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

Applicant

Name: Dow Chemical Japan Ltd.
Phillip Feil, Representative Director
Address: 2-24 Higashi Shinagawa 2-chome, Shinagawa-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Modified *cry1F*, modified *bar*, *Zea mays* subsp. *mays* (L.) Iltis) (TC6275, OECD UI：DAS-Ø6275-8)
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 preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Maize resistant to Lepidoptera and tolerant to glufosinate herbicide (modified *cry1F*, modified *bar*, *Zea mays* subsp. *mays* (L.) Iltis) (TC6275, OECD UI：DAS-Ø6275-8) is hereinafter referred to as "this recombinant maize."

The component elements were shown in Table 1.

Table 1　Component elements of the nucleic acid and their origins and functions

Component elements	Function
Modified *cry1F* cassette	
UBI1ZM	An ubiquitin promoter derived from maize (including intron and 5'-terminal untranslated sequence) (Christensen *et al*., 1992). It initiates gene transcription in the entire plant body.
Modified *cry1F*	A gene that encodes the core protein of *cry1F* gene, derived from *Bacillus thuringiensis* var. *aizawai*, allowing the expression of the modified Cry1F protein. It has increased the GC contents in the region encoding the core protein of Cry1F protein without changing any amino acid sequence in order to enhance the expression in the maize. By transferring of cloning sites, the 604th phenylalanine located in the C terminal region in the amino acid sequence of a total length of 606 has been replaced by leucine.
PINII	Proteinase inhibitor II terminator sequence from potato (An *et al*., 1989). It terminates gene transcription.
Modified *bar* cassette	
CaMV35S-1841 enhancer	An upstream enhancer derived from cauliflower mosaic virus (CaMV) 1841 strain (Pietrzak *et al*., 1986). It boosts the transcription efficiency of genes.
CaMV35S-1841 promoter	A 35S promoter derived from cauliflower mosaic virus (CaMV) 1841 strain (Pietrzak *et al*., 1986). It initiates gene transcription.
ADH1	Alcohol dehydrogenase intron 1 from maize. It enhances the expression of the modified *bar* gene and the modified PAT protein.
Modified *bar*	Phosphinothricin acetyltransferase gene isolated from *Streptomyces hygroscopicus* (Thompson *et al*., 1987) which expresses the modified PAT protein. It has modified the start codon to accommodate its expression in plants with the first amino acid replaced. Differently from wild type, the first amino acid is removed after translation only in this modified PAT protein.
PINII	Proteinase inhibitor II terminator sequence from potato (An *et al*., 1989). It terminates gene transcription.

2) Functions of component elements

(i) Functions of individual component elements of donor nucleic acid, including target gene, expression regulatory region, localization signal, and selective marker

Functions of component elements of the donor nucleic acid were shown in Table 1.

(ii) Functions of proteins produced by the expression of target gene and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

Bacillus thuringiensis var. *aizawai* (*B.t.a.*) universally exists in natural soil all over the world. All the strains of *Bacillus thuringiensis* produce an insecticidal protein known as delta-endotoxin. Most of the natural delta-endotoxins consist of nearly 120 to 140kDa protein (Schnepf *et al*., 1998). This protein, when ingested by sensitive species of insects, turns into the crystals of the full-length protein to be digested by the protease in the gut of the insects, and releases the core protein, which possesses insecticidal activity (Figure 1). The core protein is 65 to 70kDa, and it binds to the specific receptors in the midgut epithelium when ingested by insects, causing the three-dimensional coordinate structure of the protein to change and allowing the penetration through the membrane. Formation of oligomers of protein results in porous structure in the midgut cell membrane and occurrence of osmotic cytolysis, leading to death of insects (Figure 1). The modified Cry1F protein expressed by the modified *cry1F* gene constitutes a part of core protein of the Cry1F protein produced by the *B.t.* var. *aizawai*, and the modified *cry1F* gene has been synthesized to enhance its expression in maize by increasing the contents of G (guanine) and C (cytosine) in the region, which encodes the core protein of Cry1F protein, without changing any amino acid sequence. By transferring of cloning sites, in the amino acid sequence of a total length of 606, the 604th amino acid located in the C-terminal region has modified phenylalanine to leucine, though it has been confirmed to exhibit high resistance to European corn borer (*Ostrinia nubilalis*), Southwestern corn borer (*Diatraea grandiosella*), Fall armyworm (*Spodoptera frugiperda*) and other major pest insects for maize similarly as the wild-type Cry1F protein (Babcock *et al*., 2003).

