize would not release the amount of pollen necessary to affect the Coleopteran insects, because the number of individuals of the spilt recombinant maize should be extremely small.

Because the above consideration has been based on the most sensitive bioassay, and on the maximum predicted distance of pollen dispersal under the normal climate condition, it is considered that the conclusion will not be affected even if the pollen dispersal distance will be changed due to the difference of the strain of maize belonging to the same event.

Based on the above, the modified Cry3Bb1 protein produced by this recombinant maize is considered not to be detrimental to maintaining these Coleopteran species or groups of individuals.

From the above discussion, it was judged that the conclusion by the applicant that there is no significant risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is reasonable.

### 3. Crossability

In Japan, the growth of wild species that can hybridize with maize in nature has not been reported. Therefore, it was concluded that no wild species that will be affected by the crossability of this recombinant maize can be specified, and it was judged that the conclusion by the applicant that there is no significant risk of Adverse Effect on Biological Diversity attributable to hybridization is reasonable.

## 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 other than culm length and ear diameter concerning dominance in competition between this recombinant maize and the non-recombinant maize. There was statistically significant difference in culm length and ear diameter, but it revealed no significant difference in the other traits concerning dominance in competition. It cannot be considered that dominance in competition is raised with just the difference of culm length and ear diameter. In addition, it cannot be considered that dominance in competition is raised with having CP4 EPSPS protein and modified Cry3Bb1 protein in this 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.

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 this recombinant maize and the non-recombinant control maize. Also, the effects on 3 species of insects of the order Coleoptera which were regarded as wild inhabitants to be susceptible to the effect of dispersion of pollens 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 exerts an effect is estimated at within 20 m around the field and also because the nontargeted Coleopteran insects which originally inhabit the natural ecosystem do not mainly inhabit around maize cultivating fields. Based on the above understanding, it was concluded that there is no risk of Adverse Effect on Biological Diversity caused by the production of harmful substances.

In Japan, the growth of teosinte (related species of maize) and wild species in the genus *Tripsacum* has not been reported. Therefore, it was concluded that there is no risk of Adverse Effect on Biological Diversity attributable to hybridization.

Consequently, it was concluded that there is no risk that the use of this recombinant maize in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity.