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 Lepidoptera (*mEPSPS*, *cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (GA21 × MON810, OECD UI: MON-ØØØ21-9 × 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

I. Information concerning preparation of living modified organisms

The cross progeny line (*mEPSPS, cry1Ab, Zea mays* subsp. *mays* (L.) Iltis) (GA21×MON 810, OECD UI: MON-ØØØ21-9×MON-ØØ81Ø-6) (hereinafter referred to as "this stack maize") was produced by crossing maize tolerant to glyphosate herbicide (*mEPSPS, Zea mays* subsp. *mays* (L.) Iltis) (GA21, OECD UI: MON-ØØØ21-9) (hereinafter referred to as "GA21") and maize resistant to Lepidoptera (*cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (MON810, OECD UI: MON-ØØ81Ø-6) (hereinafter referred to as "MON810"), through the traditional cross-breeding method. This stack maize possesses characteristics from both the two recombinant parent lines, GA21 and MON810. Therefore, information on the GA21 and MON810, such as the methods for the preparation of them, is explained individually in the following statements.

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 GA21 are shown in Figure 1 and Table 1.

The composition of donor nucleic acid and origins of component elements used for the development of MON810 are shown in Figure 2 and Table 2.

(2) Functions of component elements

Functions of component elements which were used for the development of GA21 and MON810 are shown in Table 1 and Table 2, respectively.

[*mEPSPS* gene]

(i) 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 GA21, 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-heptulosonate-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. Moreover, it has been demonstrated that there are no differences in aromatic acid content between GA21 and the non-recombinant control maize, according to the determination of amino acid composition in seeds from recombinant crops, conducted as part of the food/feed safety evaluation of GA21. 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.

(ii) 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.

[*cry1Ab* gene]

(i) The *cry1Ab* gene, the target gene in MON810, confers resistance to Lepidoptera. This gene is derived from *Bacillus thuringiensis* subsp. *kurstaki*, which is a gram-positive bacterium universally exists in soil. The Cry1Ab protein encoded by the gene possesses an

insecticidal activity against European corn borer (*Ostrinia nubilalis*), which is one of the major Lepidopteran pest affecting maize cultivation in the US. Feeding damage by European corn borer is found widely in the above-ground part of the plant body. *B.t.* proteins which are produced by the bacterium *B.t.* including the Cry1Ab protein, bind to the specific receptors on the midgut epithelium of the target insects, and form cation selective pores, which lead to the inhibition of the digestive process and result in the insecticidal activity. *B.t.* proteins have no enzyme activity and are independent of the metabolic system of the recipient organism.

The Cry1Ab protein shows an insecticidal activity only against Lepidoptera, but not against other insects. This protein is known to show an insecticidal activity against the following major Lepidopteran pests affecting maize cultivation in the US: 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*). Among them, *O. nubilalis* and *O. furnacalis* (Asian corn borer) are known to be major Lepidopteran pests affecting maize cultivation in Japan.

(ii) 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

(1) Name and origin

The plasmid vector used for the production of GA21 and MON810 is assembled from plasmids including pUC 19 from *Escherichia coli*.

(2) Properties

The vector pDPG434 used for the production of GA21 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 permit autonomous replication of vector in *E. coli*, and iii) lacZ used as a selective marker for cloning in *E. coli*. See Table 1 for detailed information of each component element. The total numbers of base pairs of this vector is 6,128 bp. The infectivity of this vector is not known.

Each of the vectors PV-ZMBK07 and PV-ZMGT10 used to produce MON810 is composed of the followings; i) kanamycin/neomycin resistance gene (*nptII* gene) derived from transposon Tn5 as a selective marker gene for *E. coli* with the designed vector; ii) ori-pUC, a replication origin that allows the replication of the vector in *E. coli*, and iii) lacZ used as a selective marker for cloning in *E. coli*. See table 1 for detailed information of each component element. The total numbers of base pairs of the vector PV- ZMBK07 is 7,794 bp, and that of PV-ZMGT10 is 9,427 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 pDPG434 used for developing GA21. Figure 1 shows the location of the component elements and restriction sites in the vector.