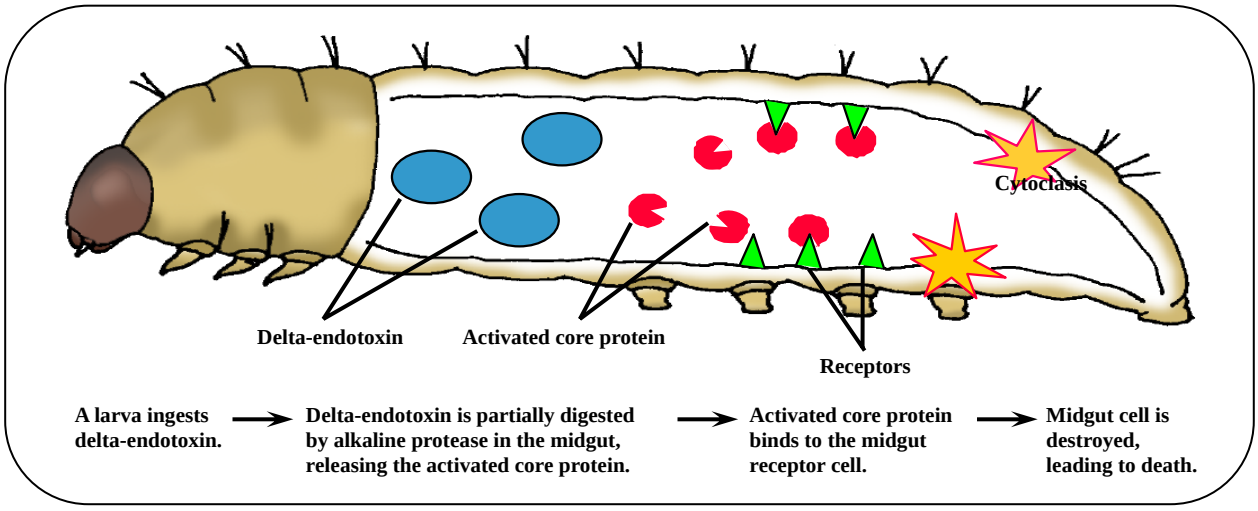

Figure 1 Mechanism of action of *B.t.* protein on the target insects including European corn borer (*Ostrinia nubilalis*)

The modified PAT protein expressed by the modified *bar* gene acetylates the herbicide glufosinate to transform it into nontoxic acetyl-glufosinate. This confers tolerance to herbicide (Figure 2).

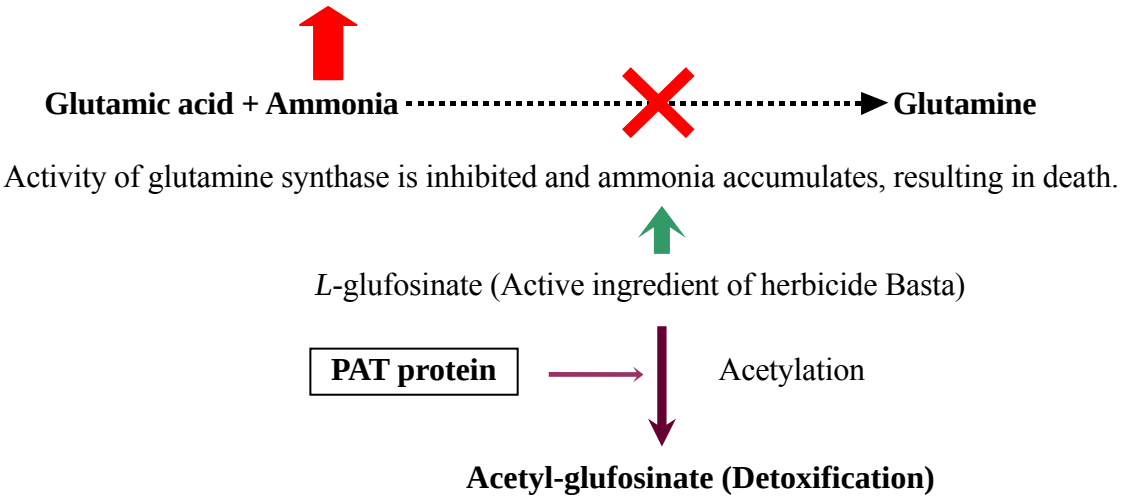

Figure 2 Mechanism of action of PAT (Phosphinothricin Acetyltransferase) protein on the herbicide glufosinate

