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	High lysine and Lepidoptera resistant maize (*cordapA, cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (LY038×MON 810, OECD UI:REN-ØØØ 38-3×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 Report

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

1. Information concerning preparation of living modified organisms

The high lysine and Lepidoptera resistant maize (*cordapA, cry1Ab, Zea mays* subsp. *mays* (L.) Iltis) (OECD UI: REN- ØØØ 38-3×MON-ØØ81Ø-6) (hereinafter referred to as "this stack line maize") produced by traditional crossbreeding of high lysine maize (*cordapA, Zea mays* subsp. *mays* (L.) Iltis) (LY038, OECD UI: REN- ØØØ 38-3) (hereinafter referred to as "LY038") and Lepidoptera resistant maize (*cry1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (MON810, OECD UI: MON-ØØ81Ø-6) (hereinafter referred to as "MON810") possesses the traits from the two genetically modified maize cultivars LY038 and MON810, the parent lines of this stack line maize. Then, the information concerning preparation of LY038 and MON810 are explained individually in the following sections.

LY038 is intended for use as feed. Typically, maize-derived livestock feeds are lacking in lysine and other essential amino acids and thus require the supplementation of lysine or other essential amino acids for proper growth of livestock (Reference 12; Reference 13; Reference 14). The development of LY038 will reduce or eliminate the need for lysine supplementation in livestock feeds and allow direct feeding of maize of the increased level of lysine compared to conventional maize to livestock. A 42-day feeding study on growing broiler chickens was conducted with diets containing this recombinant maize. As a result, it was confirmed that this recombinant maize increases the broiler growth rate as intended compared to non-recombinant maize diets without added synthetic lysine and that the growth rate was found equivalent to that obtained by feeding a diet composed of non-recombinant maize supplemented with synthetic lysine (Annex 8 of Biological Diversity Risk Assessment Report for LY038).

(1) Information concerning donor nucleic acid

[Information concerning donor nucleic acid of LY038]

i) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the production of LY038 are shown in Figure 1 and Table 1.

ii) Functions of component elements

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

Functions of component elements of donor nucleic acid that was used for the production of LY038 are shown in Table 1.

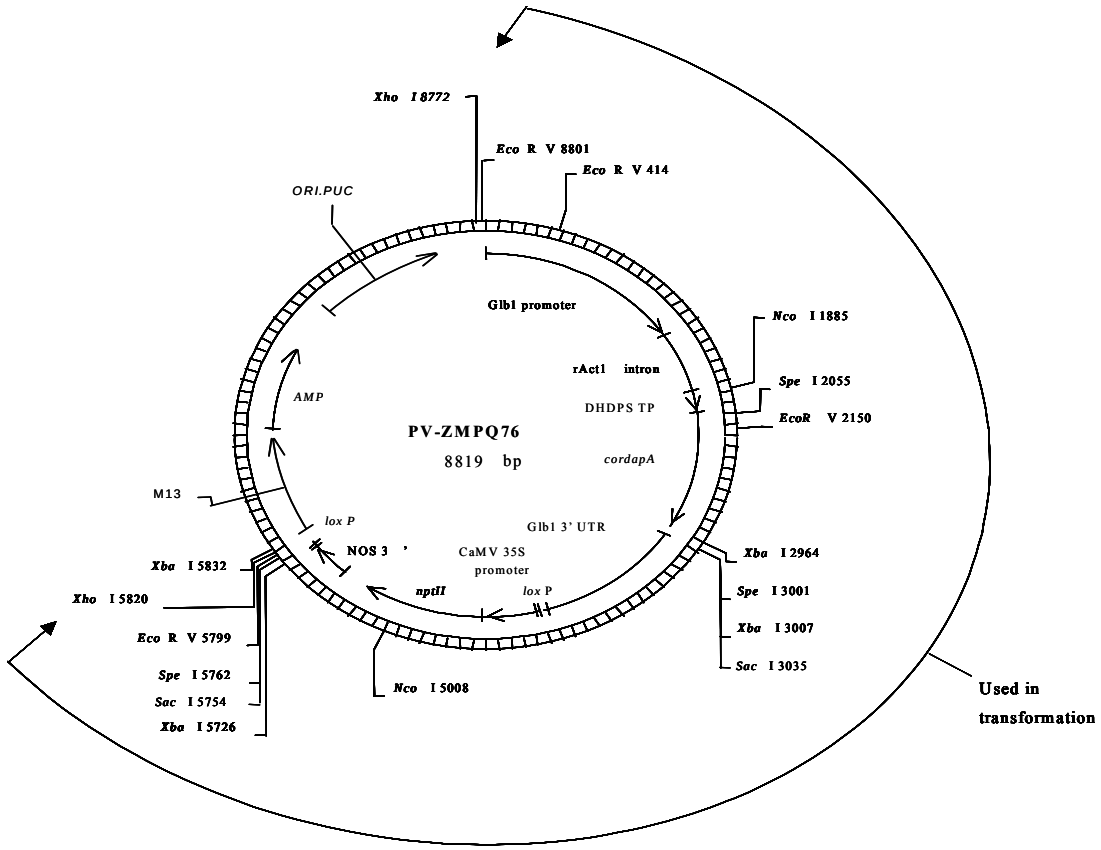

Figure 1 Plasmid vector PV-ZMPQ76 used for the production of this recombinant maize (Former designation : PV-ZMCTB331)[1]

The linear plasmid (the region indicated by the arrows in the diagram) of PV-ZMPQ76, which does not contain any plasmid backbone region in the *AMP* gene region cleaved with the restriction enzyme *Xho*I in the above diagram, was used for production of this recombinant maize.

[1] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 1 Origins and functions of the component elements of plasmid PV-ZMPQ76 (Former designation: PV-ZMCTB331) used for the production of LY038[2]

Component Elements	Origin and function
cordapA gene expression cassette	
Glb1 Promoter	The promoter region derived from the Globulin 1 (Glb1) gene from *Zea mays* L. to drive the target gene expression chiefly in grain (Reference 15).
rAct1 intron	Intron derived from the rice actin gene to enhance the efficiency of splicing and target gene expression (Reference 16).
mDHDPS TP	The chloroplast targeting sequence from the dihydrodipicolinate synthase gene in *Zea mays* L., which encodes the chloroplast transporting peptide present in the N-terminal domain of the DHDPS protein (Reference 17). It transports the target protein to the chloroplast.
cordapA	The coding region for the dihydrodipicolinate synthase gene from *Corynebacterium glutamicum* in the lysine biosynthetic pathway, conferring resistance to lysine feedback inhibition (Reference 18).
Glb1 3' UTR	The 3' untranslated region derived from Globulin 1 (Glb1) gene from *Zea mays* L., which terminates mRNA transcription and induces polyadenylation (Reference 15).
nptII gene expression cassette (was removed by the Cre-lox system in the process of rearing.)	
*lox*P	Recombination site of bacteriophage P1. It functions in pairs. DNA region present between two *lox*P sites will be removed when the two sites are recognized by Cre recombinase (DNA recombination enzyme) (Reference 19).
CaMV 35S promoter	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 20). It has the function to express transferred genes in all tissues constantly.
nptII	The gene isolated from the transposon Tn5 from *Escherichia coli* (*E. coli*), encoding neomycin phosphotransferase II (Reference 21). This gene, when expressed in maize, confers the kanamycin resistance and acts as a selective marker for transformation.
ble	A portion of bleomycin-resistant gene isolated from Tn5 (Reference 22), which, though, does not confer the bleomycin resistance.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from *Agrobacterium tumefaciens.* It induces polyadenylation of mRNA(Reference 23).
Other region (Not present in LY038)	
M13	The replication origin of bacteriophage M13 (Reference 24), permitting replication in bacteriophage.
AMP	The promoter and coding region in β-lactamase gene derived from *E. coli*, conferring ampicillin resistance in *E. coli* (Reference 25).
ORI.PUC	The plasmid replication origin permitting the replication of DNA in *E. coli* and other bacteria (Reference 25).

[2] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

2) 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

The dihydrodipicolinate synthase (hereinafter referred to as "cDHDPS") (*cordapA*) gene, expressed in LY038, is the gene isolated from *Corynebacterium glutamicum*, a typical gram-positive bacterium used normally in the commercial fermentation and production of lysine, that encodes the dihydrodipicolinate synthase, and the nucleotide sequences and amino acid sequences have been confirmed (see Annex 1 of Biological Diversity Risk Assessment Report for LY038). For the *cordapA* gene expression cassette promoter, the Glb1 promoter in *Glb1* gene derived from maize is used in order to specifically enhance the expression of target genes in grain (Reference 15). In the *cordapA* gene, the nucleotide sequence (mDHDPS TP) contains the chloroplast transporting peptide at the N-terminal of mDHDPS protein from maize, in order to function in the plastid (see Reference 17, Figure 1 and Table 1).

The cDHDPS derived from *C. glutamicum* catalyzes the reaction for synthesis of dihydrodipicolinate from aspartate semialdehyde and pyruvic acid as shown in Figure 2 (Reference 26; Reference 27).

In order to investigate whether the cDHDPS protein shares functionally important amino acid sequences with known allergens, the cDHDPS protein was compared with allergens in the database (GenBank, EMBL, PIR, NRL3D, SwissProt) using the FASTA type algorithm. As a result, the cDHDPS protein did not share structurally related homologous sequences with any of the known allergens examined.

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

Through a series of subsequent enzyme reactions with dihydrodipicolinate, lysine is synthesized. In this process, the endogenous maize mDHDPS is sensitive to lysine feedback inhibition from accumulated lysine and the production of dihydrodipicolinate is regulated. The cDHDPS enzyme is less susceptible to lysine feedback inhibition from accumulated lysine (Reference 28) (Figure 2). As a result, in LY038, the production of free lysine in the grain is enhanced compared to conventional maize (see Annex 1 of Biological Diversity Risk Assessment Report for LY038). In addition, with increasing production of free lysine, the levels of lysine catabolites, saccharopine and α-aminoadipic acid, are increased.

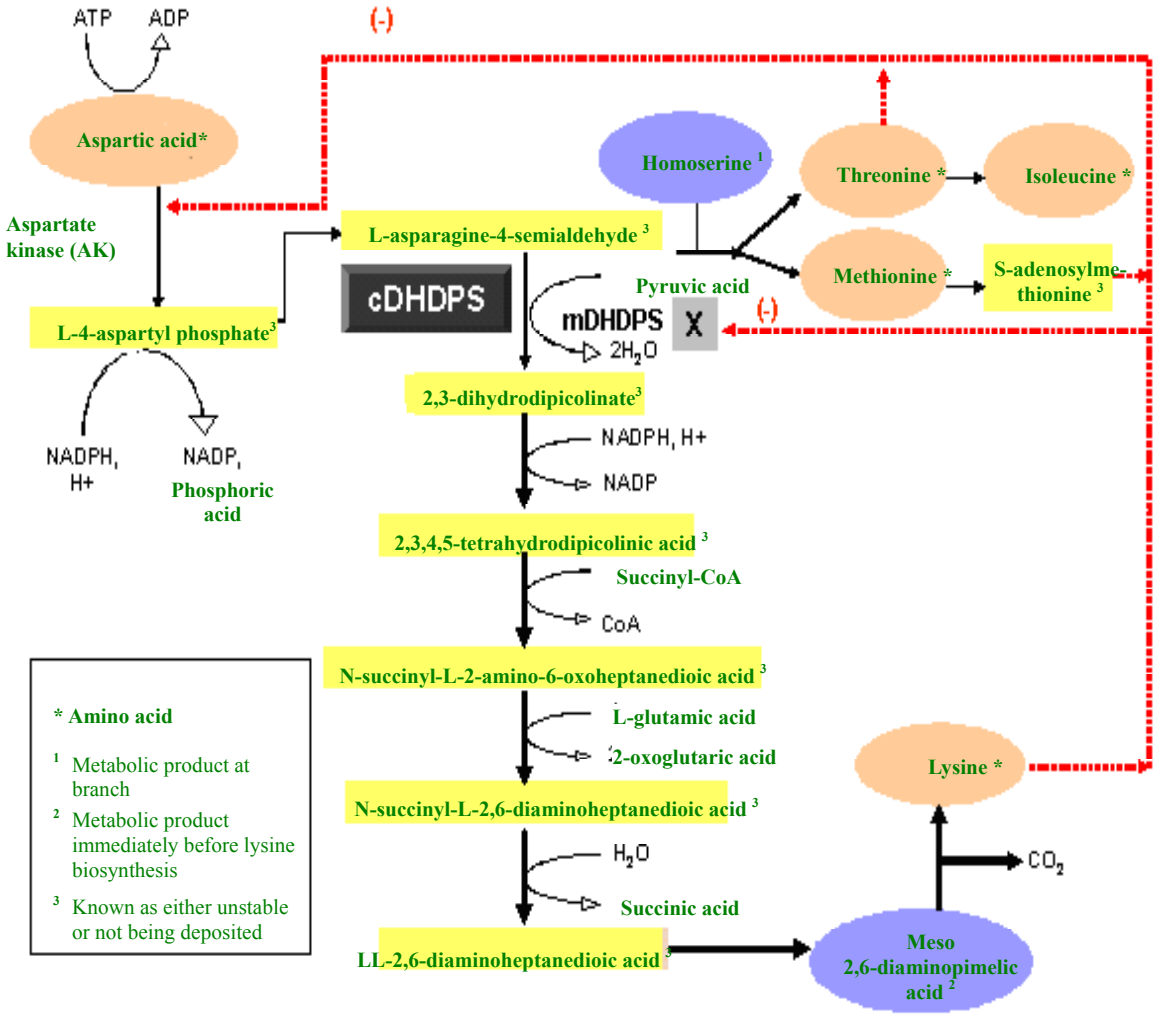

Figure 2 Lysine biosynthesis pathway[3]

(-); Lysine feedback inhibition

[3] All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Monsanto Japan Limited.