Table 2 shows the component elements of the plasmid vectors PV-ZMBK07 and PV-ZMGT10 used for developing MON810. Figure 2 shows the location of the component elements and restriction sites in the vector.

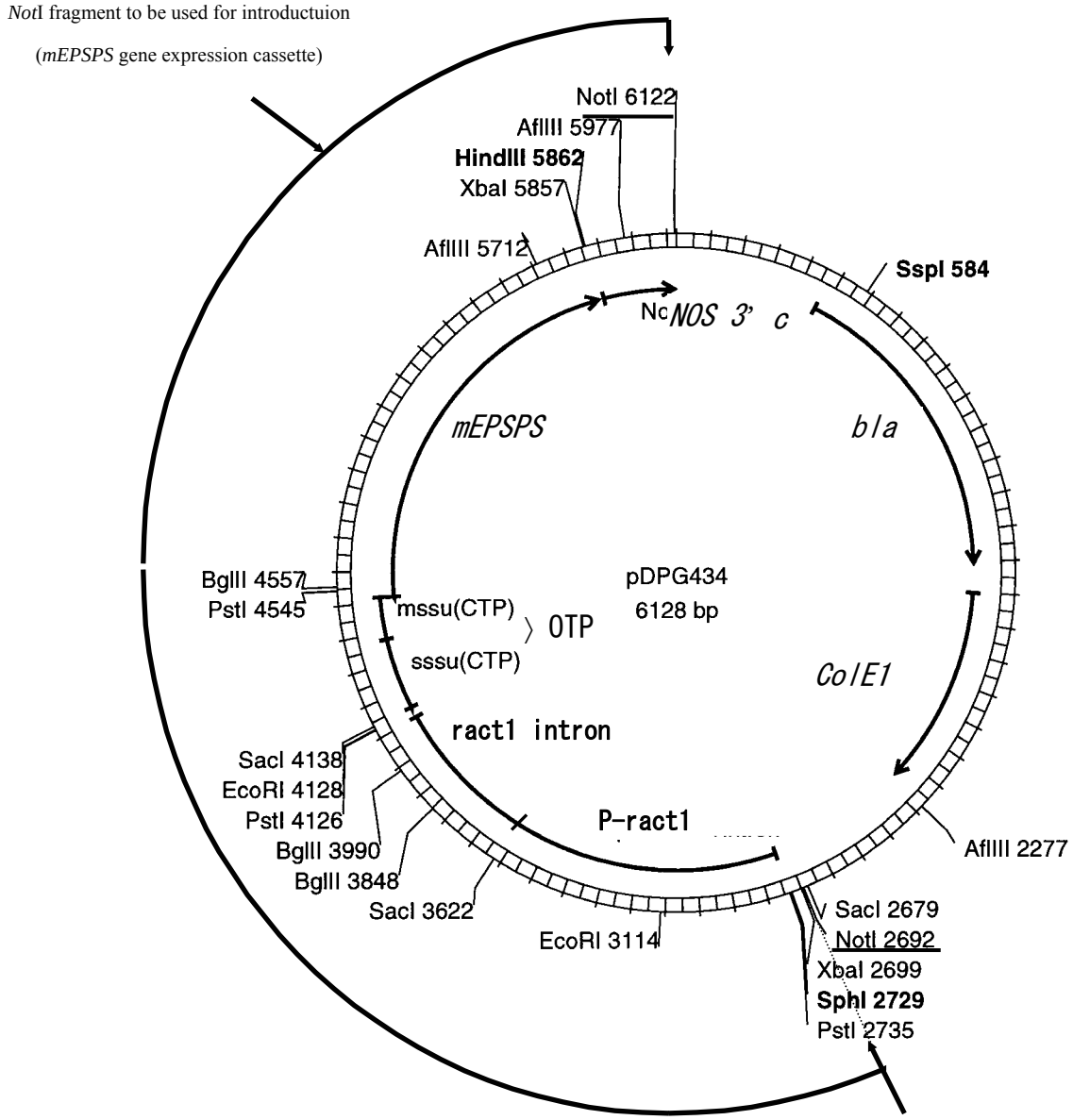

Figure 1 Plasmid vector pDPG434 used for developing GA21

Table 1 Origins and functions of the component elements of *Not*I fragment of pDPG434 used for developing GA21

Component elements	Origin and function
P-ract	Promoter region of actin 1 gene derived from rice. It makes target genes expressed (McElroy *et al*., 1990).
Ract1 intron	Intron of rice actin gene. It makes target genes expressed by enhancing splicing (McElroy *et al*., 1990).
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 (Lebrun *et al*., 1991; Padgette *et al*., 1993). 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. (Fraley *et al*., 1983).