In order to investigate whether the modified Cry1F protein and modified PAT protein share functionally important amino acid sequences with known allergens, the proteins were compared with allergens in the database (Swiss-Prot, PIR, GenPept, FARRP Protein Allergen Database). The results showed the modified Cry1F protein and the modified PAT protein did not share structurally related homologous sequences with any of the known allergens examine.

iii) Contents of any change caused to the metabolic system of recipient organism

The modified Cry1F protein is not an enzyme and thus it is considered not to affect any metabolic system of plants. In addition, the modified PAT protein is an enzyme which very specifically acetylates the glufosinate (Tompson *et al*., 1987). Consequently, it is considered unlikely that the modified PAT protein affects any other metabolic systems.

(2) Information concerning vector

1) Name and origin

The vector used for the production of the transferred PHP12537 is derived from *Agrobacterium tumefaciens* LBA 4404 strain.

2) Properties

i) The numbers of base pairs and nucleotide sequence of vector

The total number of base pairs of the expression vector PHP12537 is 49,698bp. The nucleotide sequences of the expression vector PHP12537 are shown in Annex 1.

ii) Presence or absence of nucleotide sequence having specific functions, and the functions

The *tet* and *spc* genes express the tetracycline and spectinomycin respectively, which are used for selection of the expression vector PHP12537. However, these genes are located outside the T-DNA region and thus, they have not been transferred into this recombinant maize.

iii) Presence or absence of infectivity of vector

The T-DNA region of the vector derived from the *A. tumefaciens* LBA 4404 strain, which was used for the construction of the expression vector PHP12537, has been replaced by the modified *cry1F* cassette and the modified *bar* cassette and thus, there is no sequence allowing infection of *Agrobacterium*; therefore, no infectivity has been reported.

(3) Method of preparing living modified organisms

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

The structure of the expression vector PHP12537 is shown in Figure 3. In addition, the process of production of the expression vector PHP12537 is presented in Annex 2. As a result of examination for the nucleotide sequences of the transferred gene and neighboring border regions in this recombinant maize, it is found that the nucleic acid has been transferred into genome of maize with parts of the UBI1ZM promoter and the UBI1ZM intron and the left border sequences lost in the T-DNA region on the expression vector PHP12537 (Figure 4). The nucleotide sequences of the DNA transferred to the genome of this recombinant maize and the neighboring border regions are shown in Annex 3.

2) Method of transferring nucleic acid transferred to the recipient organism

Agrobacterium method was used for transferring nucleic acid to the recipient organism.

3) Processes of rearing of living modified organisms

(i) Mode of selecting the cells containing the transferred nucleic acid

Transferred cells were selected on the medium containing herbicide glufosinate.

(ii) Presence or absence of remaining *Agrobacterium* in case of using *Agrobacterium* method for transferring nucleic acid

Agrobacterium was disinfected by addition of Carbenicillin, one of the antibiotics. Then callus was transferred to the medium for regeneration, which did not contain any antibiotic, and incubated, when it was confirmed that there was no residual *Agrobacterium*.

(iii) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity.

This recombinant maize was crossed with inbred line maize and selection was performed based on the comprehensive evaluations for resistance to European corn borer, tolerance to herbicide glufosinate and agronomic traits. Then commercial cultivar of maize was obtained through self-pollination and crossing with inbred line maize. Details are shown in Figure 5.

The following shows the approvals of this recombinant maize received from organizations in Japan.

June, 2004: The Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment granted the approval of Type I Use Regulations (isolated field test) in accordance with the "Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms."

May, 2006: The Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment granted the approval of Type I Use Regulations (isolated field test) in accordance with the "Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms."

September, 2006: An application was filed to the Ministry of Agriculture, Forestry and Fisheries for approval of the safety of use of the cultivar as feed based on the "Safety Evaluation Criteria for Feed and Additives Produced by Recombinant-DNA Techniques".