The substrates of cDHDPS are aspartate semialdehyde (hereinafter referred to as "ASA") and pyruvic acid. For the substrate specificity of cDHDPS, discussion was made as follows based on the findings obtained for DHDPS in other microorganisms.

(a) DHDPS has been shown to have a high level of substrate specificity for ASA and pyruvic acid, using enzyme preparations from two microorganisms. Enzymatic studies with DHDPS isolated from *Escherichia coli* (*E. coli*) showed that substitution of either ASA or pyruvic acid with analogues (ASA analogues = glutamate semialdehyde, N -acetyl-aspartate semialdehyde, succinate semialdehyde, and dipicolinic acid and pyruvic acid analogues = oxalacetic acid, and phosphopyruvic acid) resulted, in no DHDPS-catalyzed reactions being observed (Reference 29). Similarly, DHDPS from *Bacillus licheniformis* showed that substitution of ASA with aspartic acid, dipicolinic acid, adenylic acid, and allylglycine resulted in no reaction being observed (Reference 30). Based on the above results, it is concluded that there is high specificity of DHDPS in microorganisms for the substrates ASA and pyruvic acid.

(b) As can be seen from Table 2, a large difference in kinetic parameters of DHDPS protein is only seen in susceptibility of the cDHDPS to lysine feedback inhibition. In contrast to this, kinetic parameters were considered equivalent between *C. glutamicum* and *E. coli* for K_m^{pyr}, and between *C. glutamicum* and *Bacillus licheniformis* for K_m^{ASA}.

(c) Based on the comparison between the structures of *E. coli* DHDPS (Reference 31; Reference 32; Reference 33) and cDHDPS (Reference 34), which have been determined, the substrate-binding sites were found to be identical, and the tertiary structures were also similar to each other.

Based on the above understandings (a) through (c), the substrate specificity of cDHDPS was considered as high as that of DHDPS derived from other microorganisms.

Table 2 Kinetic parameters of DHDPS protein[4]

Origins of DHDPS protein	Kinetic parameters		
	K_m^{pyr} (mM)	K_m^{ASA} (mM)	IC_{50}^{Lys} (mM)
E.coli[1]	0.20	0.12	~0.40
Bacillus licheniformis[2]	n/d	0.765	n/d
Corynebacterium glutamicum[3]	0.32	0.70	659

K_m^{pyr}: Concentration of substrate (pyruvic acid) when the initial rate of enzyme reaction becomes a half of the maximum rate V_{max}. The smaller this value, the affinity to substrate increases [4].
K_m^{ASA}: Concentration of substrate (ASA) when the initial rate of enzyme reaction becomes a half of the maximum rate V_{max}.
IC_{50}^{Lys}: Concentration of antagonist in which reaction by antagonist alone is inhibited by 50% [5], or the concentration of lysine in which individual DHDPS proteins suffer 50% lysine feedback inhibition.
[1] Reference 29, [2] Reference 30, [3] Reference 34, [4, 5] Reference 35
n/d – not determined

On the other hand, in the production of LY038, the *nptII* (neomycin phosphotransferase type II) gene was used as a selective marker for transformed cells. However, as described later in this report, the progeny of LY038 do not contain the *nptII* gene expression cassette (see I-1-(4)-ii)). Its gene product, neomycin phosphotransferase II (NPTII), utilizes ATP to phosphorylate and inactivate kanamycin, neomycin, paromomycin and other aminoglycoside derivative antibiotics.

[Information concerning donor nucleic acid of MON810**]**

i) Composition and origins of component elements

The composition of donor nucleic acid and origins of component elements used for the production of MON810 are shown in Figure 3 and Table 3.

ii) Function of component elements

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

Functions of component elements of donor nucleic acid used for the production of MON810 are shown in Table 3.

[4] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

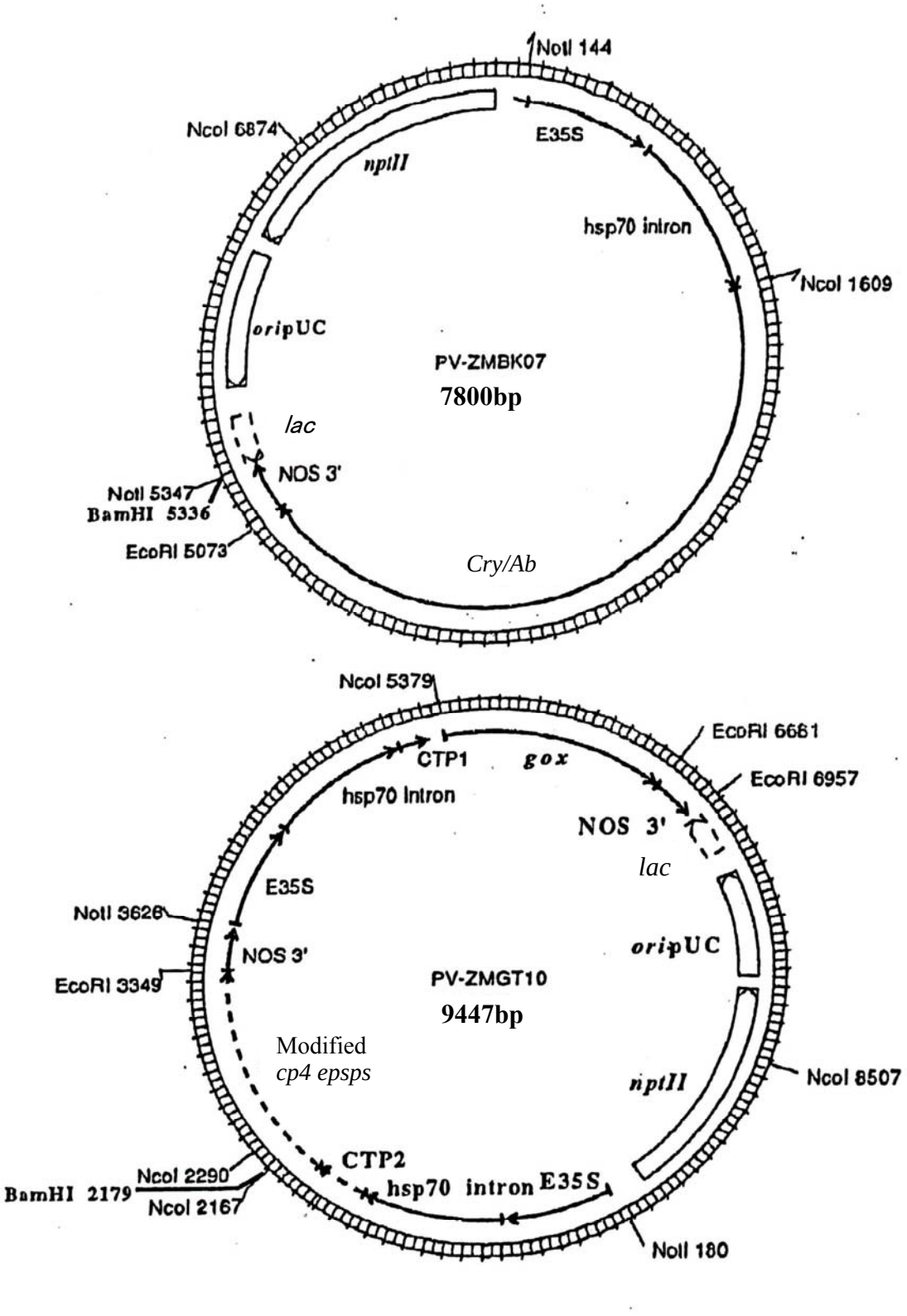

Figure 3 Plasmids PV-ZMBK07 and PV-ZMGT10 used for the production of MON810[5]

[5] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Japan Limited.

Table 3 Origins and functions of the component elements of plasmids PV-ZMBK07 and PV-ZMGT10 used for the production of MON810[6]

Component elements	Origin and function
cry1Ab gene cassette	
E35S	Contains 35S promoter (Reference 20) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 36).
hsp70 intron	Intron of heat stress protein (heat shock protein) gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 37).
cry1Ab	The gene which encodes Cry1Ab protein of *Bacillus thuringiensis* subsp. *krustaki* HD-1 strain existing in the soil (Reference 38).
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 39).
Modified *cp4 epsps* gene cassette (As a result of transferred gene analysis, this was not transferred into MON810.)	
E35S	Contains 35S promoter (Reference 20) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 36).
hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 37).
CTP2	N-terminal chloroplast transit peptide sequence in the EPSPS protein, derived from the *Arabidopsis thaliana epsps* gene (Reference 40). Transfers target proteins from cytoplasm to chloroplast in which aromatic amino acids are synthesized. In the upstream of the region, the untranslated region of 67bp exists, derived from *epsps* gene, which encodes the EPSPS protein in *Arabidopsis thaliana*.
Modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 41; Reference 42).
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 39).
gox gene cassette (As a result of transferred gene analysis, this was not transferred into MON810.)	
E35S	Contains 35S promoter (Reference 20) and duplicated enhancer from cauliflower mosaic virus (CaMV) (Reference 36).
hsp70 intron	Intron of heat shock protein gene from maize. Hsp70 intron is used to enhance the expression of foreign genes in plants (Reference 37).
CTP 1	N-terminal chloroplast transit peptide encoding sequence of the rubisco small subunit 1A of rubisco small subunit 1A gene derived from *A. thaliana* (Reference 43). Transfers target proteins from cytoplasm to chloroplast in which aromatic amino acids are synthesized.
Gox	The sequence which encodes the C-terminal of variant v247 derived from glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA (Reference 44). GOX protein degrades glyphosate.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation (Reference 39).
Other component elements (common to PV-ZMBK07 and PV-ZMGT10) (This was not transferred into MON810.)	
Lac	Partial coding sequence for β-D-galactosidase or Lac protein (Reference 45). Degrades the β-galactoside to produce the β-galactose.
ori-pUC	A segment containing replication origin for *E. coli* plasmid pUC (Reference 55). Starts the replication of the *E. coli* plasmid.
nptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II. When this gene is expressed in bacteria, it confers resistance to kanamycin and acts as a selectable marker for transformation (Reference 21).

[6] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

2) 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

The *cry1Ab* gene, the target gene in MON810 to confer Lepidoptera resistance, is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium universally exists in the soil. The Cry1Ab protein which is encoded by the *cry1Ab* gene has an insecticidal activity against European corn borer (*Ostrinia nubilalis*), which is one of the major pest insects of the order Lepidoptera to maize cultivation in the US (Reference 46). The part of the plant damaged by European corn borer is the whole plant above the ground. *B.t.* proteins which are produced by the bacterium *B.t.* including Cry1Ab protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pore, which leads to the inhibition of the digestive process and results in the insecticide activity (Reference 47; Reference 48). Consequently, it was considered that *B.t.* protein does not possess any enzyme activity and functions independently from the metabolic system of the recipient organism.

The Cry1Ab protein exhibits insecticidal activity only against insects of the order Lepidoptera and not against insects of other orders. Also, this Cry1Ab protein is known to exhibit insecticidal activity against the major pest insects of the order Lepidoptera for maize cultivation in the US, including 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*) (Reference 49; Reference 50; Reference 51; Reference 52; Reference 53). *O. furnacalis*, which belongs to the same genus as *O. nubilalis* mentioned above, is known as the major pest insect of the order Lepidoptera for maize cultivation in Japan (Reference 54).