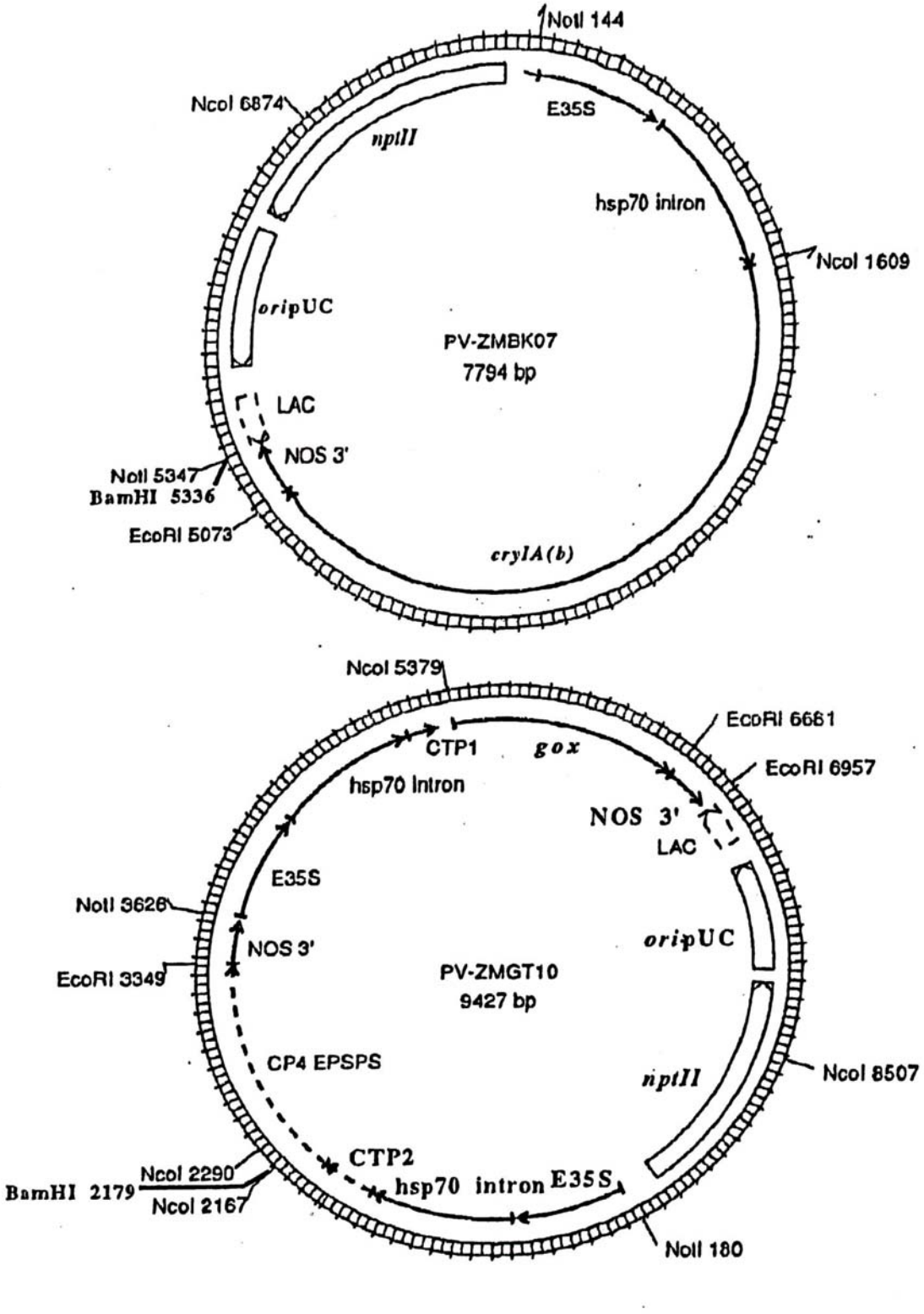

Figure 2 Plasmid vectors PV-ZMBK07 and PV-ZMGT10 used for developing MON810

Table 2 Origins and functions of the component elements of plasmid vector PV-ZMBK07 and PV-ZMGT10 used for developing MON810

Component elements	Origin and function
Cry1Ab gene cassette	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
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 page 3-4.
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 (This was not inserted to MON810 according to analysis on inserted genes.)	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
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* CP4 strain.
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 (This was not inserted to MON810 according to analysis on inserted genes.)	
E35S	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV).
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 which encodes C-terminal of variant v247 from 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.
Component elements other than above (common in both PV-ZMBK07 and PV-ZMGT10) (This was not inserted to MON810.)	
lacZ	Partial coding sequence for β-D-galactosidase or LacZ protein. Decomposes *β*-galactoside to form *β*-galactose.
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.

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

For the production of GA21, *Not*I fragment of plasmid vector pDPG434, a linear nucleic acid (shown by the arrow in Figure 1), was introduced by particle gun bombardment into a regenerated maize line derived from embryo-callus that is classified into dent type.

In developing MON810, the mixtures of plasmids PV-ZMBK07 and PV-ZMGT10 were introduced by particle gun bombardment into a maize inbred line that is classified into dent type.

(3) Processes of rearing of living modified organisms

[Processes of rearing of GA21]

i) The callus to which *Not*I fragment of pDPG434 was introduced was grown on tissue culture medium containing glyphosate for a certain period of time, and then the recombinant 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 GA21 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, an excellent line was selected. In these field experiments, the morphological and growth characteristics of this line 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 GA21 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.

June 2005: GA21 is under examination for "Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them", in line with the law concerning "Conservation and sustainable use of biological diversity through regulations on the use of recombinant -DNA plants" by the Ministry of Health, Labour and Welfare, and the Ministry of the Environmnent.