September, 2006: An application was filed to the Ministry of Health, Labour and Welfare for approval of the safety of use of the cultivar as food based on the "Safety Evaluation Criteria for Food derived from Recombinant-DNA Techniques (seed plant).

Figure 3 Structure of the expression vector PHP12537 and the section broken by restriction enzyme

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Figure 4 Outline of transfer of nucleic acid into the T-DNA region on the expression vector PHP12537

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Figure 5 Process of rearing of this recombinant maize TC6275

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

1) Location of the copy of transferred nucleic acid

The transferred nucleic acid follows the Mendel's law of inheritance once transferred in the chromosome of plant. A segregation analysis was conducted to identify how the traits introduced into this recombinant maize segregate in the populations of T1 and T1F2 generations (Annex 4). As a result of examination for presence of tolerance to herbicide glufosinate, it was found that nearly good agreement was obtained between the examination result and the segregation ratio expected from the Mendel's law for a single gene locus in the nucleus and thus, it was confirmed that the transferred nucleic acid is present on the chromosome (Table 2).

Table 2 Segregation of traits between T1 and T1F2 generations of this recombinant maize

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2) The number of copies of transferred nucleic acid

As a result of Southern blotting analysis to examine the number of copies of transferred nucleic acid in the T1F2 and BC5F1 generations, it was confirmed that one copy of both the modified *cry1F* gene and the modified *bar* gene has been transferred (Annex 5).

3) Nearby or separate location of multiple copies, if present, on the chromosome

There are no multiple copies on the chromosome.

4) The stability of the expression among individuals and generations under natural conditions

As a result of glufosinate herbicide spraying test on the leaves to identify the expression of the proteins by the transferred nucleic acid, it was confirmed that the proteins stably express in the individual generations (Annex 4).

To 338 individuals of Hybrid C (Figure 5) which exhibited tolerance to herbicide, hatchling of European corn borer was inoculated to identify the degree of insect damage. As a result, all the individuals exhibited resistance to insect damage by European corn borer. Based on the above results, it was confirmed that the expression of the modified Cry1F protein is stable across individuals.

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

This recombinant maize does not contain any sequence allowing transmission. Therefore, there is no possibility that the transferred genes to this recombinant maize could be transmitted to any other wild animals and wild plants.

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

For detection and identification of this recombinant maize, the PCR method has been developed using the nucleotide sequence specific to this recombinant maize as a primer (Annex 6). In addition, the Cry1F protein detection kit (Strategic Diagnostics Inc. in the US) and the PAT protein detection kit (EnviroLogix Inc. in the US) are commercially available.

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

1) Details of physiological or ecological properties conferred as a result of the expression of copies of the transferred nucleic acid (including the contents, if expressed specifically in specific tissue or at specific growth stage)

It was confirmed as a result of field tests in the US that this recombinant maize exhibits an excellent resistance to European corn borer (*Ostrinia nubilalis*), Southwestern corn borer (*Diatraea grandiosella*), and Fall armyworm (*Spodoptera frugiperda*), the major pest insects for maize, due to the expression of the modified Cry1F protein (Babcock *et al*., 2003). In addition, the Cry1F protein derived from *B.t*. var. *aizawai* exhibits the insecticidal activity against European corn borer, *Heliothis virescens* and *Spodoptera exigua* (Chambers *et al*., 1991).

On the other hand, it was considered unlikely that the modified Cry1F protein could affect the non-target organisms of *Daphnia magna*, earthworm, bobwhite quail, honey bee, *Chrysoperla carnea*, parasitic bee and ladybug including the non-target insects of order Lepidoptera such as *Danaus plexippus*, and *Lymantria dispar* (Annex 7).

The modified PAT protein is an enzyme that specifically acetylates the herbicide glufosinate and then, it is considered unlikely to affect any other metabolic systems.

2) Presence or absence, and if present, degree of difference between the genetically modified crop and the taxonomic species to which the recipient organism belongs with regard to the physiological and ecological characteristics

In FY 2006, isolated field tests were carried out at the National Institute of Livestock and Grassland Science, National Agriculture and Bio-oriented Technology Research Advancement Institution to identify any difference between this recombinant maize and the non-recombinant control maize (Isolated field test result report).