In order to investigate whether the Cry1Ab protein shares functionally important amino acid sequences with known allergens, the Cry1Ab protein was compared with known allergens in the database (GenBank, EMBL, PIR, NRL3D, SwissProt). As a result, the Cry1Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

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

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(2) Information concerning vector

i) Name and origin

The plasmid vector used to produce LY038 was constructed based on the pGEM (Promega Corporation, Madison, WI).

The plasmid vector used to produce MON810 was constructed based on the plasmid pUC119 derived from *Escherichia coli* (*E. coil*) (Reference 55).

ii) Properties

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

The total number of base pairs of PV-ZMPQ76 used to produce LY038 is 8,819 bp. The entire nucleotide sequence of this plasmid vector is provided in Annex 2 of Biological Diversity Risk Assessment Report for LY038.

The total number of base pairs of the vectors used to produce MON810 is 7,800 bp for PV-ZMBK07 and 9,447 bp for PV-ZMGT10. The nucleotide sequence for *cry1Ab* gene is provided in Figure 1 of Annex 1 of Biological Diversity Risk Assessment Report for MON810.

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

The vector PV-ZMPQ76 used for the production of LY038 contains the *AMP* gene as a selective marker gene, which expresses β-lactamase to confer the ampicillin resistance from *E. coli* (Reference 25).

The vectors PV-ZMBK07 and PV-ZMGT10 used for the production of MON810 contain the kanamycin/neomycin-resistant gene (*nptII* gene) as a selective marker gene (Reference 21).

3) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

The infectivity of the vectors PV-ZMPQ76, PV-ZMBK07 and PV-ZMGT10 is not known.

(3) Method of preparing living modified organisms

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

The plasmid vector PV-ZMPQ76 constructed for the production of LY038 contains the *AMP* gene region expresssing β-lactamase that confers ampicillin resistance as a selective marker gene required for construction/selection and maintenance/growth of plasmids in *E. coli*. However, the linear plasmid prepared by cleaving PV-ZMPQ76with restriction enzyme *Xho*I was used to transfer the gene cassette to maize required for expression of *cordapA* gene and *nptII* gene (see Figure 1). Therefore, in the linear plasmid transferred to the recipient organism, the plasmid backbone region containing the *AMP* gene is not present.

The linear plasmid cleaved by the restriction enzyme *Xho*I and prepared is composed of the *cordapA* gene expression cassette ([Glb1Promoter]-[rAct1 intron]-[mDHDPS TP]-[*cordapA*]-[Glb1 3′ UTR]) and the *nptII* gene expression cassette ([*lox*P]-[CaMV 35S Promoter]-[*nptII*]-[ble]-[NOS 3′]-[*lox*P]). The *lox*P at the ends of the *nptII* gene expression cassette is nucleotide sequence recognized by Cre recombinase, the topoisomerase derived from the bacteriophage P1 (see Table 1).

For the production of MON810, two plasmids, PV-ZMBK07 and PV-ZMGT10 were used as vectors. PV-ZMBK07 was constructed by connecting i) *cry1Ab* gene cassette ([E35S]-[hsp70 intron]-[*cry1Ab*]-[NOS3']), and PV-ZMGT10 was constructed by connecting ii) modified *cp4 epsps* gene cassette ([E35S]-[hsp70 intron]-[CTP2]-[*cp4 epsps*]-[NOS3']) and *gox* gene cassette ([E35S]-[hsp70 intron]-[CTP1]-[*gox*]-[NOS3']), respectively to a basic vector derived from pUC119 containing the above-mentioned *nptII* gene. Map of the plasmids PV-ZMBK07 and PV-ZMGT10 is presented in Figure 3.

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

For the production of LY038, the linear plasmid contained the *cordapA* gene expression cassette ([Glb1 Promoter]-[rAct1 intron]-[mDHDPS TP]-[*cordapA*]-[Glb1 3′ UTR]) and *nptII* gene expression cassette ([*lox*P]-[CaMV 35S Promoter]-[*nptII*]-[*ble*]-[NOS 3′]-[*lox*P]) of PV-ZMPQ76 and does not contain any plasmid backbone region in the *AMP* gene region since this plasmid was cleaved by the restriction enzyme *Xho*I. This linear plasmid was transferred to callus tissue from the inbred dent maize line H99, by the particle gun bombardment.

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

iii) Processes of rearing of living modified organisms

[Process of rearing of LY038]

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

Callus, to which the genes were transferred, was grown temporarily on tissue culture medium containing 2,4-dichloro-phenoxyacetic acid (2,4-D). Paromomycin, an aminoglycoside derivative of the kanamycin antibiotic, was added to the culture medium to select for transformed callus cells. Full plants were regenerated from the selected callus, and the regenerated individuals, which express the cDHDPS protein derived from *cordapA* gene, were selected.

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

Plasmids were transferred in LY038 by the particle gun bombardment, so confirmation of remaining *Agrobacterium* was not carried out.

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

Among the individuals regenerated on the paromomycin-added medium, [Confidential] was selected and raised, and crossed with the inbred line of conventional maize varieties [Confidential] to produce the [Confidential] generation ([Confidential] in Figure 4). Then, in order to remove the *nptII* gene expression cassette region from the transferred genes, the [Confidential] generation was crossed with another recombinant maize line (Cre event to produce the [Confidential] generation). This Cre event contains the *cre* gene expression cassette that expresses Cre recombinase, the topoisomerase derived from bacteriophage P1.

Cre recombinase recognizes the recombination site in the *lox*P nucleotide sequence used for the production of LY038 so that the *nptII* gene cassette region in the transferred gene is removed (Figure 6). The method for removing target genes in plants using the Cre-lox system has been practically applied for maize, tobacco, and tomato, and it has been confirmed to ensure accurate removal of target genes (Reference 56; Reference 57; Reference 58; Reference 19). The subsequent maize inbreeding results in the individual plants that contain the *cre* gene expression cassette and do not contain the *cre* gene in the [Confidential] generation plant body. These individual plants were screened by the Southern blotting and PCR methods for the presence of each of these genes: *cre, nptII,* and *cordapA.* At this point in time, the individual plants that contain only the *cordapA* gene were selected and the other individual plants (that contain the *cre* gene or *nptII* gene, and/or do not contain *cordapA* gene) were disposed of before flowering.

Consequently, in LY038 in the [Confidential] generation and later, there is no individual that contains *cre* gene and *nptII* gene. For the production of commercial lines of LY038, the [Confidential], which has the *cordapA* gene homogenized through inbreeding of the individuals selected in the above [Confidential] generation, was used as mother plant for breeding (Figure 4).

This [Confidential] generation has been self-pollinated and/or crossed with conventional maize varieties to produce sibling lines. Then the sibling lines have been self-pollinated and/or crossed with conventional maize varieties to obtain hybrids, which have been subjected to field tests and gene analyses (For details on the generations examined, refer to Figure 4).

This application for approval of LY038 is intended for the individuals segregated in the [Confidential] generation to have the *cordapA* gene cassette alone and their derived progeny.

In addition, the plasmid map of PV-ZM003 used for the production of the Cre event is shown in Figure 5, and the origins and functions of the component elements of PV-ZM003 are listed in Table 4. The *Agrobacterium* method was used to transfer the T-DNA region of PV-ZM003, composed of the *cre* gene expression cassette and the *nptII* gene expression cassette into maize callus cells for the production of the Cre event. Carbenicillin was used for removal of any remaining *Agrobacterium* (Reference 59) and paromomycin was added to the culture medium to select against any untransformed individuals.

[Molecular analysis of Cre event]

For analysis of the transferred gene to the Cre event, the Southern blotting analysis was conducted for the [Confidential] generation, the first generation obtained by crossing the Cre event with [Confidential] generation. As a result, it was confirmed that one copy of the T-DNA region composed of *cre* gene expression cassette and *nptII* gene expression cassette and one copy of gene fragment composed of *nptII* coding region and NOS 3' are present in the genome DNA of the Cre event (Figure 6 to Figure 13 of Annex 3 of Biological Diversity Risk Assessment Report for LY038). In addition, it was also confirmed that no gene was transferred into the Cre event in any regions other than the T-DNA region of PV-ZM003 used for the production of the Cre event (Figure 13 of Annex 3 of Biological Diversity Risk Assessment Report for LY038).

Furthermore, as a result of the Southern blotting analysis for multiple generations, it was confirmed that the one copy of T-DNA region and the one copy of gene fragment composed of *nptII* coding region and NOS 3' in the Cre event are not present in any generations after [Confidential] (Figure 14 of Annex 3 of Biological Diversity Risk Assessment Report for LY038). Also, it was demonstrated by the Southern blotting analysis for the multiple generations that there exists no region other than the T-DNA region of PV-ZM003 (Figure 22 of Annex 4 of Biological Diversity Risk Assessment Report for LY038). The Southern blotting analysis is detailed in Annex 3 of Biological Diversity Risk Assessment Report for LY038.

[Growth characteristics of Cre event]

For the growth characteristics of Cre event (cre positive isoline of Annex 5 of Biological Diversity Risk Assessment Report for LY038), an evaluation was made in 2001 of nine items (the number of established seedlings at the early stage of growth, the extent of growth on the 21st day after sowing, the number of finally established seedlings, culm length, the number of days after sowing at time of 50% silking, rate of plants suffering breakdown lodging, rate of plants suffering root lodging, rate of sterile plants, and 100-kernel weight). The control plants were individuals in which the cre gene has been removed through segregation testing during the inbreeding so that they do not contain any transferred gene (cre negative isoline of Annex 5 of Biological Diversity Risk Assessment Report for LY038). For all phenotypic characteristics that were examined, no statistically significant difference was observed between the cre positive isoline and the cre negative isoline (Table 1 of Annex 5 of Biological Diversity Risk Assessment Report for LY038).

Based on the above results, it was considered that cre positive isoline is equivalent to the control plant cre negative isoline in the growth characteristics.

The following shows the approvals of LY038 received from organizations in Japan.

April 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the conformity of the program for use (including cultivation) of recombinant in isolated fields with the "Guidelines for the use of recombinant in agriculture, forestry and fisheries".

February 2005: An application was made to the Ministry of Health, Labour and Welfare to be ensured the safety of use of the cultivar for food, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques”.

March 2005: An application was made to the Ministry of Agriculture, Forestry and Fisheries to be ensured the safety of use of the cultivar for feed, in accordance with “Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques”.

January 2006: An application was made to the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for approval of Type I Use Regulations in accordance with the “Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms.”

Figure 4 Process of rearing of high lysine maize line LY038

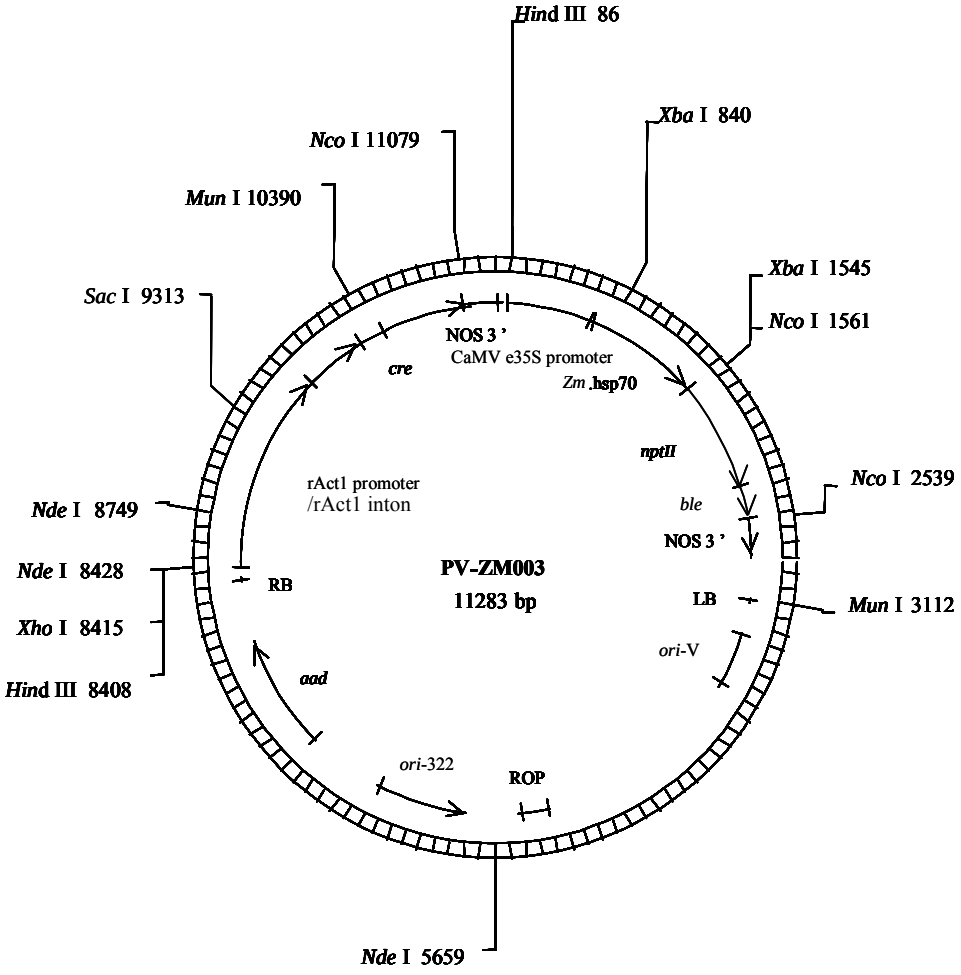

Figure 5 PV-ZM003 used for the production of Cre event [7]

The region between RB (right border sequence) and LB (left border sequence) was transferred by the *Agrobacterium* method into maize cells to produce the Cre event. It was demonstrated by the Southern blotting analysis that LY038 does not contain any T-DNA region (*cre* gene expression cassette and *nptII* gene expression cassette) derived from PV-ZM003, which was used for the production of the Cre event, nor any other regions (Figure 14 to Figure 18 of Annex 4 of Biological Diversity Risk Assessment Report for LY038, and Figure 14 of Annex 3 of Biological Diversity Risk Assessment Report for LY038).