[Processes of rearing of MON810]

(a) The callus into which PV-ZMBK07 and PV-ZMGT10 were inserted was cultured on tissue culture medium containing 2,4-D for a while, followed by cultivation on glyphosate-containing medium to screen recombinants. They were regenerated to obtain the plant body, which was used for the analysis of Cry1Ab protein expression. As a result of Southern blotting analysis to examine genes inserted into MON810, it was confirmed that *nptII* gene, *cp4 epsps* gene and *gox* gene expression cassettes were not present. However, MON810 was screened by glyphosate though the *cp4 epsps* gene was not inserted in MON810. It was possibly because of the segregation of the inserted genes in the following generation (BC0F1) of the regenerated individual (R0). However, since MON810 was selected as maize resistant to pests and neither glyphosate test nor Southern blotting analysis was performed for the following generation of the regenerated individuals, we did not specify the reason why MON810 was screened by glyphosate.

(b) DNA fragment was introduced to MON810 by particle gun bombardment, so confirmation of remaining Agrobacterium was not carried out.

(c) The pedigree selection was started in 1992. We conducted field tests between 1993 and 1995, and selected superior lines. For these lines, we examined the introduced genes and the expression levels of the Cry1Ab protein, as well as morphological and growth

properties in six fields of the US in 1994. Based on the results from the tests, the MON810 gained approval from the US authorities, and has been cultivated for commercial purposes since 1997.

The situation of approval of MON810 in Japan is the following.

October, 1996: 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.

May, 1997: Based on 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 (the Ministry of Health and Welfare, at that time).

June, 1997: 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, 2001: 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.

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.

April, 2003: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being cultivated in Japan was certified by the Ministry of Agriculture, Forestry and Fisheries

June, 2004: MON810 was approved for "Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them", in line with the law concerning "Conservation and sustainable use of biological diversity through regulations on the use of recombinant-DNA plants" by the Ministry of Health, Labour and Welfare and the Ministry of the Environment.

