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 (*mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (GA21, OECD UI: MON-ØØØ21-9)
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 concerning preparation of living modified organisms

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of maize tolerant to glyphosate herbicide (*mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (GA21, OECD UI: MON-ØØØ21-9) are shown in Table 1.

(2) Functions of component elements

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

Glyphosate is the active ingredient in Roundup, a nonselective herbicide, and inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis by specifically binding to the enzyme. 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 *mEPSPS* gene, which is the inserted gene of this recombinant maize, is the modified EPSPS gene of the original maize. It has been shown that the produced mEPSPS protein possesses lower sensitivity to glyphosate, but has equal level of the other functions being compared to EPSPS protein. The activity of the mEPSPS protein is not inhibited even under the presence of glyphosate, thus, the recombinant plants that express this protein have normal functions of shikimate synthesis and grow normally.

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-heptulonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It has been clarified to be extremely unlikely that the stages from DAHP, through the production of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSP) which is catalyzed by EPSPS, 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 that are tolerant to the Roundup herbicides. Those results confirmed that there is no difference in the content of aromatic amino acids, which are the end product of the shikimate pathway between the original non-recombinant plants and the 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.

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

2. Information concerning vector

(1) Name and origin

The plasmid vector pDPG434 used for the production of this recombinant maize is assembled from plasmids including pUC19 from *Escherichia coli*.

(2) Properties

This vector is composed of the followings; i) ampicillin resistance gene (*bla* gene) as a selective marker gene of the vector assembled in *E. coli*, ii) ori-pUC, the replication origin region and permits autonomous replication of vectors in *E. coli*, and iii) lacZ used as a selective marker for cloning in *E. coli*. The details of the component elements are shown in Table 1.

The total numbers of base pairs of the pDPG434 used for the production of this recombinant maize are 6,128 bp.

The infectivity of this vector is not known.

3. Method of preparing living modified organisms

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

Table 1 shows the component elements of the plasmid vector transferred in the recipient organism.

Figure 1 shows the location of the component elements in the vector and restriction sites.