[7] All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 4 Origins and functions of the component elements of plasmid PV-ZM003 used for the production of Cre event[8]

Component elements	Origin and function
cre gene expression cassette (Not present in LY038)	
rAct1 promoter/ rAct1 intron	Promoter region and intron from the rice actin 1 gene. The promoter region makes target genes expressed (Reference 16). The intron enhances the efficiency of splicing, thereby activating the expression of target genes (Reference 16; Reference 60).
cre	The coding region for Cre recombinase (rec3) gene, the topoisomerase of bacteriophage P1 (Reference 61). The recombinase recognizes two *lox*P sites (Table 2) to remove the DNA region present between the sites (Reference 62).
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from *A. tumefaciens.* It induces polyadenylation of mRNA (Reference 23).
nptII gene expression cassette (Not present in LY038)	
CaMV e35S promoter	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 20) and including enhancer region (Reference 63). It has the function to express the target genes in all tissues constantly.
Zm.hsp70	Intron from the maize *hsp* 70 gene, maintaining the gene transcription level (Reference 37).
nptII	A gene isolated from transposon Tn5 of *E. coli*, encoding the neomycin phosphotransferase II (Reference 21). This gene confers the kanamycin resistance and acts as a selective marker for transformation when expressed in maize.
ble	A portion of bleomycin-resistant gene isolated from Tn5 (Reference 22), though not sufficient for conferring the bleomycin resistance.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from *A. tumefaciens.* It contains a polyadenylation sequence for mRNA (Reference 23).
Other region (Not present in LY038)	
LB	A DNA fragment containing the left border sequence derived from Ti plasmid pTil5955 (Reference 64). Defines the T-DNA transferred from *A. tumefaciens* to the plant genome.
ori-V	A part of ABI *Agrobacterium* isolated from the broad-recipient range plasmid RK2 (Reference 65). Permits autonomous replication in vectors.
ROP	A coding sequence to repress primer protein to maintain the number of copies of plasmids in *E. coli* (Reference 66).
ori-322	The replication origin region derived from pBR322, for maintaining the plasmid in *E. coli* (Reference 25).
aadA	The bacterial promoter and coding region for 3'(9)-O-nucleotidyltransferase, an aminoglycoside modifying enzyme, derived from transposon Tn 7, conferring resistance to spectinomycin and streptomycin (Reference 67). (GenBank accession X03043)
RB	A DNA fragment containing right border sequence of nopaline type T-DNA derived from Ti plasmid pTiT37. Defines the T-DNA transferred from *A. tumefaciens* to plant genome (Reference 68).

[8] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

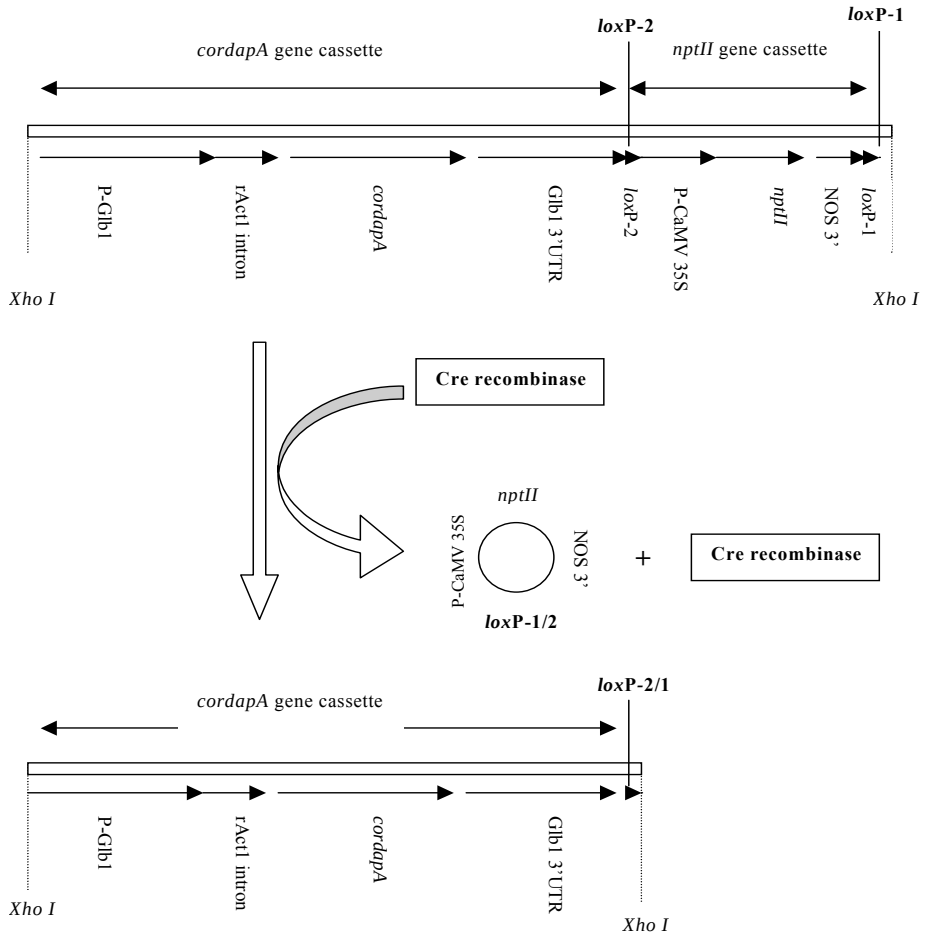

Figure 6 Schematic diagram for removal of *nptII* gene expression cassette by Cre-*lox*P[9]

About 5.9 kb portion of the region from *Xho* I (8772) to *Xho* I (5820) in the clockwise direction in PV-ZMPQ76 (Figure 1) was transferred by transformation into the maize genome. The transgenic plants were crossed with the individuals containing *cre* gene cassette to remove the *nptII* gene cassette. The ring-shaped *nptII* gene cassette and Cre recombinase shown in the diagram are removed by the subsequent inbreeding as follows: individual plants in the [Confidential] generation either contain or do not contain the *cre* gene expression cassette. These individual plants were screened by PCR and then the Southern blotting for the presence of each of these genes: *cre*, *nptII*, and *cordapA*. Only those individual plants were selected that do not contain the *cre* gene and *nptII* gene but contain the *cordapA* gene. All other individual plants (that contained either or both the *cre* gene or *nptII* gene) were disposed of before flowering. As a result, retained by LY038 plant was the *cordapA* gene expression cassette and two half portion of single recombined *lox*P locus. But the LY038 plant lacked the *nptII* gene cassette and *cre* gene cassette. This has been further confirmed by the Southern blotting analysis.

The mechanism of recombination by the Cre-*lox*P system has been well described in the scientific literature (Reference 62; Reference 69; Reference 56) and then it is outlined below. The Cre-*lox*P recombination system consists of the 38.5 kDa Cre recombinase and two *lox*P recognition sequences that flank the two ends of a linear portion of DNA that is to be removed. Each *lox*P sequence comprises a total of 34 bp composed of an 8 bp spacer sequence in between two 13 bp inverted replicate sequences (Reference 70). The Cre recombinase recognizes 13 bp inverted replicate sequences and catalyzes recombination across the 8 bp spacer sequence in the middle of the two individual *lox*P sites. As a result, the intervening linear DNA segment is removed and recombined into the ring shape while a half of individual *lox*P sites are connected at the both ends of the straight-chain DNA, and the rest half of individual *lox*P sites (*lox*P-1/2) remain on the genome.

[9] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Japan Limited.

[Process of rearing of MON810]

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

Callus to which PV-ZMBK07 and PV-ZMGT10 were transferred was grown on a tissue culture media containing 2,4-D for a certain period of time, and then the recombinant plant was selected on a glyphosate-containing medium. From the selected callus, the regenerated plant was obtained and the expression of Cry1Ab protein was analyzed. As a result of the Southern blotting analysis for transferred genes to MON810, it was confirmed that *nptII* gene, modified *cp4 epsps* gene and *gox* gene expression cassettes were not present (Figure 4 of Annex 2 of Biological Diversity Risk Assessment Report for MON810). For the reason why the recombinant plant was selected for glyphosate despite the fact that modified *cp4 epsps* gene was not transferred in MON810, it was considered likely that in the next generation ([Confidential]) of the regenerated plant ([Confidential]), segregation for the transferred gene could take place. However, for MON810, selection was repeated to obtain the pest insect resistant maize and the glyphosate assay and the Southern blotting analysis were not carried out in the next generation of the regenerated plant, so no definite reason was identified.

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

Plasmids were transferred in MON810 by the particle gun bombardment, so confirmation of remaining *Agrobacterium* was not carried out.

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

Pedigree selection was started in 1992, and field experiments were carried out from 1993 to 1995. Finally, MON810 was selected as an excellent event. In field experiments carried out at 6 sites in the US in 1994, the morphological and growth characteristics of this event were investigated and also analysis of the expressions of the Cry1Ab protein and transferred genes were implemented (For the events used in the tests, see Figure 7). Based on these results, necessary approvals were obtained in the US and general commercial cultivation has been conducted since 1997. An application for approval of MON 810 is intended for the individuals selected in the [Confidential] generation, which has the *cry1Ab* gene cassette, and their posterity.

Confidential: Not made available or disclosed to unauthorized person

Figure 7 Rearing process of MON810

The following shows the approvals of MON810 received from organizations in Japan.

March 1996: The Ministry of Agriculture, Forestry and Fisheries ensured the conformity of the program for use (use for processing and provision as feed) of recombinant in isolated fields with the "Guidelines for the use of recombinant in agriculture, forestry and fisheries".

October 1996: The Ministry of Agriculture, Forestry and Fisheries ensured the conformity of the import to Japan (use for processing and provision as feed) with the "Guidelines for the use of recombinant in agriculture, forestry and fisheries".

May 1997: The Ministry of Health, Labor and Welfare (Ministry of Health and Welfare, at that time) ensured the conformity of use as food with the “Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4”.

June 1997: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of use of the cultivar for feed with “Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)” .

March 2001: The Ministry of Health, Labour and Welfare ensured the safety of use for food, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques.”

May 2001: The Ministry of Agriculture, Forestry and Fisheries ensured the conformity of the program for use (including cultivation) of recombinant in isolated fields with the "Guidelines for the use of recombinant in agriculture, forestry and fisheries".

March 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of the use as feed, following “Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques.”

April 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the conformity of cultivation in Japan with the "Guidelines for the use of recombinant in agriculture, forestry and fisheries."

June 2004: The approval was received from the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use Regulations (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) in accordance with the “Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms”.

[Rearing process of LY038×MON810]

This stack line maize was produced by a crossbreeding between the two inbred lines LY038 and MON810 (Figure 8).

Figure 8 Rearing process of this stack line maize LY038×MON810

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

i) Place where the replication product of transferred nucleic acid exists

The gene transferred to LY038 is inherited in subsequent generations in agreement with Mendel's laws; therefore the transferred gene exists on the chromosome (Annex 7 of Biological Diversity Risk Assessment Report for LY038).