[Processes of rearing of GA21×MON810]

This stack maize was developed by crossing the two recombinant maize lines, GA21 and MON810, through the traditional crossing method.

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

[State of existence of nucleic acid transferred in GA21 and stability of expression of traits]

As a result of Southern blotting analysis of GA21, the inserted genes were present at one site in the genome of this recombinant maize. In addition, more detailed analysis was performed based on nucleotide sequencing and Southern blotting analysis. It revealed that this inserted gene consisted of the following components; i) one 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 concerned 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.

[State of existence of nucleic acid transferred in MON810 and stability of expression of traits]

Southern blotting analysis confirmed that the genome of MON810 contained an inserted copy of a DNA fragment essential for the expression of the *cry1Ab* gene derived from PV-ZMBK07. Southern blotting analysis conducted for multiple generations confirmed that the inserted gene was inherited stably in progeny. In addition, it was also confirmed by biological examination during the screening process that resistance to Lepidoptera was stably

expressed in multiple generations.

Southern blotting analysis for MON810 indicated that only a region essential for the expression of the *cry1Ab* gene derived from PV-ZMBK07 was inserted into the maize genome, and the *cp4 epsps* gene and *gox* gene cassettes derived from PV-ZMGT10 as well as the *nptII* gene were not.

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

For detection and identification of GA21, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and neighboring areas of plant genome are used as primers. This method makes it possible to specifically detect GA21.

At present, http://www.maff.go.jp/sogo_shokuryo/jas/manual00.htm provides standard analysis methods for the detection and identification of MON810.

In order to detect and identify this stack maize, each grain of corn seeds needs to be examined by the two methods mentioned above.

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

This stack maize is expected to express mEPSPS and Cry1Ab proteins in the plant body, by the function of genes inserted into its parent lines, GA21 and MON 810. As mentioned in (ii)-(1)-2-I, the Cry1Ab protein do not possess enzyme activity, and functions independently of the metabolic system of the recipient organism. Similarly, it is indicated that the EPSPS protein, which has a function equivalent to the mEPSPS protein, is not a rate-determining enzyme in the shikimate cycle. Moreover, GA21 is unlikely to have an effect on the metabolic pathway of the recipient organism because it has been confirmed that there are no differences in aromatic amino acid content between recombinant and non-recombinant control maize lines, according to the determination of amino acid composition in seeds from recombinant crops, conducted as part of the food/feed safety assessment of GA21. In addition, the mEPSPS protein has high substrate specificity. For these reasons, it is unlikely that these two proteins will interact with each other.

In order to actually confirm this stack maize's tolerance to glyphosate herbicide, a glyphosate spray test was carried out at a field in the US. It demonstrated that tolerance to glyphosate was expressed in this stack maize (Table 3). In order to confirm this stack maize's resistance to Lepidoptera, the levels of feeding damage by Lepidopteran pests was examined at a field. It demonstrated that resistance to

Lepidoptera was expressed in this stack maize (Table 4).

Based on the above understanding, regarding the difference between this stack maize and the maize which is the taxonomic species to which the recipient organism belongs, it is guessed with the use of the results of individual examinations for the various characteristics of GA21 and MON810.

Table 3 Herbicide tolerance examination by spraying glyphosate herbicide (product name: RoundUp Ultra) on crossbred progeny cultivar of GA21×MON810

Crossbred progeny cultivar	Rate (%) of tolerance to glyphosate herbicide [1]	
	Spray at 4th leaf stage	Spray at 8th leaf stage
GA21×MON810	100	97
GA21	100	100

[1] Glyphosate herbicide was sprayed at the 4th and the 8th leaf stages at 32 oz/A (=250 mL/10a), the maximum recommended dosage, and then etiolation levels were observed 7-10 days later. A row of plants of each crossbred progeny cultivar were examined in a single biological test. This test was repeated six times. The rate of tolerance was evaluated based on the comparison of bleaching levels between plants on which glyphosate herbicide was or was not sprayed.

