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

Du Pont Kabushiki Kaisha
Akio Kobayashi, President

Sanno Park Tower
2-11-1, Nagata-chou, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the type of Living Modified Organism	Maize tolerant to glufosinate herbicide and resistant to Lepidoptera (*pat*, *cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (T25×MON810, OECD UI: ACS-ZMØØ3-2×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

One of the parent lines, T25, was developed by Hoechst Schering Company (today's Bayer CropScience) (Germany), while the other, MON810, was developed by Monsanto Company (USA). T25 and MON810 were bred based on the conventional F1 hybrid breeding method, and inbred lines of them were crossbred to create a hybrid variety, stack line T25×MON810, based on the conventional F1 hybrid breeding method.

Stack line T25×MON810 is given both tolerance to glufosinate herbicide, by the function of the *pat* gene derived from T25, and resistance to Lepidoptera, by the function of the *cry1Ab* gene derived from MON810.

Information about T25 and MON810, both of which gained approval respectively on November 22, 2004 and on June 1, 2004, is provided in this evaluation report, quoted from the following documents open to the public: the opinion of experts of the Conference on Adverse Effect on Biological Diversity, and a summary of application. Access the following websites to obtain these documents.

<T25>
Opinion of experts
http://www.bch.biodic.go.jp/download/lmo/public_comment/T25sp.pdf
Summary of application
http://www.bch.biodic.go.jp/download/lmo/public_comment/T25ap.pdf

<MON810>
Opinion of experts
http://www.bch.biodic.go.jp/download/lmo/public_comment/MON810sp.pdf
Summary of application
http://www.bch.biodic.go.jp/download/lmo/public_comment/MON810ap.pdf

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

Composition of donor nucleic acid that was used for the development of T25 and origins of component elements are shown in Table 1, and composition of donor nucleic acid that was used for the development for MON810 and origins of component elements are shown in Table 2.

Table 1 Composition of donor nucleic acid that was used for the development of T25 and origins of component elements

Component elements	Size (kbp)	Origin and function
Pat gene expression cassette		
P-35S	0.52	A 35S promoter derived from the cauliflower mosaic virus (CaMV). It has the function to express introduced genes in all tissues constantly.
Pat	0.53	A gene derived from *Streptomyces viridochromogenes* that encodes phosphinothricin acetyltransferase (PAT protein)
T-35S	0.20	35S RNA terminator derived from cauliflower mosaic virus. Terminates mRNA transcription and induces polyadenylation.
Others		
Bla	0.86	It is an ampicillin resistant gene derived from *E.coli*. It expresses β-lactamase only in bacteria.
ori-pUC	0.55	It is the replication origin (ColE1) of pUC18, and initiates replication of plasmid.

Table 2 Composition of donor nucleic acid that was used for the development of MON810 and origins of component elements

Component elements	Size (kbp)	Origin and function
Cry1Ab gene expression cassette		
E35S	0.61	Contains 35S promoter and duplicated enhancer derived from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 intron	0.80	Intron of heat shock protein gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
Cry1Ab	3.46	The gene which encodes Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain in the soil.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
Cp4 epsps gene expression cassette (This was not inserted to MON810 according to analysis on inserted genes.)		
E35S	0.61	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 intron	0.8	Intron of heat shock protein gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 2	0.31	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
Cp4 epsps	1.4	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation

(Continued on the next page)

(Continued from the previous page)

Component elements	Size (kbp)	Origin and function
gox gene expression cassette (This was not inserted to MON810 according to analysis on inserted genes.)		
E35S	0.61	Contains 35S promoter and duplicated enhancer derived from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 intron	0.80	Intron of heat shock protein gene derived from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 1	0.26	Sequence that encodes N-terminal chloroplast transit peptide of the small subunit 1A gene of rubisco in *Arabidopsis*. Transfers target proteins from cytoplasm to chloroplast.
Gox	1.3	A synthetic sequence generated based on glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein degrades glyphosate.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation
Component elements other than above (common in both PV-ZMBK07 and PV-ZMGT10) (These were not inserted to MON810 according to analysis on inserted genes.)		
LacZ	0.24	Partial coding sequence for β-D-galactosidase or LacZ protein. Used as a selectable marker in cloning experiments in *E.coli,* since blue color appears as the substrate Xgal is degraded by β-D-galactosidase.
ori-pUC	0.65	A segment containing replication origin for *E. coli* plasmid pUC. Starts the replication of the plasmid.
nptII	0.79	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) Functions of component elements

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

Table 1 and Table 2 show functions of individual component elements of donor nucleic acid, such as target genes, expression regulation regions, localization signals and selective markers, in T25 and MON810.