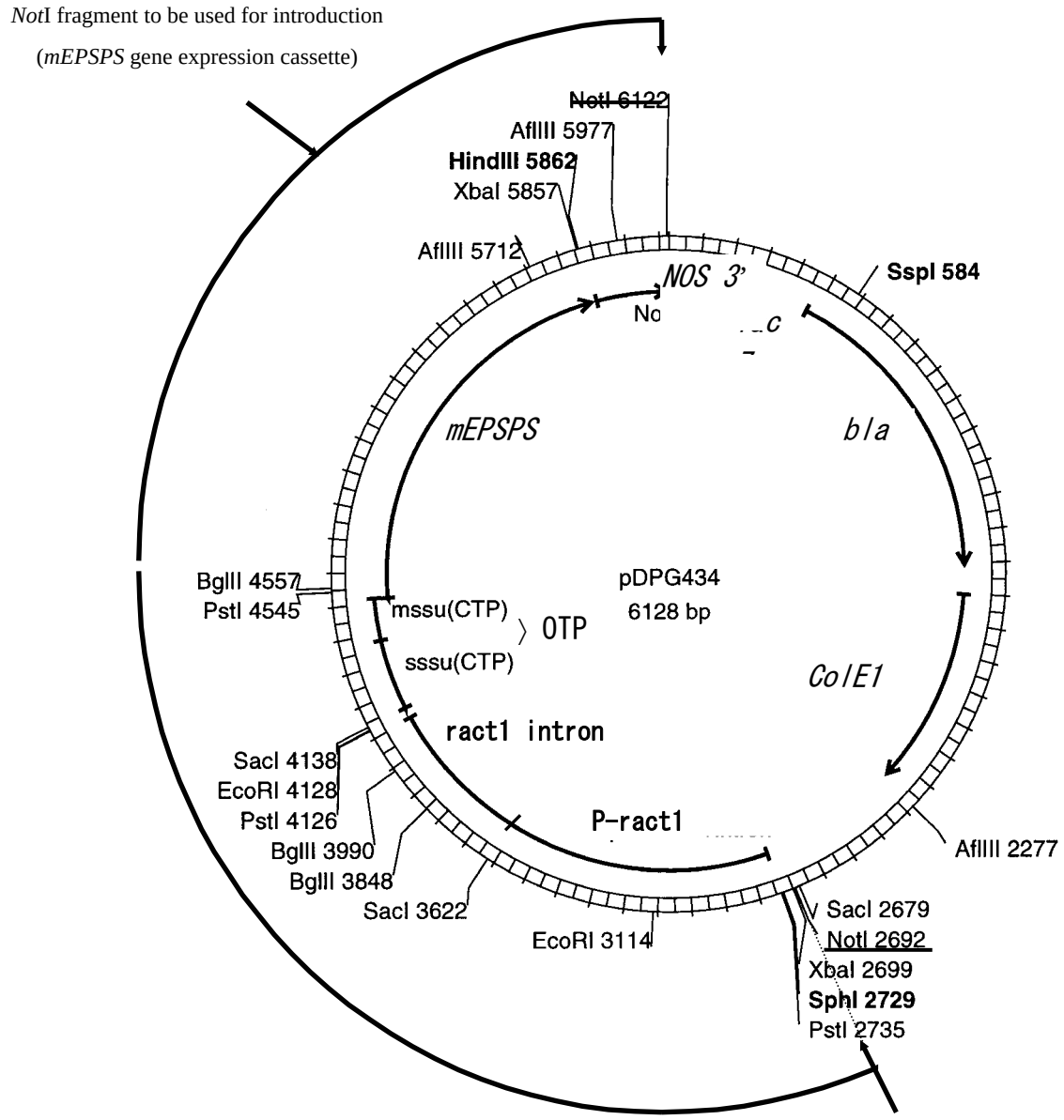

Figure 1 Plasmid vector

Table 1 Origins and functions of the component elements of *Not*I fragment of pDPG434 used for the development of this recombinant maize

Component elements	Origins and functions
P-ract	Promoter region of actin 1 gene derived from rice. It makes target genes expressed.
ract1 intron	Intron of rice actin gene. It makes target genes expressed by enhancing splicing.
OTP	OTP sequence created based on the chloroplast transportation peptide (CTP) sequences at N-terminal of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCo) of sunflowers (*Helianthus annus*) and maize (*Zea mays*). Transports the mEPSPS protein to chloroplasts, where synthesis of various aromatic amino acids occur.
mEPSPS	A modified gene of 5-enol-pyruvyl-shikimate-3-phosphate synthase gene (*epsps)* of *Zea mays* by region-specific mutation. The details of the function are shown in page 1 - 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.

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

For the production of this recombinant maize, *Not*I fragment of pDPG434, a linear nucleic acid (shown by the arrow in Figure 1) was introduced by particle gun bombardment to embryo culture callus that is classified into dent type.

(3) Processes of development of living modified organisms

i) The callus to which *Not*I fragment of pDPG434 was introduced was grown on a tissue culture medium containing glyphosate for a certain period of time, and then the recombinant plant was selected. From the selected callus, the regenerated plant was obtained and the gene introduced line was selected by ELISA and glyphosate tolerance analysis.

ii) DNA fragment was introduced to the recombinant maize by particle gun bombardment, so confirmation of remaining Agrobacterium was not carried out.

iii) Pedigree selection was started in 1992, and field experiments were carried out from 1994 to 1997. Finally, excellent lines were selected. In these field experiments, the

morphological and growth characteristics of these lines were investigated and also analysis of the expression level of the gene and inserted genes were implemented. Based on these results, necessary approval was obtained in the US and general commercial cultivation began in 1998.

The status of approval of this recombinant maize in Japan are the following.

December, 1998: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was certified by the Ministry of Agriculture, Forestry and Fisheries.