Table 4 Examination of feeding damage by Lepidoptera based on biological examination for crossbred progeny cultivar of GA21×MON810

Crossbred progeny cultivar	Levels of feeding damage [1]		
	Number of entrance holes (holes)	Length of entrance holes (cm)	Number of larvae (larvae)
GA21×MON810	0.0	0.0	0.0
MON810	0.0	0.0	0.0

[1] The presence of entrance holes by Lepidoptera, the length of entrance holes and the number of larvae were observed in this examination conducted at a field. The length of entrance holes were 15-30cm in an examination for a non-recombinant control maize line which was conducted in the same condition though not simultaneously.

(1) Tolerance to glyphosate herbicide is conferred to GA21, by the function of the mEPSPS protein encoded by the *mEPSPS* gene which is constantly expressed in every part of the plant body. It was actually confirmed that the mEPSPS protein was expressed at leaves and grains. It was also confirmed that, when glyphosate herbicide was sprayed, GA21 grew normally, while the non-recombinant control maize died.

Resistance to European corn borer (*Ostrinia nubilalis*), a major Lepidopteran pest affecting maize cultivation in the US, is conferred to MON810, by the function of the expression of the Cry1Ab protein encoded by the *cry1Ab* gene, resulting in the reduction of feeding damage by European corn borer. Feeding damage by them is found widely in the above-ground part of maize. However, it was confirmed that the Cry1Ab protein was constantly expressed in every part of the plant body. In addition, Southern blotting analysis indicated that the *nptII* gene, *cp4 epsps* gene and *gox* gene expression cassettes are not present in MON810, and showed no signs of traits derived from such genes.

For these reasons, it is believed that the mEPSPS and Cry1Ab proteins are also constantly expressed at every part of the plant body in this stack maize.

(2) A field tests were conducted at the National Institute for Agro-Environmental Sciences in 1998, for GA21 and a variety used for back-crossing (hereinafter referred to as "C2") as the control line.

A field tests were conducted at the National Institute for Agro-Environmental Sciences in 1996 and 2001-2002, for MON810AX and MON810BX—lines belonging to MON810—and MON810AC and MON810BC as the control lines.

1) 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, height of ear, maturation date, total number of ears, number of effective ears, and fresh plant weight at harvesting time. No significant difference was observed in all items between GA21 and the non-recombinant control maize.

In addition, evaluation was conducted regarding the germination rate, uniformity of germination, date of tassel exertion, silking date, maturation date, plant type, tiller number, total number of ears, number of effective ears, culm length, height of ear, and fresh plant weight at harvesting time. No significant difference was observed in all items except culm

length between MON810 and the non-recombinant control maize. A statically significant difference was found in culm length between MON810BX, a recombinant maize, and MON810BC, as the non-recombinant control maize. The average culm length was 248.1 cm in MON810BX and 229.3 in MON810BC.

2) Chilling-tolerance at the early stage of growth

Chilling tolerance tests at the early growth stage of GA21 have not been conducted at the isolated field. 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 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.

We evaluated the chilling tolerance of seedlings of MON810 and the non-recombinant control maize (maximum air temperature: 12-14°C; minimum air temperature: 2°C). As a result, all the fully developed leaves had wilted by the 21st day after the start of cold treatment. Chilling tolerance showed no differences between MON810 and the non-recombinant control maize.

It is therefore unlikely that there are differences in chilling tolerance between this stack maize and the taxonomic maize species to which the recipient organism belongs.

3) 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 the parent lines GA21 and MON810 were beginning to die at the end of the isolated filed test. For this reason, no wintering ability tests were carried out for matured plants.

4) Fertility and size of the pollen

No adverse effects are expected from pollen dispersal because it was only herbicidal tolerance that was conferred to the GA21. In addition, there are no close wild species that can hybridize with the GA21. For these reasons, no tests were conducted for the fertility and size of pollen.

We observed the fertility (amount of pollen in anthers) and size of pollen from MON810 and the non-recombinant control maize under a microscope, after staining the pollen with 0.1% neutral red solution and iodine-potassium iodide solution. No difference was observed between MON810 and the non-recombinant control maize.