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.

a. PAT protein

PAT protein (phosphinothricin acetyltransferase) confers the tolerance to glufosinate herbicide. The glufosinate herbicide contains *L*-glufosinate, the active ingredient, which inhibits the activity of glutamine synthase that synthesizes glutamine from glutamic acid and ammonia. As a result, ammonia is accumulated in the plant body, causing the plant to die. The PAT protein acetylates and detoxifies *L*-glufosinate, thereby conferring the glufosinate tolerance to the plant body. It is reported that the PAT protein shows extremely high substrate specificity and catalyzes the acetylation of free amino groups in *L*-glufosinate, and that it does not accept other *L*-amino acids or select *D*-glufosinate for substrates. It has not been reported that the PAT proteins share amino acid sequence homology with any of the known allergenic proteins.

b. Cry1Ab protein

The *cry1Ab* gene, which encodes the Cry1Ab protein, is derived from *Bacillus thuringiensis* (hereinafter referred to as "*B.t*") subsp. *kurstaki*, or a gram-positive bacterium universally exists in soil. The Cry1Ab protein has an insecticidal activity against European corn borer (*Ostrinia nubilalis*), a major Lepidopteran pest that affects maize cultivation in the US. Feeding damage by European corn borer is found extensively in the above-ground part of the plant body. *B.t.* proteins produced by *B.t.* bacteria, including the Cry1Ab protein, exhibit an insecticidal activity by binding to specific receptors on target insects' midgut epithelial cells to form cation-selective micro-pores, resulting in the inhibition of their digestive process. *B.t.* proteins have no enzyme activity and are independent of the metabolic system of the recipient

organism.

The Cry1Ab protein has an insecticidal activity only against Lepidoptera, and not against other insects. This protein is known to show an insecticidal activity against the following major Lepidopteran pests that affect 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* (European corn borer) and *O. furnacalis* (Asian corn borer) are known to be major Lepidopteran pests that affect maize cultivation in Japan. The Cry1Ab protein was compared with known contact allergens in the database in order to investigate whether it shares functionally important amino acid sequence with them. As a result, the Cry1Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

It is expected that T25×MON810 allows the effective control of Lepidopteran pests and weeds for maize cultivation, and that it provides growers with a new option against Lepidopteran pests and a more effective option against weeds.

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

It is reported that the PAT protein shows extremely high substrate specificity and catalyzes the acetylation of free amino groups in *L*-glufosinate, the active ingredient. Also, it is reported that the PAT protein does not accept other *L*-amino acids or select *D*-glufosinate for substrates. Like other Cry proteins, it is not reported that the Cry1Ab protein has an enzyme activity in plant bodies.

Consequently, the PAT protein exhibits very high substrate specificity and the Cry1Ab protein possesses no enzyme activity. Therefore, it is unlikely that, in stack line T25×MON810, the introduced genes will have effects on the recipient organism's metabolic systems, or interact with each other.

2. Information concerning vector

(1) Name and origin

The plasmid pUC/Ac was used for a vector to create T25 (Figure 1). To construct this plasmid pUC/Ac, the synthetic *pat* gene was inserted at Sal I cleavage sites between the 35S promoter and the 35S terminator of pDH51 developed based on pUC18 derived

from *Escherichia coli* K12 strain. The plasmids PV-ZMBK07 and PV-ZMGT10 derived from the *E. coli* plasmid pUC119 were used for a vector to create MON810.