November, 1999: Based on the "Chapter 4 of the Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare.

December, 1999: Based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

March, 2003: Based on the "Procedure to confirm the safety of feed and additives derived from recombinant-DNA plants", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

March, 2003: Based on the "Procedure for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants", safety of use for food was approved by the Ministry of Health, Labour and Welfare.

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

As a result of Southern blotting analysis, the inserted gene was present at one site in the genome of this recombinant maize. In addition, more detailed analyses were performed based on nucleotide sequencing by PCR and Southern blotting analysis. It revealed that this inserted gene consisted of the following components; i) a copy of the *mEPSPS* gene cassette lacking part of promoter 5' terminal, ii) three copies of the *mEPSPS* gene cassette in the intact form, iii) part of the *mEPSPS* gene cassette, and iv) part of the ract promoter.

"Part of the *mEPSPS* gene cassette" in iii) was a truncated *mEPSPS* gene consisting of the ract promoter, ract1 intron, OTP, the first 289 bp of the *mEPSPS* gene, and a termination codon at its end. Since it was cocerned that the truncated *mEPSPS* gene might produce a shorter mRNA

than expected, we carried out Northern blotting analysis, which demonstrated that the gene did not produce a detectable amount of RNA. As a result of Western blotting analysis, in addition, GA21 showed only a single band with a size expected from the complete mEPSPS protein.

"Part of the ract promoter" in iv) contains the ract promoter only; it was truncated upstream of the beginning of the intron, with its 3' terminal fused with the maize genome DNA. There were possible two open reading frames, ORF-1 (97 amino acids) and ORF-2 (19 amino acids), in the maize genome DNA sequences downstream of the 3' terminal of the inserted gene. Northern blotting analysis detected no RNAs derived from this maize genome DNA sequence.

In addition, as a result of Southern blotting analysis of multiple generations, the inserted genes were stably inherited in the progeny. Also, it was confirmed during the selection process that the tolerance to glyphosate herbicide was stably expressed in multiple generations.

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

For the methods for detection and identification of this recombinant maize, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and neighboring regions of plant genome are used as primers. This method makes it possible to specifically detect this recombinant maize.

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

(1) With the constant expression of the mEPSPS protein, which is encoded by the *mEPSPS* gene, in various parts of the plant, tolerance to glyphosate herbicide is conferred to this recombinant maize. In practice, the expressions of mEPSPS protein in leaves and grains were confirmed. In addition, it was confirmed that the non-recombinant control maize died due to the influence of glyphosate herbicide, while the recombinant maize grew normally.

(2) The isolated field tests were carried out in the National Institute for Agro-Environmental Science in 1998, for this recombinant maize and the recurrent parent used for GA21 development as the control line.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, evaluation was conducted regarding the germination rate, uniformity of germination, date of tassel exertion, silking date, culm

length, plant shape or plant type, tiller number, ear height , maturation date, number of ears, number of effective ears, and fresh plant weight at harvesting time. No significant difference was observed in all items between this recombinant maize and the non-recombinant control maize.

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

Chilling tolerance tests at the early growth stage of this recombinant maize have not been conducted at the isolated field. However, in field tests conducted in the US between 1994 and 1997 and in commercial cultivation conducted since 1998, no cases were reported where this recombinant maize had shed seeds in the field at harvest time and then their seedlings grew to winter and survived until the following spring.

(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. Also, it never shows regrowth, vegetative reproduction, or seed production. It was actually observed that this recombinant maize was beginning to die at the end of the isolated filed test. For this reason, no wintering ability tests were carried out for matured plants.

(d) Fertility and size of the pollen

No adverse effects are expected from pollen dispersal because it was only herbicidal tolerance that was conferred to this recombinant maize. In addition, there are no close wild species that can cross with this recombianant maize. For these reasons, no tests were conducted for the fertility and size of pollen.