It is therefore unlikely that there are differences in the fertility and size of pollen between this stack maize and the taxonomic maize species to which the recipient organism belongs.

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

For ears obtained from the sibling crossing of GA21, 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 GA21 and the non-recombinant control maize in all items except the row number per ear and 100-kernel weight. Therefore, regarding production of the seed, it was expected that there would be no differences between GA21 and the non-recombinant control maize.

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.

For ears obtained from the sibling crossing of MON810, we examined ear length, ear diameter, the row number per ear, the grain number per row, and 100-kernel weight. As a result, no statistically significant differences were observed in all items between MON810 and the non-recombinant control maize. Therefore, regarding production of the seed, it was expected that there would be no differences between MON810 and the non-recombinant control maize.

Shedding habit under the natural conditions was not observed because, in any of GA21, MON810 or their non-recombinant controls, ears are covered with husk at harvest time.

The germination rate of collected seeds has not been examined at isolated fields for GA21. 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 GA21 and the non-recombinant control maize in the number of GA21 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 GA21 seeds would be as low as those of the non-recombinant control maize.

Between MON810 and the non-recombinant control maize, there was no difference in germination rate evaluated on the 4th day after the sowing of collected seeds, and no dormancy was noted in seeds of MON810.

Therefore, it is unlikely that there are differences in seed production, shedding habit, dormancy and germination rate of the seed between this stack maize and the taxonomic species to which the recipient organism belongs.

6) Crossability

Crossability test was not performed for the parent line GA21 and MON810, since no wild relatives that can be crossed grow in Japan.

7) Productivity of harmful substances

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

A plow-in test, a succeeding cropping test and a soil microflora test were carried out for MON810 and the non-recombinant control maize. They indicated no differences between MON810 and the non-recombinant control maize in all items.

It is therefore unlikely that there are differences in the productivity of harmful substances between this stack maize and the taxonomic species to which the recipient organism belongs.

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.

This stack maize was produced by crossing maize tolerant to glyphosate herbicide (MON-00021-9) and maize resistant to Lepidoptera (MON-00810-6) through the traditional crossing method. The Committe on Adverse Effect on Biological Diversity judged that each of these parent lines would not result in Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stack maize.

It is reported that the mEPSPS protein encoded by the glyphosate-tolerant gene (*mEPSPS*) derived from MON-00021-9 shows high substrate specificity, and that the Cry1Ab protein encoded by the Lepidoptera-resistant gene (*cry1Ab*) possesses no enzyme activity. It is therefore unlikely that traits conferred by *mEPSPS* and *cry1Ab* will interact with each other.

It has been confirmed that this stack maize expresses tolerance to glyphosate herbicide and resistance to Lepidoptera, by a herbicide spray test and a biological test using European corn borer (*Ostrinia nubilalis*), respectively.

Based on the above understanding, it is unlikely that notable changes in traits have occurred in this stack maize, except for the traits it received from both the parent lines.

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

(1) Competitiveness

This stack maize has both tolerance to glyphosate herbicide derived from MON-00021-9 and resistance to Lepidoptera derived from MON-00810-6. However, it is hard to consider that the glyphosate becomes a selection pressure in the natural environment. moreover, feeding damage by Lepidopteran pests is not a major factor that hinders the growth of maize in Japan in the natural environment. Both of these traits will not enhance the competitiveness of the stack maize; it is therefore unlikely that this stack maize will be more competitive than its parent lines.

Based on the above understanding, it was judged that the conclusion by the applicant that the use of this recombinant maize poses no unacceptable risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack maize has both the mEPSPS protein productivity derived from MON-00021-9 and the Cry1Ab protein productivity derived from MON-00810-6. The Cry1Ab protein shows an insecticidal activity against Lepidoptera, while mEPSPS protein is known not to be a harmful substance to animals and plants. For this reason, even though this stack maize has both the proteins, it is unlikely that the productivity of harmful substance will be greater in this stack line than its parent lines.

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 unacceptable 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 unacceptable 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 unacceptable risk that the use of mEPSPS line GA21 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.