(2) Properties

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

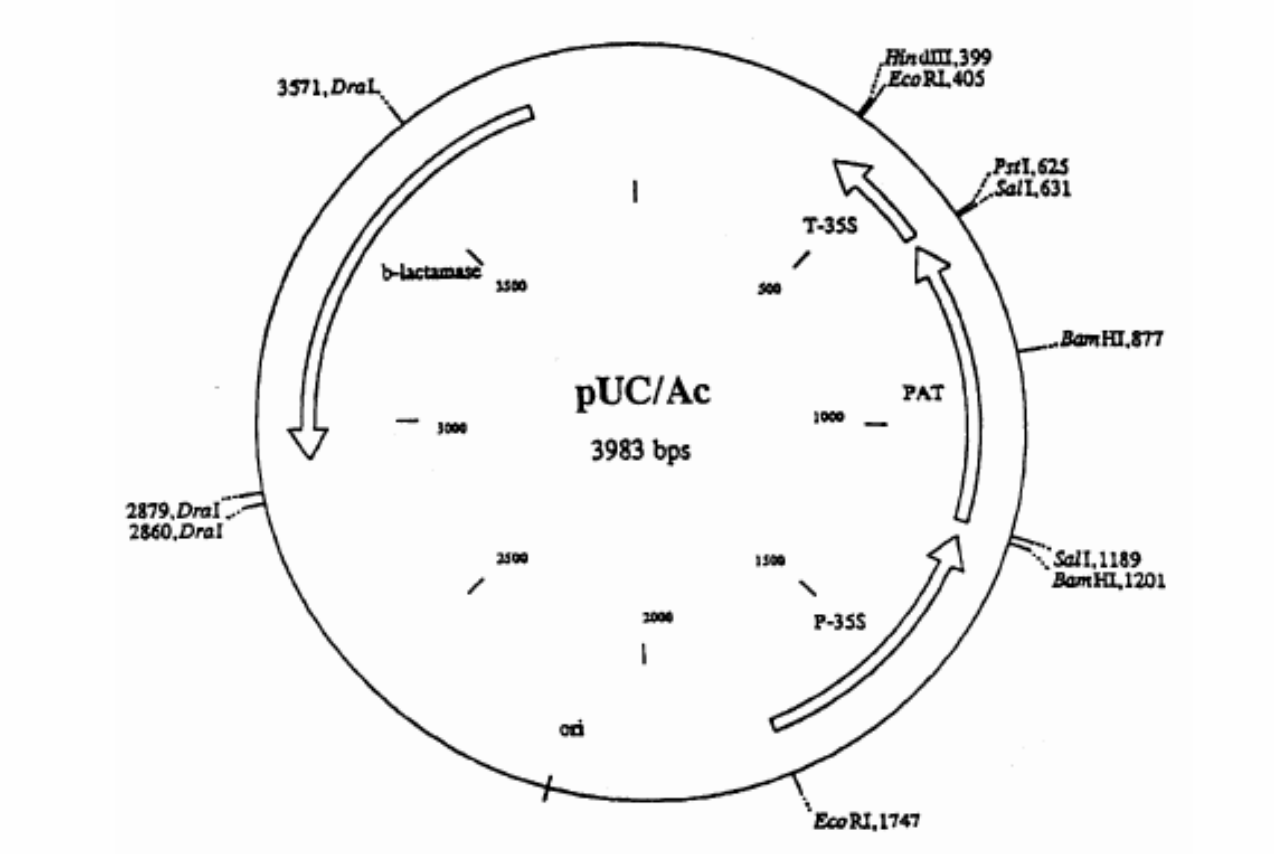

Figure 1 Plasmid pUC/Ac used for developing T25

Quoted from the webpage providing a summary of an evaluation report for T25:
http://www.bch.biodic.go.jp/download/lmo/public_comment/T25ap.pdf

The number of base pairs of the vector used to create T25 is 3,983 bp. The number of base pairs of PV-ZMBK07 and PV-ZMGT10, both of which were used to create MON810, were 7,794 bp and 9,427 bp, respectively. The sequence of base pairs of component elements of each vector is confirmed.

ii) Types of any nucleotide sequence having specific functions

All the genes in the plasmid pUC/Ac used to create T25 were characterized. They contain none of known harmful nucleotide sequences.

Plasmids PV-ZMBK07 and PV-ZMGT10, which were used to create MON810, each contain kanamycin/neomycin-resistant gene (*nptII* gene) derived from the transposon Tn5 as a selective marker gene for *E. coli* with the designed vector.

iii) Presence or absence of infectious characteristics of vector

The plasmid pUC/Ac used to create T25 is not transferable, and therefore noninfectious. Besides, it is reported that it is only in *E. coli* and a few gram-negative bacteria that the vector pUC18 can sustain antonymous replication.

It is not reported that plasmids PV-ZMBK07 and PV-ZMGT10 used to create MON810 are infectious.