The gene transferred to MON810 is inherited in susequent generations in agreement with Mendel's laws; therefore the transferred gene exists on the chromosome (Annex 1).

ii) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations

As a result of the Southern blotting analysis to examine the genes transferred to LY038, it was confirmed that one copy of an intact *cordapA* gene expression cassette is transferred into the genome of LY038 at one site (see Annex 1 of Biological Diversity Risk Assessment Report for LY038 and Annex 4 of Biological Diversity Risk Assessment Report for LY038). In addition, it was also confirmed that there are no *nptII* gene expression cassette region, other regions of plasmid vector PV-ZMPQ76, or T-DNA region (*cre* gene expression cassette and *nptII* gene expression cassette) derived from the recombinant maize Cre event (the plasmid map of PV-ZM003 used for production is shown in Figure 5, and the origins and functions of component elements of PV-ZM003 are listed in Table 4) used for crossing in the [Confidential] generation and other regions (see Annex 1 of Biological Diversity Risk Assessment Report for LY038, Annex 4 of Biological Diversity Risk Assessment Report for LY038 and Annex 3 of Biological Diversity Risk Assessment Report for LY038). Moreover, it was demonstrated that the transferred gene is stably inherited in multiple generations (Figure 19 and Figure 20 of Annex 4 of Biological Diversity Risk Assessment Report for LY038). For the T-DNA region derived from the Cre event, additional studies across multiple generations have confirmed the absence of the cre gene expression cassette (Annex 3 of Biological Diversity Risk Assessment Report for LY038). In addition, it was also confirmed as a result of the Southern blotting analysis for the multiple generations that regions other than the T-DNA region of PV-ZM003 also do not exist (Annex 4 of Biological Diversity Risk Assessment Report for LY038). The schematic diagram of the transferred gene in LY038 is shown in Figure 9.

As a result of the Southern blotting analysis to examine the gene transferred to MON810, it was confirmed that one copy of DNA fragment derived from PV-ZMBK07 which is necessary for the expression of *cry1Ab* gene is transferred into the genome of MON810 at one site (Figure 3 of Annex 2 of Biological Diversity Risk Assessment Report for MON810). The schematic diagram of the transferred gene, which became clear from the results of the analysis and the analytical result of nucleotide sequence, is shown in Figure 10. In addition, as a result of the Southern blotting analysis in multiple generations (the generations identified with the superscript ^{d} in Figure 4), it was also proved that the transferred gene is descended stably to the progeny (Figure 6 of Annex 2 and Figure 2 of Annex 3 of Biological Diversity Risk Assessment Report for MON810).

As a result of the Southern blotting analysis of the transferred gene in MON810, it was confirmed that the gene transferred in the genome of maize is limited to the region necessary for the expression of the *cry1Ab* gene derived from PV-ZMBK07 and that there exist no expression cassettes of the modified *cp4 epsps* gene and *gox* gene derived from *nptII* gene and PV-ZMGT10 respectively (Figure 4 and Figure 5 of Annex 2 of Biological Diversity Risk Assessment Report for MON810). The schematic diagram of transferred gene in MON810 is shown in Figure 10.

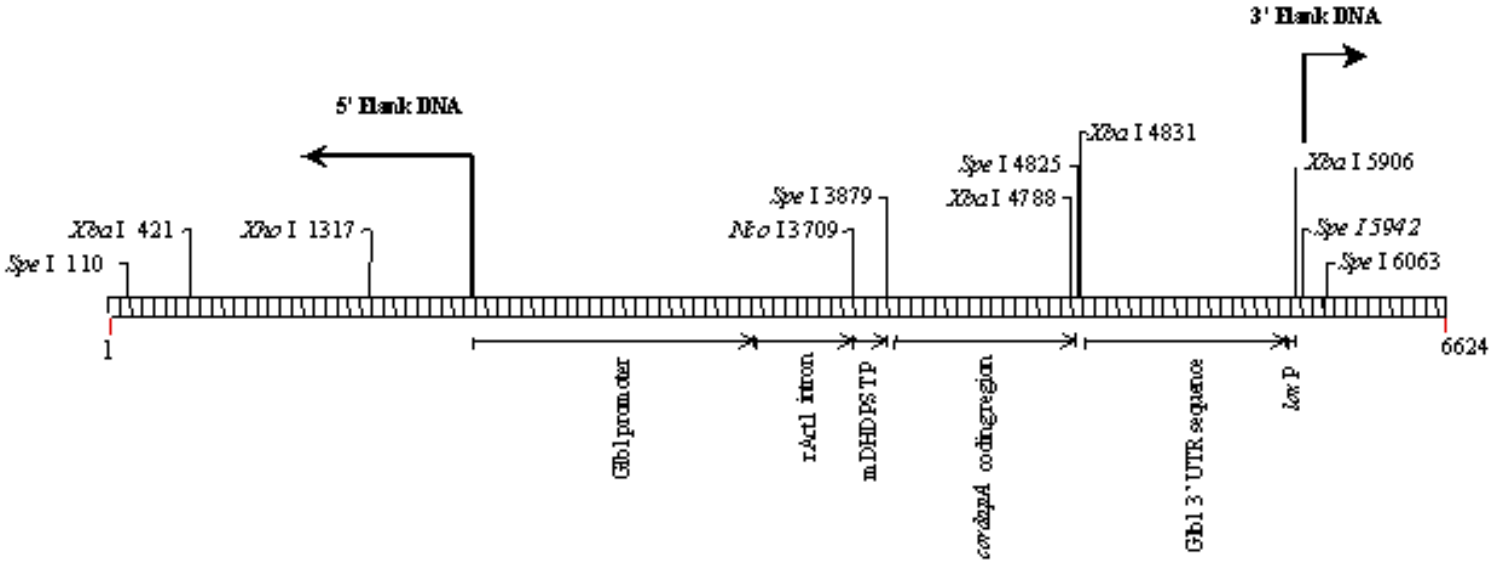

Figure 9 Map of transferred gene in LY038 and diagram of cleavage by restriction enzyme [10]

[10] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Japan Limited.

Figure 10 Map of transferred gene in MON810 [11]

[11] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Japan Limited.

iii) The position relationship in the case of multiple copies existing in chromosome

This item does not apply due to one copy for both LY038 and MON810.

iv) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

The Western blotting analysis was conducted using the polyclonal antibody specific to the cDHDPS protein for the protein extracted from the seeds in several generations of LY038. As a result, the seeds from all the generations subjected to the Western blotting analysis exhibited the band in agreement with the molecular weight of cDHDPS protein and it was confirmed that the target traits are stably expressed (see Figure 3 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

For MON810, it was confirmed in the process of selection based on bioassay that the resistance to the order Lepidoptera is stably expressed through multiple generations.

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

Regarding PV-ZMPQ76 used for the production of LY038 and PV-ZMBK07 and PV-ZMBK10 used for the production of MON810, the region of recipient organism, which allows autonomous replication, is limited to gram-negative bacterium such as *E. coli*. Consequently, there is no possibility that the plasmids might be transmitted to any wild animals and wild plants in the natural world.

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

For the detection and identification of LY038, the transferred genes and the DNA sequences in the flanking plant genome are used as primers. This method makes it possible to specifically detect LY038 (see Annex 4 of Biological Diversity Risk Assessment Report for LY038).

For the detection and identification of MON810, a standard analysis method is disclosed at the web site of the Ministry of Agriculture, Forestry and Fisheries (http://www.maff.go.jp/sogo_shokuryo/jas/manual00.htm) (Annex 4 of Biological Diversity Risk Assessment Report for MON810).

For the detection and identification of this stack line maize, the above-mentioned two methods must be applied to each grain of maize seeds.

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

It is estimated that the cDHDPS protein and Cry1Ab protein are expressed in the plant body of this stack line maize by the function of genes that were transferred in the parent lines LY038 and MON810.

As mentioned in I - 1 - (1) - 2), the cDHDPS protein expressed in LY038 is considered to have high substrate specificity; therefore, it is suggested that except for free lysine and the secondary metabolic products, saccharopine and α-aminoadipic acid, are enhanced in levels in the lysine biosynthesis pathway, any other metabolic pathway would not be affected. This conclusion is likely supported by the findings that in the component analysis of LY038, the amounts of free lysine and the secondary metabolic products, saccharopine and α-aminoadipic acid, were increased exceeding the variable ranges for conventional varieties but all the other components were found to fall within the variable ranges for the conventional maize varieties, and that in the morphological and growth characteristics tests of LY038, no specific difference from the control Null type plant was observed.

On the other hand, the Cry1Ab protein expressed in MON810 event does not possess any enzyme activity and functions independently from the metabolic system of the recipient organism, so it is considered not to affect the metabolic pathway of plant. This conclusion is likely supported by the findings that in the component analysis of MON810, all the components examined fell within the variable ranges for the conventional maize varieties and that in the morphological and growth characteristics test of MON810, no specific difference from the non-recombinant control maize was observed.

As discussed above, the LY038, the parent line of this stack line, has modified the lysine biosynthesis pathway, a part of the metabolic system of the recipient organisms, though the Cry1Ab protein expressed in the other parent line MON810 does not possess enzyme activity and does not affect the metabolic system of the recipient organism. Therefore, it was considered that even if LY038 and MON810 are crossed with each other based on the crossbreeding method, cDHDPS protein and Cry1Ab protein do not interfere with each other and the amount of free lysine and Lepidoptera resistance in this stack line maize do not cause any changes compared to the parent lines LY038 and MON810.

To confirm the above understanding in practice, the concentration of free lysine contained in this stack line maize was analyzed in grain from field trials conducted in Argentina. As a result, it was confirmed that there was no statistically significant difference in the concentration of free lysine between this stack line maize and LY038 in which only cDHDPS protein is expressed (Table 5). In addition, regarding resistance to Lepidoptera, a corn earworm (*Helicoverpa zea*) bioassay was conducted in Argentina field trials. As a result, it was confirmed that there was no statistically significant difference regarding damage to this stack line maize by corn earworm and damage to MON810 in which only Cry1Ab protein is expressed (Table 6).

Based on the above results, it was considered that the individual proteins expressed in this stack line maize function independently from each other. Then, for the information used in the Item-by-item assessment of Adverse Effects on Biological Diversity in II about the difference between this stack line maize and the taxonomic maize species to which the recipient organism belongs, the results of individual examinations for the various characteristics of LY038 and MON810 were attached as follows.

Table 5 Investigational results on free lysine concentration (μg/g dwt) in grains of this stack line maize based on component analysis[12]

	Free lysine concentration (μg/g dwt) (Mean value ± S.E.)
LY038×MON810	1,515 ± 50
LY038	1,439 ± 50
LY038(-)	25± 53

Grains were harvested from the fields at 4 sites in Argentina for component analysis (3 replicates/field, 2001/2002). As a result of statistical treatment, no statistically significant difference was observed between LY038 x MON810 and LY038 based on the analysis of variance (ANOVA) ($p<0.05$).

dwt = Dry weight

Table 6 Investigational results of the severity of damage by the order Lepidoptera (*Helicoverpa zea,* Corn earworm) based on bioassay of this stack line maize[13]

	Nodal injury score (NIS) (Mean value ± S.E.)
LY038 × MON810	0.14 ± 0.01
LY038(-) × MON810	0.13 ± 0.03
LY038(-)	2.68 ± 0.04

LY038×MON810, LY038(-)×MON810, and LY038(-) were cultivated in the fields in Argentina (4 replicates/field, 2002/2003). A total of 20 plants at R4 stage selected at random from each replicate were investigated for the severity of damage by *Helicoverpa zea* (corn earworm). The insect damage severity was evaluated on a scale from 0 to 3 as follows: (0: No damage, and no larvae 1: Minor damage observed in the first layer of grain 2: Damage observed in the second and third layers 3: Damage observed in the fourth and more layers). As a result of statistical treatment, no statistically significant difference was observed between LY038×MON810 and LY038(-)×MON810 ($p<0.05$).

[12] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.
[13] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

i) Specific contents of physiological or ecological characteristics that were accompanied by the expression of replication products of transferred nucleic acid

(a) Specific contents of physiological or ecological characteristics that were accompanied by the expression of *cordapA* gene in LY038

The grain harvested from the cultivation fields at 5 sites in the US in 2002 (3 replicates/maize under test/field) were analyzed for lysine, free lysine and downstream metabolic products after lysine biosynthesis (Figure 11). As the control plant, Null-type maize obtained by segregation in the process of development of LY038 was used. In addition, for comparison between the values measured by analysis, commercial maize varieties (20 varieties) cultivated concurrently in the individual fields were also analyzed.

As a result, it was demonstrated that, due to the cDHDPS protein expressed by the transferred *cordapA* gene, the percent total lysine content (%) and the level of free lysine (μg/g dwt) in the grain of LY038 in relation to the total amino acid level are enhanced and the levels of saccharopine and α-aminoadipic acid, the catabolites of lysine, are also increased as expected (Table 7 and Table 8). In addition, for pipecolic acid, a catabolite of lysine, the levels were found to fall within the ranges measured for commercial maize varieties, though it was found to be increased by about two times compared to the control Null-type maize (Table 7).