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

For ears obtained from the sibling crossing of this recombinant maize, we examined ear length, ear diameter, the number of rows per ear, the number of grains per row, and 100-kernel weight. As a result, no statistically significant differences were observed between this recombinant maize and the non-recombinant control maize in all items except the row number per ear and 100-kernel weight. Therefore, it was expected that there would be no differences between this recombinant maize and the non-recombinant control maize regarding production of the seed.

We failed to carry out an appropriate examination for the row number per ear and 100-kernel weight because the seeds used for this examination were inbred seeds, which produced fewer kernels.

Shedding habit under the natural conditions was not observed because, in any of this recombinant maize or the non-recombinant control maize, ears are covered with husk at harvest time.

The germination rate of the collected seeds from this recombinant maize at the isolated fields has not been examined. However, in a field test conducted in the US between 1994 and 1997 and in commercial cultivation conducted since 1998, no cases were reported where a difference was found between this recombinant maize and the non-recombinant control maize in the number of seeds that shed in the field after harvest time and then germinated and grew. For this reason, it was expected that the dormancy and germination rate of the seed would be as low as that of the non-recombinant control maize.

(f) Crossability

Crossability test was not performed since no wild relatives that can be crossed grow in Japan.

(g) Productivity of harmful substances

A succeeding cropping test, a soil microflora test and a plow-in test were carried out for this recombinant maize and the non-recombinant control maize. They indicated no differences between this recombinant maize and the non-recombinant control maize in all items. GA21 has been cultivated in the US for commercial purposes since 1998. After GA21 was harvested, the plant body of GA21 was plowed in, and soy beans and wheat were cultivated at the same field the following year. However, no growth inhibition has been reported.

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

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

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

(1) Competitiveness

Regarding maize (*Zea mays* subsp. *mays*（L.）Iltis.), to which the recipient organism belongs, cultivation has been conducted in Japan, but there is no report that it has grown voluntarily in Japanese natural environment so far.

This recombinant maize has been conferred the tolerance to glyphosate herbicide because of the transferred *mEPSPS*, but it is hard to consider that the glyphosate becomes a selection pressure in the natural environment. In addition, as a result of examination in the isolated fields in Japan, it was confirmed that there is no significnant difference between this recombinant maize and the non-recombinant control maize with regard to various traits relating to competitiveness except that difference was observed in the grain number per row and 100-kernel weight.

Based on the above understanding, it was judged that the conclusion by the applicant that there are no specific wild animals and wild plants possibly affected by this recombinant maize and that the use of this recombinant maize poses no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

Regarding the maize, to which the recipient organism belongs, there is no report that it produces harmful substances to affect wild animals and wild plants.

This recombinant maize produces mEPSPS protein that offers the tolerance to glyphosate, though there is no report that this protein is any harmful substance. Furthermore, mEPSPS protein is an enzyme that catalyzes the shikimate pathway where aromatic amino acids are synthesized. However, it is clarified that the mEPSPS protein is not a rate-determining enzyme in the pathway, and actually it is confirmed that the

amount of aromatic amino acid content of this recombinant maize is same as that of non-recombinant control maize. In addition, based on the fact that EPSPS protein is an enzyme which specifically reacts with phosphoenolpyruvate and shikimate-3-phosphate, it is unlikely that the mEPSPS protein catalyzes reactions of other substances to produce different substances.

In addition, as a result of examination on the productivity of harmful substances of the recombinant maize (the effects of the secretion from roots on other plants, the effects of the secretion from roots on the microorganisms in soil, and the effects of the possession in the plant body on other plants), no significant difference from the non-recombinant maize was observed at the isolated fields in Japan.

Based on the above understanding, no wild animals or wild plants are specified to be possibly affected, and it was concluded that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances.

(3) Crossability

In Japan, the growth of wild species that can be crossed with maize in natural environment has not been reported.

Based on the above understanding, no wild species can be specified as having some effects, the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability is valid.

2. Conclusion

Based on 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 valid.