3. Method of preparing living modified organisms

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

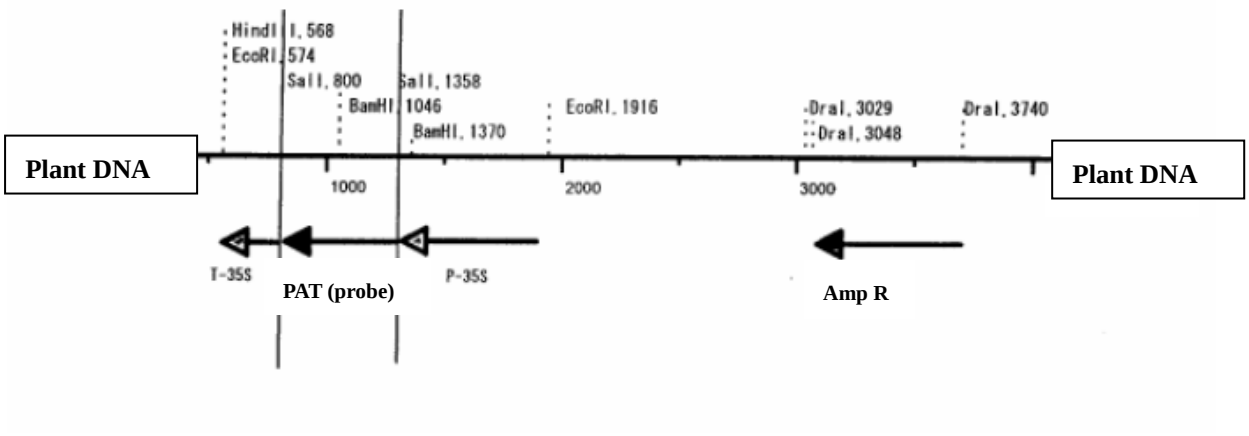

Figure 2 Structure of nucleic acid transferred into T25

Quoted from the evaluation report on Adverse Effect on Biological Diversity of T25

Two plasmids were constructed based on a vector derived from pUC119 having the *nptII* gene: a) the plasmid PV-ZMBK07 to which the *cry1Ab* gene cassette ([E35S]-[hsp70 intron]-[*cry1Ab*]-[NOS3']) is connected, and b) the plasmid PV-ZMGT10 to which the *cp4 epeps* gene cassette ([E35S]-[hsp70 intron]-[CTP2]-[*CP4EPSPS*]-[NOS3']) and the *gox* gene cassette ([E35S]-[hsp70

intron]-[CTP1]-[*GOX*]-[NOS3']) are connected. These two plasmids were used as vectors to create MON 810.

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

Protoplast of He/89, a tissue culture-derived strain, was used to transfer the genes into the recipient organism for creating T25, and the polyethylene glycol was used as a fusion agent.

In order to create MON810, the mixture of plasmids PV-ZMBK07 and PV-ZMGT10 was introduced by particle gun bombardment into the F2 generation of the maize inbred line A188 X B73 that is classified into dent type.

(3) Processes of rearing of living modified organisms

The rearing processes of T25 and MON810 are described in respective evaluation reports on Adverse Effect on Biological Diversity. We crossed the recombinant maize T25 with a yellow dent corn-type commercial variety and with a variety possessed by Bayer CropScience, followed by selective breeding. For MON810, the evaluation of line screening was started in 1992. We conducted field tests between 1993 and 1995, and selected MON810 as a superior line. For this lines, we examined the introduced genes and the expression levels of the Cry1Ab protein at six fields in the US in 1994, as well as morphological and growth properties. Based on the results from the tests, the MON810 gained an approval from the US authorities, and has been cultivated for commercial purposes since 1997.

The rearing processes of this stack line T25×MON810 is described in Table 3. As can be seen, this line is reared based on the conventional F1 breeding method by Pioneer Hi-Bred International, Inc.

In Japan, it was confirmed in 1995 that the use of T25 in an open system met the "Guidelines for the Use of Recombinant in Agriculture, Forestry, and Fisheries" (hereinafter referred to as "Guidelines"). In addition, its safety as food and as feed was confirmed in 2001 and in 1997, respectively (the safety as feed was reconfirmed in 2003 in accordance with the legislation of the examination system). Similarly, it is confirmed in 1996 that a project to use MON810 in an open system met the Guidelines. In addition, its safety as food and as feed was confirmed in 2001 and in 1997, respectively (the safety as feed was reconfirmed in 2003 in accordance with the legislation of the examination system). Also, it is confirmed in March 2002 that a project to use stack line T25×MON810 in an open system met the Guidelines. In addition, its safety as food and as feed was confirmed in June 2003 and in December

2001, respectively.