Moreover, regarding the total amino acid level other than lysine, a statistically significant difference was observed in glutamic acid, histidine, isoleucine, and phenylalanine compared to the control Null-type maize (Annex 10 of Biological Diversity Risk Assessment Report for LY038). However, the ranges of the measured values for the amino acids with observed statistically significant differences fell within the tolerance interval (99% T.I.) for the 20 concurrently cultivated non-recombinant commercial maize varieties (Annex 10 of Biological Diversity Risk Assessment Report for LY038). Therefore the level of amino acids other than lysine in LY038 was judged to fall within the variable ranges for the conventional maize varieties.

Based on the above results, the following was confirmed: In LY038, due to the cDHDPS protein expressed by the *cordapA* gene, the percent lysine content and free lysine level in the grain are enhanced in relation to the total amino acid level, exceeding the ranges for conventional maize, and resultantly, there was an increase in the levels of saccharopine, and α-aminoadipic acid and pipecolic acid, the downstream catabolites after lysine biosynthesis, exceeding the levels of conventional maize.

In order to evaluate any effects on wild animals of saccharopine, α-aminoadipic acid, and pipecolic acid, which are increased in LY038, a feeding test, review of the literature and acute toxicity test were conducted and the results are summarized below.

[Feeding test]

The following diets were fed to growing broiler chickens for 42 days to examine 24 items regarding their growth, 13 items regarding the weight of individual body parts at the end of the study, and 6 items regarding the composition of fillet and dark meat: The diet formulated to contain LY038 in which saccharopine, α-aminoadipic acid and pipecolic acid are accumulated (hereinafter referred to as "LY038 diet"); the diet with the control Null-type maize and added synthetic lysine formulated to the equal total lysine level as LY038 (hereinafter referred to as "LY038(-)L diet"); diets formulated with different non-recombinant commercial varieties and similarly added synthetic lysine (hereinafter referred to as "commercial variety L diets"); the diet formulated to contain the control Null-type maize without added synthetic lysine (hereinafter referred to as "LY038(-)NL diet"); and diets formulated with different non-recombinant commercial variety without added synthetic lysine (hereinafter referred to as "commercial variety NL diets") (Table 1 to Table 3 of Annex 8 of Biological Diversity Risk Assessment Report for LY038).

As a result, in all the items examined, no statistically significant difference was observed between the LY038 diet and the control LY038(-)L diet (Table 1 to Table 3 of Annex 8 of Biological Diversity Risk Assessment Report for LY038). In addition, in the comparison between the commercial variety L diets and the LY038 diet examined concurrently for reference, no statistically significant difference was observed for any items evaluated except for weight of fillets after treatment/total carcass weight (%).

Regarding the weight of fillet after treatment/total weight after treatment (%), in which a statistically significant difference was observed, no statistically significant difference was observed between the LY038 diet and the control LY038(-)L diet. Based on the results, it was considered that the differences would not compromise the safety as feed of LY038.

Based on study results, it was judged that the LY038 diet is equivalent to the control LY038(-)L diet and that saccharopine, α-aminoadipic acid and pipecolic acid contained in LY038 are not expected to affect the growth of broilers.

[Literature articles review]

1) Saccharopine

Saccharopine is synthesized by the reaction of lysine and lysine-ketoglutarate reductase (Figure 11). In addition, it is confirmed that saccharopine is present in asparagus (4 μg/g FW), lettuce (4 μg/g FW) (Reference 71), mushroom (102 μg/g) (Reference 72), broccoli (122 μg/g DW)*, cauliflower (97 μg/g DW)* and other plants.

* Monsanto in-house data

Regarding saccharopine, it is considered that saccharopine is produced in the body of human or livestock consuming typical lysine-containing foods or feeds. Additionally, saccharopine is metabolized at a high rate and as a result, it is not considered to accumulate in the body of humans or livestock (Reference 73). As a result of the comparison between the amount of saccharopine taken as feed, which is increased in LY038, and the amount of saccharopine considered to be metabolized in humans or livestock by the saccharopine catabolic enzyme (saccharopine dehydrogenase; SDH), it was concluded that an adequate amount of SDH is present in the liver of humans or livestock to metabolize the saccharopine derived from LY038 (Table 9). This amount of SDH refers to the value converted by Fellow *et al*. for 24 hours based on the experimental results. However, even if the amount of SDH varies much or less in a day, the saccharopine catabolic activity defined by the rate of saccharopine metabolized to the amount of saccharopine ingested per day from LY038 is found to be approx. 487,000 times more for human, approx. 36 times more for swine, and 56 times more for bovine. Therefore, it has been determined that higher metabolizm activity is still maintained in humans over the amount consumed.

There are some reports that saccharopine was actually detected in livestock, though the detected level is low (Reference 74; Reference 75), which supports the previous discussion by Fellow *et al*. that saccharopine does not accumulate in the body of animals. Based on the above understanding, the safety of LY038 is ensured since animals or livestock rapidly metabolize the saccharopine even if they take in LY038 as feed.

2) α-aminoadipic acid

α-aminoadipic acid is synthesized by the reaction of α-aminoadipate semialdehyde and aminoadipate-semialdehyde dehydrogenase, after saccharopine is metabolized by the α-aminoadipate semialdehyde (Figure 11). In addition, α-aminoadipic acid is confirmed to be present in lentil (7.90 μg/g FW), green peas (3.1 μg/g FW), lettuce (3.2 μg/ g FW)(Reference 76; Reference 77), broccoli (490 μg/g DW)*, cauliflower (175 μg/g DW)*, string bean (141 μg/g DW)*, mushroom (637 μg/g DW)* and other plants.

The amount of α-aminoadipic acid, contained in individual tissues of swine of 10.5 kg body weight consuming a lysine-containing basal diet (total lysine content of 1.15%) has been determined as 204.72 mg (Table 10) based on the report (Reference 78). This suggests that swine are regularly exposed to the levels of α-aminoadipic acid as a result of metabolizing the lysine contained in basal diet. On the other hand, assuming that the maize contained in the basal diet used in this test is

* Monsanto in-house data

composed entirely of LY038, an additional 21.8 mg α-aminoadipic acid could be ingested compared to the level ingested with the basal diet. Possible toxic effect of the additional 21.8 mg α-aminoadipic acid has been investigated as described below.

In another experiments of reference 78, the amount of α-aminoadipic acid in the blood plasma of swine given the basal diet (total lysine content of 1.15%) was 16 nmol/ml, whereas the amount of α-aminoadipic acid in the blood plasma of swine given the feed with 1.15% lysine added to the basal diet (total lysine content of 2.30%) was 108 nmol/ml, showing an approx. 6.8 times increase. However, of the examined parameters of increase in swine body weight, feed intake and feed efficiency, no statistically significant difference was observed (Reference 78). Based on the results, it was considered unlikely that the additional intake of 21.8 mg α-aminoadipic acid, approx 11% increase of 204.72 mg α-aminoadipic acid to which the swine is expected to be exposed as a result of feeding of the above described basal diet, would provide any toxic effect.

3) Pipecolic acid

Pipecolic acid is biosynthesized by the reaction of piperideine-6-carboxylic acid and piperideine-6-carboxylic reductase (Figure 11). In addition, it is confirmed that pipecolic acid is present in adzuki bean (16.94 μg/g), pinto bean (43.42 μg/g), broccoli (12.25 μg/g), cabbage (19.23 μg/g), cauliflower (10.57 μg/g), potato (2.43 μg/g) and other plants (Reference 79).

There is a report that no specific toxic effect was observed as a result of the actual dosing of pipecolic acid at 300 to 330 mg per 1kg body weight to rats (Reference 80). This dose was compared to the amount of pipecolic acid possibly consumed by livestock when ingesting LY038. Assuming that adult swine (body weight ranging from 70 to 115 kg) ingests approx. 3 kg of formula feed per day (Reference 81), of which maize accounts for 55.8% (Reference 82) which is all taken from LY038, the amount of pipecolic acid that swine would ingest per day from the grain of LY038 is determined to be approx. 0.845 mg[14]. Based on these calculations, the maximum dose of 330 mg/kg, at which no adverse effect was observed in rats, corresponds to approx. 391 times the amount of pipecolic acid that the swine would ingest from LY038 on a daily basis.

Based on the above results, it was considered unlikely that saccharopine, α-aminoadipic acid, and pipecolic acid would have any effect on the growth of animals.

[14] (35.35 x 3,000(g) × 0.558)/70 = 845 μg = 0.845 mg/1kg body weight, 330/0.845 = 391

[Acute toxicity test]

Regarding the pipecolic acid, the toxicity information using the rats has been obtained from literature review as discussed above and, acute toxicity tests using mice was conducted for the saccharopine and α-aminoadipic acid, for which no toxicity information has been obtained.

1) Saccharopine

Saccharopine was forced-administered orally in four (4) doses, 50, 150, 450, and 2,000 mg/kg to mice. As a result, even at the maximum dose of 2,000 mg/kg, no adverse effect was observed in the mice (Annex 11-a of Biological Diversity Risk Assessment Report for LY038).

Based on the above results, a calculation was made to determine how the 2,000 mg/kg dose of saccharopine, which is found to cause no toxic effect, corresponds to the amount expected to be consumed by livestock (swine taken as an example) from LY038 per day.

The maximum amount of saccharopine observed in the grain of LY038 harvested in the US field tests was 818.42 µg/g dwt (Table 7). Adult swine (body weight of 70 kg) consume approx. 3 kg formula feed per day (Reference 81). Assuming that the maize content of formula feed is 55.8% (Reference 82) and all of the maize in the feed is from LY038, the amount of saccharopine the swine consume per day from the grain of LY038 is found to be approx 19.6 mg per 1 kg body weight[15]. Consequently, the maximum dose of 2,000 mg/kg at which no adverse effect is observed in the mice in the test is found to correspond to approx. 102 times as large as the amount of saccharopine the swine would consume from LY038 on a daily basis.

2) α-aminoadipic acid

α-aminoadipic acid was forced-administered orally in four (4) doses, 50, 150, 450, and 2,000 mg/kg to mice. As a result, even at the maximum dose of 2,000 mg/kg, no adverse effect was observed in the mice (Annex 11-b of Biological Diversity Risk Assessment Report for LY038).

Based on the above results, a calculation was made to determine how the 2,000 mg/kg dose of α-aminoadipic acid that was found to cause no toxic effect corresponds to the amount expected to be consumed by livestock (swine taken as an example) from LY038 per day.

The maximum amount of α-aminoadipic acid observed in the grain of LY038 harvested in the US field tests was 89.32 µg/g dwt (Table 7). Adult swine (body weight of 70 kg) consume approx. 3 kg formula feed per day (Reference 81). Assuming that the maize content of formula feed is 55.8% (Reference 82) and all of the maize in the feed is from LY038,

[15] (818.42×3000(g)×0.558)/70=19,571µg = 19.6 mg/1 kg body weight, 2000/19.6 = 102

the amount of α-aminoadipic acid the swine consumes per day from the grain of LY038 is approx 2.1 mg per 1 kg body weight[16]. Consequently, the maximum dose of 2,000 mg/kg at which no adverse effect is observed in the mice in the test is found to correspond to approx. 936 times the amount of α-aminoadipic acid the swine would consume from LY038 on a daily basis.

Based on the above results, it was confirmed that saccharopine and α-aminoadipic do not have any toxic effect on mice.