(i) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between this recombinant maize and the non-recombinant control maize regarding the uniformity of germination, germination rate, time of tasseling, time of silking, culm length, plant shape, tiller number, height of formed ear, maturation time, total number of ears, number of productive ears, ear length, ear diameter, row number per ear, grain number per row, ear grain color, 100-kernel weight, ear grain shape, and fresh weight of the above-ground part at harvesting. This recombinant maize and the non-recombinant control maize both exhibited excellent uniformity of germination and no difference was observed between the both plants in time of tasseling and time of silking. The plant shape of both this recombinant maize and the non-recombinant control maize was found upright without any tiller, and no difference was observed also regarding the yellow-ripening time. There was no difference between this recombinant maize and the non-recombinant control maize in the total number of ears and the number of productive ears. In addition, this recombinant maize and the non-recombinant control maize both showed yellow ear grain color and wedge-like ear grain shape without any significant difference. Moreover, no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize regarding culm length, height of formed ear, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, and fresh weight of the above-ground part at harvesting. Regarding germination rate, a statistically significant difference was observed between this recombinant maize and the non-recombinant control maize, and the germination rate was lower for this recombinant maize compared to the non-recombinant control maize. However, the germination rate of harvested seeds was as high as 98.0% for both plants, this recombinant maize and the non-recombinant control maize.

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

Cold-tolerance at the early stage of growth of this recombinant maize and the non-recombinant control maize was examined. The seeds harvested in the field tests were sown in pots. The seedlings raised to second to third leaf stage were left to stand outdoors on December 30, 2006. As a result, all the individuals of both this recombinant maize and the non-recombinant control maize died in around 18 days and no difference was observed between the both plants.

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

Maize is a summer type annual plant, and after ripening the matured plant body usually withers and dies out. For this reason, overwintering tests were not carried out.

(iv) Fertility and size of the pollen

To examine the fertility (maturity) and size of the pollens of this recombinant maize and the non-recombinant control maize, the pollens were stained with the aceto-carmine solution and observed. As a result, regarding the fertility and size of the pollen, no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize.

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

Regarding production of the seed, the number of productive ears, row number per ear, grain number per row, and 100-kernel weight were compared between this recombinant maize and the non-recombinant control maize. As a result, no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize in all of the items examined; therefore, it was judged that there is no difference in production of the seed between this recombinant maize and the non-recombinant control maize. Regarding shedding habit of the seed, the ears of this recombinant maize and the non-recombinant control maize were covered with bracts at the time of harvesting and thus, shedding of the seeds was not observed. Regarding dormancy of the seed, the seed parents and the harvested seeds from both this recombinant maize and the non-recombinant control maize were found to offer higher germination rates, no difference was observed between the both plants and thus it was judged that dormancy of the seeds would be extremely low.

(vi) Crossability

Crossability test was not performed since there is no report that any wild relatives that can be crossed with maize are growing voluntarily in Japan.

(vii) Productivity of harmful substances

To compare the productivity of harmful substances of this recombinant maize to the non-recombinant control maize, succeeding crop test, plow-in test and soil microflora test were conducted.

<Succeeding crop test>

As a result of succeeding crop test of radish (test plant) using the soil cultivated with this recombinant maize and the non-recombinant control maize, no statistically significant difference was observed between the both plants regarding germination rate, plant height, fresh weight and dry weight of radish (test plant).

<Plow-in test>

As a result of plow-in test using the plant body of this recombinant maize and the non-recombinant control maize, no statistically significant difference was observed between the both plants regarding germination rate, plant height, fresh weight and dry weight of radish (test plant).

<Soil microflora test>

Soil at harvest time was collected from the cultivation fields of this recombinant maize and the non-recombinant control maize, and the number of filamentous fungi, bacteria and actinomyces was measured based on the dilution plate technique. As a result, no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize.

Based on the above results, regarding the productivity of harmful substances, it is considered that this recombinant maize could not produce any unexpected harmful substances.

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

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

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

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis), the species to which the recipient organism belongs, has been long cultivated and used in Japan, though there is no report that it has become self-seeding in a natural environment in Japan.