For both T25 and MON810, applications were filed for the Type 1 Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" enacted in February 2004. As a result, MON810 and T25 gained approvals respectively on June 1, 2004 and on November 22, 2004. The Conference on Adverse Effect on Biological Diversity has judged that neither of these lines would result in Adverse Effect on Biological Diversity when used in line with Type 1 Use.

Table 3 Rearing processes of stack line T25×MON810

Maize Hybrid lines	Varieties used for hybrid
T25 [1)]	Inbred A + T25 × Inbred B
MON810 [2)]	Inbred A × Inbred B + MON810
Stack line T25×MON810 [3)]	Inbred A + T25 × Inbred B + MON810

1) As for T25 hybrid, seeds were obtained by hybridizing Inbred A + T25 (herbicidal resistance is conferred to Inbred A) and Inbred B, based on the conventional crossbreeding method.
2) As for MON810 hybrid, seeds were obtained by hybridizing Inbred B + MON810 (pest tolerance is conferred to Inbred B) and Inbred A, based on the conventional crossbreeding method.
3) As for stack line T25×MON810, seeds were obtained by hybridizing Inbred A + T25 (T25 hybrid) and Inbred B + MON810 (MON810 hybrid), based on the conventional crossbreeding method.

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

It was confirmed that, in both T25 and MON810, the target nucleic acids were introduced into the maize genome.

(2) The number of copies of transferred nucleic acid and stability of its inheritance through multiple generations

Southern blotting analysis to examine the genes transferred to T25 confirmed that a copy of pUC/Ac was present in the genome of T25. Also, Southern blotting analysis conducted for multiple generations confirmed that the inserted genes were inherited stably in offspring. A part of the *bla* gene was lost because it was split into two fragments when inserted into the maize genome. A 35S promoter-like sequence was located at the 5'-terminal of the *bla* gene fragment. However, both the *bla* gene fragment and the 35S promoter-like sequence were found to be incomplete. Northern blotting analysis indicated that the *bla* gene did not function. Also, *β*-lactamase activity was analyzed and found to be undetectable. Overall, these results suggest that neither of the nucleotide fragments transferred to T25, the incomplete *bla* gene nor the 35S promoter-like sequence, functions.

As for MON810, southern blotting analysis confirmed that a copy of a DNA fragment derived from the plasmid PV-ZMBK07 and essential for the expression of the *cry1Ab* gene was present at a single site on the genome of MON810. Furthermore, southern blotting analysis conducted for multiple generations confirmed that the inserted gene was inherited stably in offspring. It was also confirmed during the screening process that resistance to Lepidoptera was stably expressed in multiple generations.

Southern blotting analysis for MON810 confirmed that only a region derived from the plasmid PV-ZMBK07 and essential for the expression of the Cry1Ab protein was inserted into the maize genome, and that the *cp4 epsps* gene and *gox* gene cassettes derived from the plasmid PV-ZMGT10 and the *nptII* gene were not present.

We also confirmed that the introduced genes derived from T25 and MON810 were stably transmitted in stack line T25×MON810 by southern blotting analysis. We collected leaves from four plants of each of T25×MON810, T25 and MON810, and treated genome DNA extracted from the respective leaves with restriction enzymes. Then, we conducted Southern blotting analysis using the *pat* gene and *cry1Ab* gene as proves. As a result, this stack line showed the same band pattern as T25 and MON810, indicating that the genes from T25 and MON810 were stably inherited in stack line T25×MON810.

(3) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid

ELISA analysis also confirmed that the PAT and Cry1Ab proteins were stably produced

in this stack line T25×MON810. For this ELISA analysis, we used maize plants cultivated at three fields in France, at two fields in Italy and at one field in Bulgaria, in 2000 by Pioneer Hi-Bred International, Inc (USA). For the analysis, leaves at the V9 stage were collected from five maize plants at each field. After collected, these samples were freeze-dried for dehydration, ground, suspended in PBST (phosphate buffered saline containing the detergent Tween20), and then finely ground. The suspension was centrifuged, and the supernatant was used in ELISA analysis. The PAT and Cry1Ab proteins were measured by the double antibody sandwich assay using polyclonal antibodies, each specific to the respective proteins. For the detection of each protein, the secondary antibodies labeled with horseradish peroxidase (HRP) were used. The HRP substrate was added to allow coloring, followed by the addition of reaction termination solution, and then absorbance at 450 nm was measured.