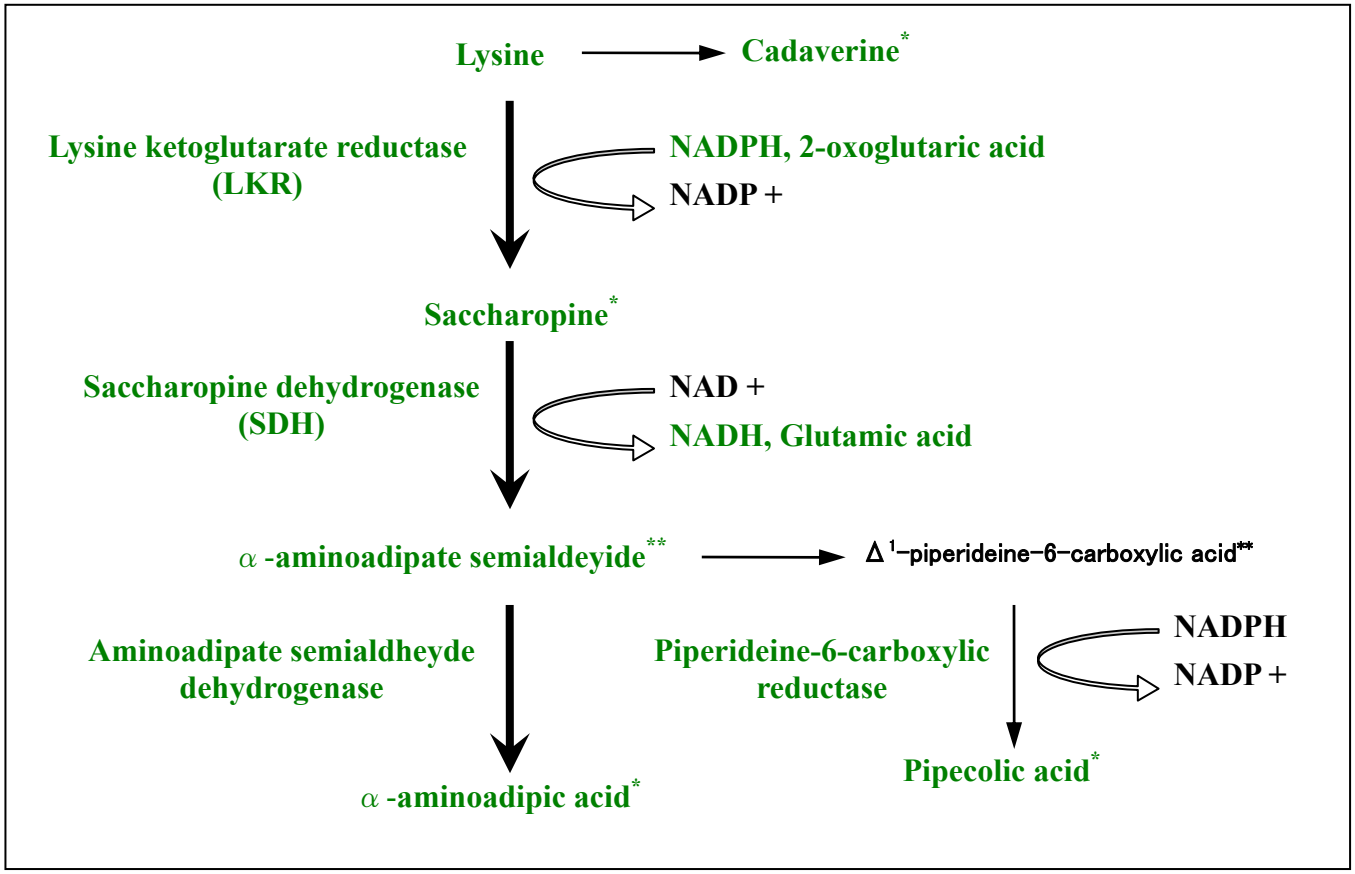

Figure 11 Metabolic pathway of lysine catabolism (Reference 83)[17]

* Metabolic products after biosynthesis of lysine analyzed
** Known to be unstable and then, left out from component analysis

[16] (89.32×3000(g)×0.558)/70=2,136 μg = 2.136 mg/1 kg body weight, 2000/2.136 = 936
[17] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 7 Analytical results on free lysine, lysine and related catabolites in grain of LY038[18]

Component (Unit)[1]	LY038 Mean value ± S.E.[2] (Range)[3]	Control plant Mean value ± S.E. (Range)	p-value	Commercial variety (Range) [99% T.I.[4]]
Lysine (% total AA)	3.81 ± 0.14 (3.08－4.50)	2.70 ± 0.14 (2.14－3.23)	<0.001	(2.38－4.07) [1.85, 4.29]
Free lysine (μg/g dwt)	1351.13 ± 109.52 (921.86－1696.61)	25.99 ± 3.18 (18.39－40.21)	<0.001	(14.69－108.52) [0, 104.89]
L-pipecolic acid (μg/g dwt)	28.72 ± 1.37 (22.37－35.35)	14.96 ± 1.58 (10.06－21.82)	<0.001	(2.71－42.15) [0, 45.15]
Saccharopine (μg/g dwt)	650.29 ± 36.40 (499.30－818.42)	5.88 ± 0.90 (2.75－8.26)	<0.001	(2.71－20.85) [0, 23.00]
Reference: Lysine in stem and leaf (% total prot. dwt)	4.70 ± 0.21 (4.00－6.46)	4.54 ± 0.21 (3.70－5.94)	0.379	(3.28－6.11) [3.17, 5.56]

Grains, stems and leaves harvested from fields at 5 sites in the US were analyzed for components (3 replicates/field; in 2002)

1 total AA = Total amino acid level, dwt = Dry weight, total prot = Total protein level
2 S.E. = Standard error
3 Difference between minimum and maximum values of measured values
4 Range defined as containing 99% of population of commercial varieties with 95% confidence. The lower limit defined at 0.

Table 8 Analytical results on α-aminoadipic acid in grain of LY038[19]

Field	LY038 Mean value (μg/g DW[2]) (Range)	Control plant Mean value (μg/g DW) (Range)	Commercial variety Mean value (μg/g DW) (Range)
1	82.34 (78.58 - 89.32)	6.33 (6.19 - 6.46)	8.76 (5.59 - 13.45)
2	39.65 (36.59 - 42.41)	≦ LOQ (-- - --)	≦ LOQ (-- - --)
3	50.66 (46.56 - 54.68)	≦ LOQ (-- - --)	≦ LOQ (-- - --)
4	59.93 (44.62 - 67.74)	≦ LOQ (-- - --)	8.59 (7.83 - 9.36)
5	50.36 (48.27 - 51.79)	≦ LOQ (-- - --)	≦ LOQ (-- - --)
Average	56.59 (36.59 - 89.32)	6.33 (6.19 - 6.46)	8.73 (5.59 - 13.45)

1 Statistical treatment was not conducted since most of control plant and commercial varieties exhibited the results below the limit of quantitation (LOQ).
2 DW = Dry wei

[18] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.
[19] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

Table 9 Metabolism of saccharopine in human and other animals[20]

	Metabolism amount of saccharopine by SDH (g /whole liver/day)	Estimated daily intake of saccharopine from LY038 (g/day)	Estimated intake of saccharopine from major components other than maize in the formula feed
Swine	39[a]	1.09 [b]	15.8 mg/day[e]
Bovine	131[a]	2.35[c]	63.6 mg/day[f]
Human	114[a]	0.000234[d]	87 mg/day[g]

a Reference 84

b Based on the assumption that adult swine (body weight ranging from 70 to 115 kg) ingests approx. 3 kg formula feed per day (Reference 81), of which maize accounts for 55.8% (Reference 85), which is all taken in from LY038.
3 kg formula feed × Maize content 0.558 × 650.29 mg /kg grain = 1.09 g saccharopine/day

c Based on the assumption that adult bovine ingests grain of maize as feed in approx. 9 kg formula feed per day (Reference 86), of which maize accounts for 40.1% (Reference 85), which is all taken in from LY038.
9 kg formula feed × Maize content 0.401× 650.29 mg/kg grain = 2.35 g saccharopine/day

d From the findings that the content of saccharopine in LY038 is 650.29 µg/g dwt, daily intake of maize and its processed goods = 0.4 g fwt, and water content of maize grain = 8.9%, a Japanese per capita daily intake of saccharopine is found 234 µg: (0.4 x (100-8.9)/100 = 0.36 g dwt, 0.36 g dwt x 650.29 µg/g dwt = 234 µg). This corresponds to 0.000234 g in terms of gram.

e Of 3 kg formula feed that swine takes in, the components other than maize are found 3 x (1-0.558) = 1.32 kg. Thus, estimated amount of saccharopine is found 12 x 1.32 = 15.8 mg.

f Of 9 kg formula feed that bovine takes in, the components other than maize are found 9x (1-0.441) = 5.30 kg. Thus, estimated amount of saccharopine is found 12 x 5.3 = 63.6 mg.

g For the major foods (cereal, pulse, vegetables, fruits, mushrooms, seafood, meat, dairy products, etc.) listed in Reference 86, the content of saccharopine was calculated based on literature and other available data. When no data is available for any foods from literature, the foods concerned are assumed to contain relatively similar amount of saccharopine as the foods for which data is available from literature.

[20] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

Table 10 Amount of α-aminoadipic acid detected in individual tissues of swine ingesting the lysine-containing basal diet, and the amount of α-aminoadipic acid contained in the basal diet derived from LY038[21]

(Calculated based on the data from experiments in which young swine weighing 10.5 kg ingests 1,057 g basal diet per day (Reference 78).)

	Proportion of individual tissues to the total body weight (1)	Amount of α-aminoadipic acid detected (2)	Total weight of α-aminoadipic acid in individual tissues	Content of α-aminoadipic acid derived from LY038 in basal diet
Blood plasma	5 .00 %	35 nmol/ml	2.13 mg(3)	—
Liver	1.14 %	2,268 nmol/g	31.48 mg(4)	—
Kidney	0.36 %	769 nmol/g	3.38 mg(5)	—
Muscle	49.00 %	281 nmol/g	167.73 mg(6)	—
Total	—	—	204.72 mg	21.77 mg(7)

(1) Based on the literature and other data including "A Digest of Statistics" by the Ministry of Agriculture, Forestry and Fisheries (Reference 87)
(2) Reference 78
(3) Calculation of the content of α-aminoadipic acid【molecular weight = 116.16, namely 1 mol = 116.16 g】 in blood plasma: The content of α-aminoadipic acid in 1 mL of blood plasma is found 35 x 10-9 x 116.16 = 4,066 x 10-9 g. The volume of blood plasma in a body weight of 10.5 kg becomes 10,500 g x 0.05 = 525 g. Therefore, the total amount of α-aminoadipic acid contained in the blood plasma is found 4,066 x 10-9 x 525 = 2.13 x 10-3 g = 2.13 mg.
(4) Calculation of the content of α-aminoadipic acid in the liver: The content ofα-aminoadipic acid in 1 g liver is found 2,268 x 10-9 x 116.16 =2.63 x 10-4 g. The mass of liver in a body weight of 10.5 kg becomes 10,500 g x 0.0114 = 119.7 g. Therefore, the total amount of α-aminoadipic acid contained in the liver is found 2.63 x 10-4 x 119.7 = 314.8 x 10-4 g = 31.48 mg.
(5) Calculation of the content of α-aminoadipic acid in the kidney: The content of α-aminoadipic acid in 1 g kidney is found 769 x 10-9 x 116.16 = 8.93 x 10-5 g, and the mass of kidney in a body weight of 10.5 kg is 10,500 x 0.0036 = 37.8 g. Therefore the amount of α-aminoadipic acid contained in the kidney is found 8.93 x 10-5 x 37.8 = 3.376 x 10-3 g = 3.38 mg.
(6) Calculation of the content of α-aminoadipic acid in muscle: The content of α-aminoadipic acid in 1 g muscle is found 281 x 10-9 x 116.6 = 3.26 x 10-5 g, and the mass of muscle in a body weight of 10.5 kg is 10,500 x 0.49 =5,145 g. Therefore, the amount of α-aminoadipic acid in the muscle is found 3.26 x 10-5 x 5,145 = 16,773 x 10-5 g = 167.73 x 10-3 g = 167.73 mg.
(7) 1,057 (the weight of feed ingested) x 0.364 (the content of feed derived from maize) x 56.59μg (the measured content of α-aminoadipic acid in 1 g of LY038) = 21,772 μg = 21.77 mg

[21] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Japan Limited.

(b) Specific contents of physiological or ecological characteristics that were accompanied by the expression of *cry1Ab* gene in MON810

It was confirmed that with the expression of Cry1Ab protein, which is encoded by the *cry1Ab* gene, resistance to insect damage by *Ostrinia nubilalis*, the major pest insect of the order Lepidoptera for maize cultivation in the US, was conferred to MON810 and then insect damage by *Ostrinia nubilalis* was decreased (Annex 2 of Biological Diversity Risk Assessment Report for MON810).

ii) Differences between the recombinant plant and the taxonomic species to which the recipient organism belongs[22]

Isolated field tests were carried out in Kawachi Research Farm (KRF), Monsanto Japan Limited, in 2003 using the maize lines LY038-A and LY038-B, which belongs to LY038, and the control maize lines Cont-38A and Cont-38B. The LY038-A and LY038-B are two F1 hybrids obtained by crossing the [Confidential] generation of the progeny for generations of LY038 with two different non-recombinant maize inbred lines respectively (see Figure 4). The control lines Cont-38A and Cont-38B are the F1 hybrids obtained by crossing the Null-type maize segregated in the [Confidential] generation of LY038 with two different non-recombinant maize inbred lines respectively (see Figure 4) It has been confirmed by the Southern blotting analysis and PCR analysis that *cordapA* gene, *nptII* gene, and *cre* gene are not present in the genome of Null-type maize (Figure 5 to Figure 23 of Annex 4 of Biological Diversity Risk Assessment Report for LY038).