As a result of examination in the isolated fields in Japan regarding the morphological and growth characteristics of this recombinant maize, there was no significant difference or difference observed from the non-recombinant control maize in all the items examined but germination rate. In addition, even for the germination rate, for which a significant difference was observed, the germination rate of harvested seeds from this recombinant maize and the non-recombinant control maize was found as high as 98.0% for the both plants and thus, it is considered unlikely that this difference could cause this recombinant maize to become dominant in competition.

This recombinant maize is given traits to be resistant to the insects of order Lepidoptera due to the transferred modified *cry1F* gene and also to be tolerant to herbicide glufosinate due to the modified *bar* gene. However, it is not generally considered that

the insect damage by Lepidopteran insects is the major factor to inhibit the growth of maize and the herbicide glufosinate functions as a selective pressure under the natural environment in Japan; therefore it is hard to consider that these characteristics enhance the competitiveness of this recombinant maize.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant maize poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

There has been no report that maize, the species to which the recipient organism belongs, produces any harmful substances that could affect wild animals and wild plants.

In the isolated field tests in Japan, this recombinant maize has been investigated for productivity of any harmful substance (the substances secreted from the roots which can affect other plants, the substances secreted from the roots which can affect microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying) with the result that there is no significant difference observed from the non-recombinant control maize.

This recombinant maize produces the modified Cry1F protein which possesses the insecticidal activity against the insects of order Lepidoptera and the modified PAT protein (phosphinothricin acetyltransferase) which confers tolerance to glufosinate.

The modified Cry1F protein possesses the insecticidal activity against the insects of order Lepidoptera. This suggests that there is a possibility that the modified Cry1F protein expressed in the pollens of this recombinant maize when cultivated could affect the Lepidopteran insects inhabiting around the cultivation fields. In practice, however, based on the results of examination for possibility that wild species listed in the Red Data Book of the Ministry of the Environment (2006 edition) could ingest the pollens, bioassay on the ingestion of pollens in conjunction with food plants, and investigations on the degree of pollen dispersion around the cultivation fields, the extent of the effects, if present, is limited; therefore, it is considered extremely low that the pollens dispersed from this recombinant maize could affect wild animals and wild plants at levels of their individuals.

On the other hand, there has been no report that the modified PAT protein could adversely affect wild animals and wild plants. In addition, the modified PAT protein has high substrate specificity and then, it is considered not to affect the metabolic system of the recipient organism.

As a result of searching for homology with amino acid sequence, it has been confirmed that the modified Cry1F protein and the modified PAT protein have no sequence which is structurally homologous with any known allergens.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that

the use of this recombinant maize poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

3) Crossability

In the Japanese natural environment, there are no wild species which can cross with maize. Therefore, it was judged that there are no specific wild plants or wild animals that are possibly affected by this recombinant maize, and that the use of such maize poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by applicant is valid.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant maize in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Reference

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Annex List

Annex 1: Nucleotide sequence of the expression vector PHP12537
(Confidential: Not made available or disclosed to unauthorized person)

Annex 2: Process for production of the expression vector PHP12537
(Confidential: Not made available or disclosed to unauthorized person)

Annex 3: Sequences of transferred genes in the maize line 6275 and neighboring regions
(Confidential: Not made available or disclosed to unauthorized person)

Annex 4: Traits in individual generations of this recombinant maize based on the Mendel's Law of Segregation
(Confidential: Not made available or disclosed to unauthorized person)

Annex 5: Identification test for the presence of transferred nucleic acid in the cell
(Confidential: Not made available or disclosed to unauthorized person)

Annex 6: Methods for detection and identification of genetically modified organisms and validation tests for the sensitivity and reliability of the methods
(Confidential: Not made available or disclosed to unauthorized person)

Annex 7: Possible effects on non-target species
(Confidential: Not made available or disclosed to unauthorized person)

Annex 8: Letter for approval in the US
(Confidential: Not made available or disclosed to unauthorized person)

Annex 9: Environmental safety evaluation of Cry1F insect-resistant and glufosinate-tolerant maize line 1507 in isolated field (Excerpts)
(Confidential: Not made available or disclosed to unauthorized person)

Isolated field test report：

Isolated filed test report for the maize resistant to Lepidoptera and tolerant to glufosinate herbicide
(Modified *cry1F,* modified *bar, Zea mays* subsp. *mays* (L.) Iltis)(TC6275, OECD UI : DAS-Ø6275-8)(Confidential: Not made available or disclosed to unauthorized person)