As a result, the average expression levels of the PAT and Cry1Ab proteins in stack line T25×MON810 were equivalent to those of respective proteins in T25 and MON810 as shown in Table 4. This result confirmed that each of the introduced genes derived from T25 and MON810 were stably expressed in stack line T25×MON810.

Table 4 Results of measurement of the PAT and Cry1Ab proteins in leaves by ELSA analysis

	Stack line T25×MON810		T25		MON810	
	Average	Minimum – Maximum	Average	Minimum - Maximum	Average	Minimum - Maximum
Pat protein	33.3	17.1-54.5	33.9	11.9-64.6	-	-
Cry1Ab protein	118	43-224	-	-	117	48-202

(ng/mg dry weight)

We conducted a field evaluation test on resistance to European corn borer as well as a glufosinate herbicide spraying test. As a result, no changes were found in the function, in phenotype, of each protein expressed in this stack line T25×MON810.

In 1998, resistance to European corn borer was evaluated at fields in Iowa and Minnesota states where Pioneer Hi-Bred International, Inc. (USA) cultivated this stack line T25×MON810, one of its parent lines, MON810, and the non-recombinant maize. A test evaluated 30 individual plants, and this test was performed in duplicate or triplicate. Feeding damage was visually observed for leaves at the V6 stage and for stems about two weeks before harvesting, and expressed as feeding index. As a result,

feeding damage was observed in leaves and stems of the non-recombinant maize, while virtually not in leaves and stems of this stack line T25×MON810 and its parent line MON810. This result confirmed that this stack line T25×MON810 had as much resistance to European corn borer as its parent line MON810, and that the function of the Cry1Ab protein was not changed.

In 2001, a herbicide spraying test was performed in the US for this stack line T25×MON810 and the non-recombinant maize cultivated by Pioneer Hi-Bred International, Inc. (USA). The glufosinate herbicide was sprayed at a standard dosage (Liberty^{R}: 0.48 kg ai/ha) and it was visually observed whether this stack line T25×MON810 was given tolerance to glufosinate herbicide derived from one of its parent lines, T25. As a result, in this stack line T25×MON810, too, none of individuals was affected by glufosinate herbicide. In addition, no significant differences were observed in yields between the stack line and the non-recombinant maize, as shown in Table 5. These results confirmed that the function of the PAT protein produced in this stack line T25×MON810 was not changed.

Table 5 Examination of yields of stack line T25×MON810

	Yields				LSD (-0.5)	Pr>F	Significant differences
	Stack line T25×MON810 (bu/acre) (Figures in parenthesis are expressed in t/ha.)	Number of Replications	Non-recombinant maize (bu/acre) * (Figures in parenthesis are expressed in t/ha.)	Number of Replications			
Field 1	160.81 (10.13)	13	154.99 (9.76)	13	19.16	0.5697	Not any
Field 2	190.01 (12.00)	17	189.32 (11.93)	17	14.79	0.9403	Not any
Field 3	193.00 (12.22)	17	192.22 (12.16)	17	22.41	0.8433	Not any
Field 4	185.83 (11.71)	11	186.05 (11.72)	11	28.78	0.9620	Not any

* Basis ^{R} Gold, a herbicide generally used for maize cultivation, was sprayed on the non-recombinant maize at a dosage of 0.14 kg ai/ha to prevent the reduction of yields due to weed damage.

Overall, these results confirmed that the acquired traits derived from its parent lines, T25 and MON810, were stably expressed in this stack line T25×MON810, and that both the traits conferred by the parent lines did not interact with each other in this stack line T25×MON810.

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

Transmission of this item is absence, because the transferred nucleic acid does not contain any sequence allowing transmission.

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

Detailed information about methods to detect T25 and MON810 by PCR is described in "Testing for Foods Produced by Recombinant DNA Techniques" in a website for Foods and Food Additives Produced by Recombinant DNA Techniques provided by the Ministry of Health, Labour and Welfare. (http://www.mhlw.go.jp/topics/idenshi/kensa/kensa.html) Applying these public detection methods for T25 and MON810 to every maize grain enables this stack line T25×MON810 to be detected.