Isolated field tests were carried out in National Institute for Agro-Environmental Sciences in 1996 and from 2001 to 2002 using MON810AX and MON810BX which belong to the event of MON810, as well as MON810AC and MON810BC as the control lines. MON810AX and MON810BX are the hybrid progeny lines which are derived from the different rearing processes starting from the first generation (R0) of the MON810 as indicated by the pedigree in Figure 7. The control lines MON810AC and MON810BC are the hybrid progeny lines of the non-recombinant maize crossed in a specific way to attain the same genetic background with MON810AX and MON810BX.

(a) Morphological and growth characteristics

For the morphological and growth characteristics of LY038, evaluation was conducted on a total of 19 items (uniformity of germination, germination rate, time of tassel exertion, time of silking, flowering period, culm length, plant shape or plant type, tiller number, height of ear, maturation period, number of ears, ear length, ear diameter, row number per ear, grain number per row, grain color, 100-kernel weight, grain shape and plant weight in the harvest time). For the recombinant maize LY038-A evaluated, a statistically significant difference from the control Null-type maize Cont-38A was observed in culm

[22] All the rights pertinent to the information in (a) through (g) in this section and the responsibility for the contents rest upon Monsanto Japan Limited.

length, height of ear, ear diameter and row number per ear ($p<0.05$). For the other recombinant maize LY038-B evaluated, a statistically significant difference from the control Null-type maize Cont-38B was observed in row number per ear and 100-kernel weight ($p<0.05$), but in all the other items evaluated, no difference was observed (see Annex 6 of Biological Diversity Risk Assessment Report for LY038). For the characteristics evaluated in which a difference was observed between the respective combinations of recombinant maize and control Null-type maize, their mean values are all found to fall within the variable ranges for the conventional maize when comparison is made between the maximum and minimum mean values of non-recombinant maize used as control for the recombinant maize (MON863 line, MON810 line, NK603 line, DLL25 line, MON88001 line, MON88012 line and MON88017 line) subjected to the isolated field tests and the variable ranges for conventional maize (see Table 2 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

For MON810 and the non-recombinant control maize, evaluation was conducted on germination rate, uniformity of germination, time of tassel exertion, time of silking, time of flower initiation, time of flower completion, flowering period, maturation period, plant type, tiller number, total number of ears, number of effective ears, culm length, height of ear, grain color and grain shape of ear, and fresh weight at harvesting time. In any of the characteristics except in culm length, statistically significant difference was not observed between MON810 and the non-recombinant maize (Table 2 of Annex 2 and Annex 3 of Biological Diversity Risk Assessment Report for MON810). Regarding culm length, statistically significant difference was found between the recombinant maize MON810BX and the non-recombinant control maize MON810BC, and the average value of culm length was 248.1 cm for MON810BX and 229.3 cm for MON810BC (Table 4 of Annex 3 of Biological Diversity Risk Assessment Report for MON810). Meanwhile no statistically significant difference was observed between the recombinant maize MON810AX and the non-recombinant control maize MON810AC (Table 4 of Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

For the recombinant maize LY038-A and its control Null-type maize Cont-38A, and the recombinant maize LY038-B and its control Null-type maize Cont-38B, the seedlings at 3rd leaf stage were left to stand in the condition at 5℃ for 22 days. As a result, the seedlings were all found completely dead, and no difference was observed in the degree of death (see Annex 6 of Biological Diversity Risk Assessment Report for LY038).

Sensitivity to cold temperatures (between highest temperatures of 12 to 14℃ and lowest temperature of 2℃) of the seedlings of MON810 and the non-recombinant control maize was evaluated. All the fully developed leaves and had wilted on the 21st day after the start of cold treatment. No difference was observed between MON810 and the non-recombinant control maize in cold tolerance (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not regrow and propagate vegetatively. It was observed that dying started after ripening at the end of isolated field experiments for the parent lines LY038 and the relevant control Null-type maize, and MON810 and the relevant non-recombinant control maize in practice. Based on the above, an overwintering test for the matured plant of this recombinant maize was not carried out.

(d) Fertility and size of the pollen

Between the recombinant maize LY038-A and its control Null-type maize Cont-38A, and between the recombinant maize LY038-B and its control Null-type maize Cont-38B, no statistically significant difference was observed in fertility of the pollen, but high fertility of pollen was observed (see Table 3 of Annex 6 of Biological Diversity Risk Assessment Report for LY038), and no difference was observed in the shape and size of the pollen (see Annex 6 of Biological Diversity Risk Assessment Report for LY038).

To examine the fertility (maturity) and size of the pollens of MON810 and the non-recombinant control maize, pollens were stained with 0.1% neutral red solution and iodine-potassium iodide solution and observed under a microscope. As a result, no difference was observed between MON810 and the non-recombinant control maize (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

Regarding the production of seeds for LY038, an examination was conducted to identify the number of grains formed per ear for the ears harvested from crossing between siblings. The number of grains formed per ear was observed to be statistically significant different between the recombinant maize LY038-B and its control Null-type maize Cont-38B ($p<0.05$) (see Table 3 of Annex 6 of Biological Diversity Risk Assessment Report for LY038). However, between the recombinant maize LY038-A and its control Null-type maize Cont-38A, no difference was observed (see Table 3 of Annex 6 of Biological Diversity Risk Assessment Report for LY038). For the number of grains formed per ear of the recombinant maize LY038-B, their mean values are all found to fall within the variable ranges for the conventional maize when comparison is made between the maximum and minimum mean values of the Null-type maize used as control for the recombinant maize (MON863 line, MON810 line, NK603 line, DLL25 line, MON88001 line, MON88012 line and MON88017 line) subjected to the isolated field tests and the variable ranges for conventional maize (see Table 3 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

Regarding the production of the seed of MON810, ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight of the ears harvested after sib-mating were examined. As a result, in all of the

characteristics examined, no statistically significant difference was observed between MON810 and the non-recombinant control maize (Annex 2 and Annex 3 of Biological Diversity Risk Assessment Report for MON810).

Regarding shedding habit of the seed, shedding habit was not observed in the natural conditions, since the ears of LY038 and its control Null-type maize and MON810 and its non-recombinant control maize were both covered with husks at the time of harvesting.

Regarding the germination rate of harvested seeds of LY038, no statistically significant difference was observed between the recombinant maize LY038-A and its control Null-type maize Cont-38A, and between the other recombinant maize LY038-B and its control Null-type maize Cont-38B (see Table 4 of Annex 6 of Biological Diversity Risk Assessment Report for LY038). The both recombinant maize exhibited a high germination rate of 95% or more, and no dormancy of the seeds was observed.

Regarding the germination rate 4 days after sowing of harvested seeds of MON810, no statistically significant difference was observed between the recombinant maize and the non-recombinant maize, and no dormancy of the seeds was observed (Annex 3 of Biological Diversity Risk Assessment Report for MON810).

(f) Crossability

A crossability test of the parent lines LY038 and MON810 was not performed, since no wild relatives that can be crossed grow in Japan.

(g) Productivity of harmful substances

[Productivity of harmful substances by LY038]

Regarding the number of bacteria, actinomyces and filamentous fungi in soil before and after cultivation, no statistically significant difference was observed between the recombinant maize LY038-A and its control Null-type maize Cont-38A, and between the recombinant maize LY038-B and its control Null-type maize Cont-38B (see Table 5 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

In addition, as a result of plant body plow-in test using radish, no statistically significant difference was observed between the recombinant maize LY038-A and its control Null-type maize Cont-38A, and between the recombinant maize LY038-B and its control Null-type maize Cont-38B in the germination rate, fresh weight and dry weight of radish (see Table 6 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

Moreover, the *cordapA* gene transferred to LY038 is regulated by the Glb1 promoter which makes target genes expressed primarily in grain, so there was a concern about possible production of any unintended harmful substances in grain. Therefore, an additional plow-in test using the grain of LY038 and the

control Null-type maize was conducted. As a result, no statistically significant difference was observed between LY038 and its control Null-type maize in the number of radish plants germinated, plant height, fresh weight and dry weight ($p<0.05$) (see Table 2 of Annex 9 of Biological Diversity Risk Assessment Report for LY038).

As a result of succeeding crop tests using radish, no statistically significant difference was observed between the soil for cultivation of recombinant maize LY038-A and the soil for cultivation of the control Null-type maize Cont-38A in all the items examined, radish germination rate, fresh weight and dry weight. In contrast to this, between the soil for cultivation of the recombinant maize LY038-B and the soil for cultivation of the control Null-type maize Cont-38B, a statistically significant difference was observed in radish dry weight, though no statistically significant difference was observed in germination rate and fresh weight ($p<0.05$) (see Table 7 of Annex 6 of Biological Diversity Risk Assessment Report for LY038).

In the succeeding crop tests, no statistically significant differences were observed, although it was observed that the germination rate of radish (Product name: Icicle) used as the test plant was mumerically slightly lower when cultivated on the soil from LY038 (see Table 7 of Annex 6 of Biological Diversity Risk Assessment Report for LY038). This was considered to result from the possibility that the germination property of radish (Product name: Icicle) used in the succeeding crop tests is not uniform inherently. Then, the preliminary germination test was conducted using 5 varieties of radish (Shirasagi (white heron), Comet, Icicle, White-Mini, and French Breakfast), and an additional succeeding crop test was followed using the French Breakfast variety which was found best for uniformity of germination in the preliminary germination test (see Table 1 of Annex 9 of Biological Diversity Risk Assessment Report for LY038). The watering to pots was provided by drawing up water from the bottom of pots rather than watering to the surface, and the number of replicates was also increased to 4 replicates from 3.

This study resulted in no statistically significant difference observed between LY038 and the control Null-type maize in the number of radish plants germinated, plant height, fresh weight, and dry weight (see Table 3 of Annex 9 of Biological Diversity Risk Assessment Report for LY038).

[Productivity of harmful substances by MON810]

Plow-in tests, succeeding crop tests, and soil microflora tests were performed for MON810 and the non-recombinant control maize. Statistically significant difference was not observed in any of the items (Tables 13-1 to 13-2, Table 14, Table 15, Table 5 and Table 6 of Annex 3 of Biological Diversity Risk Assessment Report for MON810).

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

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

This stack line maize was produced by traditional crossbreeding of high lysine maize (LY038) and Lepidoptera resistant maize (MON810), and the parent lines had been individually judged at the Meeting for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied for the Type I Use same as the stack line maize.

It is reported that the cDHDPS protein which is encoded by dihydrodipicolinate synthase gene (*cordapA* gene) derived from LY038 is the enzyme that possesses high substrate specificity, and that the Cry1Ab protein which is encoded by Lepidoptera resistant gene (*cry1Ab* gene) derived from MON810 does not possess enzyme activity. Consequently, it is considered to be extremely low that the characteristics conferred by *cordapA* and *cry1Ab* could interact with each other.

This stack line maize has been confirmed not to be significantly different from the parent lines based on the component analysis of grains for free lysine content and based on the bioassay with corn earworm for Lepidoptera resistance.

Based on the above understanding, it is considered that there is no specific change in the characteristics in this stack line maize except it possesses the same characteristics as the parent lines do.

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

(1) Competitiveness

In this stack line maize, the content of free lysine is enhanced specifically in the grains due to the cDHDPS protein which is encoded by *cordapA* gene derived from LY038 and at the same time, the resistance to Lepidoptera is expressed due to the Cry1Ab protein which is encoded by *cry1Ab* gene derived from MON810. However, the enhanced free lysine content in the grains is considered unlikely to cause this stack line maize to become competitive. In addition, the insect damage by the order Lepidoptera is not the major cause to make the maize difficult to grow in the natural environment in Japan. Consequently, it is considered that these characteristics do not increase the competitiveness and thus this stack line maize is not predominant over the parent lines in the competitiveness.

Based on the above understanding, it was 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 line maize possesses the productivity of cDHDPS protein derived from LY038 and the productivity of Cry1Ab protein derived from MON810. It has been confirmed that the Cry1Ab protein possesses the insecticidal activity against insects of the order Lepidoptera and also that the cDHDPS protein enhances lysine content in the grains of maize though it is not a harmful substance to animals and plants. Thus, it is considered that cDHDPS protein and Cry1Ab protein do not interact with each other. Consequently, it is considered unlikely that the productivity of harmful substances of this stack line maize could become higher than that derived from the combined characteristics of parent lines even though this stack line maize contains the both proteins.

Therefore, the conclusion that the use of this stack line maize poses no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances, which was made by the applicant, is valid.

(3) Crossability

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

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

[Bibliography]