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

(1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acids

i) Tolerance to glufosinate herbicide

The stack line T25×MON810 is given tolerance to glufosinate herbicide, due to the *pat* gene derived from *Streptomyces viridochromogenes* introduced into T25. The PAT protein encoded by the *pat* gene acetylates and transforms the glufosinate herbicide to nontoxic acetylglufosinate, thereby conferring the glufosinate tolerance to the plant body.

ii) Resistance to Lepidoptera

The stack line T25×MON810 produces the Cry1Ab protein, due to the *cry1Ab* gene derived from *Bacillus thuringiensis* subsp. *kurstaki*yurai introduced into MON810. As a result, the stack line is given resistance to European corn borer (*Ostrinia nubilalis*) and other Lepidopteran pests which cause feeding damage to maize.

European corn borer is a pest that causes the greatest damage to maize cultivation in the USA. The hatched larvae eat leaves for growth, and get into stems from the base of leaves. Once they eat into stems, it is generally difficult to control them because in general an agent sprayed hardly reaches them. They hollow stamens out by eating the inside of them. Larvae which get into female flowers damage growing ears. The total cost of pest control against European corn borer is estimated at about $ 1 billion per year.

(2) Presence or absence of difference between recombinant plant and the species to which recipient organism belongs, and the degree of difference, if any

The stack line T25×MON810 is a hybrid variety created by inbred lines of T25 and MON810, which were bred with conventional crossbreeding methods. It is anticipated that the crossbreeding of these two lines results in showing heterosis. Since the PAT protein has high substrate specificity and the Cry1Ab protein has no enzyme activity, it is unlikely that, in this stack line, the introduced genes have effects on the recipient organism's metabolic system, or interact with each other. Therefore, the influence of heterosis in this stack line over other traits is expected to fall within the fluctuation range in hybrid varieties between recombinant and non-recombinant maize lines as well as in hybrid varieties between conventional non-recombinant maize lines.

For these reasons, the differences between this stack line and the taxonomic maize species to which recipient organism belongs are evaluated based on the results of individual examinations for each trait of T25 and MON810.

An isolated field test was conducted for T25 in 2003 at the Research Center of Grassland Science, National Institute of Livestock and Grassland Science, National Agriculture and Bio-oriented Research Organization. A field test was conducted for MON810 in 1996 and 2001-2002 at the National Institute for Agro-Environmental Sciences, an Independent Administrative Institution. Here are summaries of the results from these tests.

a) Morphological and growth characteristics

For the recombinant maize T25 and the non-recombinant control maize, evaluation was conducted regarding uniformity of germination, germination rate, time of tasseling, time of silking, culm length, tiller number, height of ear, maturation time, number of ears, number of effective ears, ear length, ear diameter, row number per ear, grain number per row, grain color, 100-kernel weight, grain shape and fresh weight at harvesting time. As a result, no statistically significant difference was observed between recombinant and non-recombinant control maize in any of the

characteristics.

MON810 was evaluated for the following examination items: germination rate, the uniformity of germination, time of tasseling, time of silking, culm length, plant type, the number of tillers, ear height, maturation time, the number of ears, the number of effective ears, live weight at harvest time. As a result, no statistically significant differences were observed between MON810 and the non-recombinant maize in any of the examination items except culm length. As for culm length, one variety (MON810BX) among the two hybrid varieties tested showed statistically significant differences in culm length from the non-recombinant control maize (MON810BC): the average culm length was 248.1 cm in MON810BX and 229.3 cm in MON810BC. However, the other hybrid variety showed no statistically significant difference.

Additionally according to an actual germination test for this stack line T25×MON810, its germination rate was 98.4%, falling within 92%-100%, the fluctuation range of the germination rate of the conventional hybrid seeds.

It is therefore unlikely that there are differences in morphological and growth characteristics between this stack line T25×MON810 and the taxonomic maize species to which the recipient organism belongs.

b) Chilling tolerance at the early stage of growth

Chilling tolerance of the recombinant maize T25 and the non-recombinant control maize was evaluated. Chilling exposure of seedlings of both plants were conducted in an incubator at 4°C, and their growth was observed. As a result, the elongation of all of the seedlings of both plants was retarded. Whreras, all of the seedlings which were left in the field in winter were observed to dye in a few days. No difference between the recombinant maize T25 and the non-recombinant control maize was observed on chilling tolerance.

Similarly, we evaluated the cold tolerance of seedlings of MON810 and the non-recombinant control maize under the conditions of maximum air temperature (12-14°C) and minimum air temperature (2°C). All the fully developed leaves had wilted on the 21st day after the start of cold treatment. No difference between MON810 and the non-recombinant control maize was observed on chilling tolerance,

c) Wintering ability and summer survival of the matured plant

It is well known that maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not re-grow and propagate vegetatinvely, or produce seeds. T25 and the non-recombinant maize left at an isolated field after bearing were actually observed to die due to the coldness of winter. It was also observed that MON810 was beginning to die at the end of the isolated filed test. It is unlikely that there are differences in the wintering ability of matured plants between this stack line T25×MON810 and the taxonomic maize species to which the recipient organism belongs.

d) Fertility and size of the pollen

The fertility (amount of pollen in anthers) and size of pollen taken from T25 and the non-recombinant control maize, after staining the pollen with 0.1% neutral red solution and iodine-potassium iodide solution were microscopically observed. No difference was observed between T25 and the non-recombinant maize.

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

It is therefore unlikely that there are differences in the fertility and size of pollen between this stack line T25×MON810 and the taxonomic maize species to which the recipient organism belongs.

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

For T25, we examined 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 T25 and the non-recombinant maize. For MON810, 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 MON810 and the non-recombinant maize in any of the examination items.

Maize ears are covered with husk at harvest time, and a cob hold grains tightly. For these reasons, shedding habit is believed to be extremely low. Therefore, no tests have been carried out for the shedding habit of T25. The same goes for

MON810. Actually, no shedding habit under the natural conditions was observed in MON810.

In a germination test, both seeds harvested from T25 and from the non-recombinant maize soon germinated at 100% germination rates, exhibiting no dormancy. And the germination rates of harvested seeds on 4th day after sowing were high enough in both MON810 and the non-recombinant maize, and no difference was observed between them, indicating no domancy of MON810.

It is therefore unlikely that there are differences in the production and other relevant characters between this stack line and the taxonomic maize species to which the recipient organism belongs.

f) Crossability

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

g) Productivity of harmful substances

It is not known that maize secretes any harmful substances from the roots that could have adverse effects on the surrounding plants and/or microorganisms in soil. Also it is not known that maize produces any allelochemicals after dying that could affect other plants.

A succeeding cropping test, a plow-in test and a soil microflora test were carried out for T25 and the non-recombinant maize. These results indicated no statistically significant differences between T25 and the non-recombinant maize in all examination items.

Similarly, a succeeding cropping test, a plow-in test and a soil microflora test were carried out for MON810 and the non-recombinant maize. These results indicated no statistically significant differences between MON810 and the non-recombinant maize in all examination items.

It is therefore unlikely that there are differences in the productivity of harmful substances between this stack line T25×MON810 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 created by crossbreeding maize tolerant to glufosinate herbicide (ACS-ZM003-2:T-25) and maize resistant to Lepidoptera (MON-00810-6:MON810) through the conventional crossbreeding method. The Conference on Adverse Effect on Biological Diversity has judged that neither of these parent lines would 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 PAT protein encoded by the glufosinate-tolerant gene (*pat*) derived from ACS-ZM003-2 shows high substrate specificity, and that the Cry1Ab protein encoded by the Lepidoptera-resistant gene (*cry1Ab*) derived from MON-00810-6 possesses no enzyme activity. It is therefore unlikely that traits conferred by *pat* and *cry1Ab* will interact with each other.

ELISA protein analysis conformed that the expression levels of the PAT and Cry1Ab proteins in this stack maize were equivalent to those of respective proteins in ACS-ZM003-2 and MON-00810-6. According to a biological test using European corn borer (*Ostrinia nubilalis*), resistance to Lepidoptera in this stack maize was equivalent to that in MON-00810-6.

For these reasons above, 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 glufosinate herbicide derived from ACS-ZM003-2 and resistance to Lepidoptera derived from MON-00810-6. But it is hard to consider that the glufosinate tolerance becomes a selection pressure in the natural environment. Feeding damage by Lepidopteran pests, however, is not a major factor that hinders the growth of maize in Japan in the natural environment. Neither of

these traits will enhance the competitiveness of the stack maize; it is therefore unlikely that this stack maize will be more competitive than are its parent lines. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack maize has both the PAT protein productivity derived from ACS-ZM003-2 and the Cry1Ab protein productivity derived from MON-00810-6. The Cry1Ab protein shows an insecticidal activity against Lepidoptera, while the PAT protein is known not to be a harmful substance to animals and plants. For these reasons, 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 in its parent lines. Based on the above understanding, the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is judged valid.

(3) Crossability

In Japan, the growth of wild relatives (teosinte) 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 stack line T25×MON810 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.