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 maize (*cordapA*, *Zea mays* subsp. mays (L.) Iltis) (LY038, OECD UI : REN-ØØØ38-3)
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

This recombinant maize 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 this recombinant maize 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 (Reference 8).

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the production of high lysine maize (*cordapA*, *Zea mays* subsp. *mays* (L.) Iltis) (LY038, OECD UI: REN-ØØØ38-3) (hereinafter referred to as "this recombinant maize") are shown in Figure 1 and Table 1.

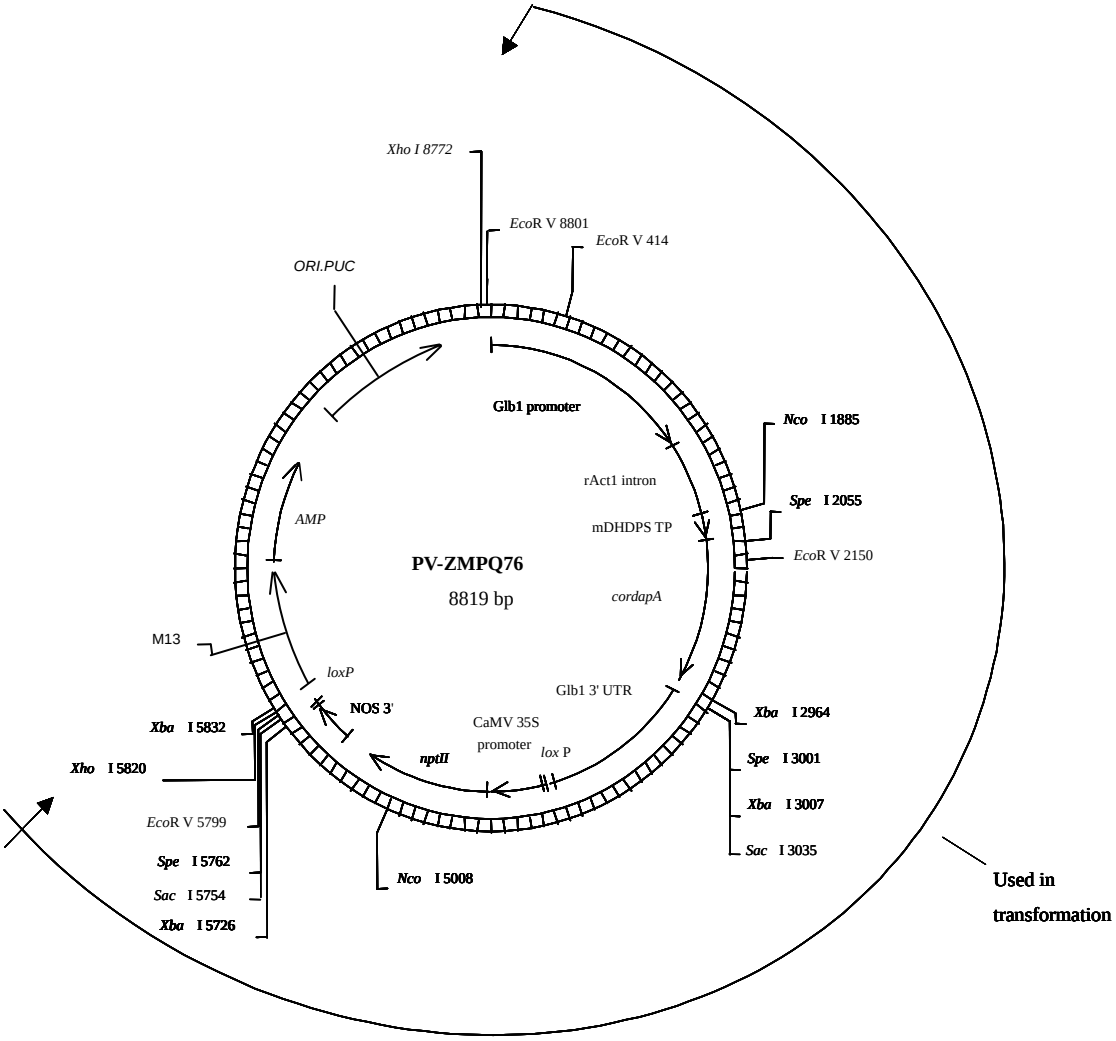

Figure 1 Plasmid vector PV-ZMPQ76 used for the production of this recombinant maize (Former designation：PV-ZMCTB331)[1]

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 this recombinant maize[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 this recombinant maize)	
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.

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

The functions of component elements of the donor nucleic acid that were used for the production of this recombinant maize are shown in Table 1.

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 this recombinant maize, 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). 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 this recombinant maize, the production of free lysine in the grain is enhanced compared to conventional maize (see Annex 1). In addition, with increasing production of free lysine, the levels of lysine catabolites, saccharopine and α-aminoadipic acid, are increased.

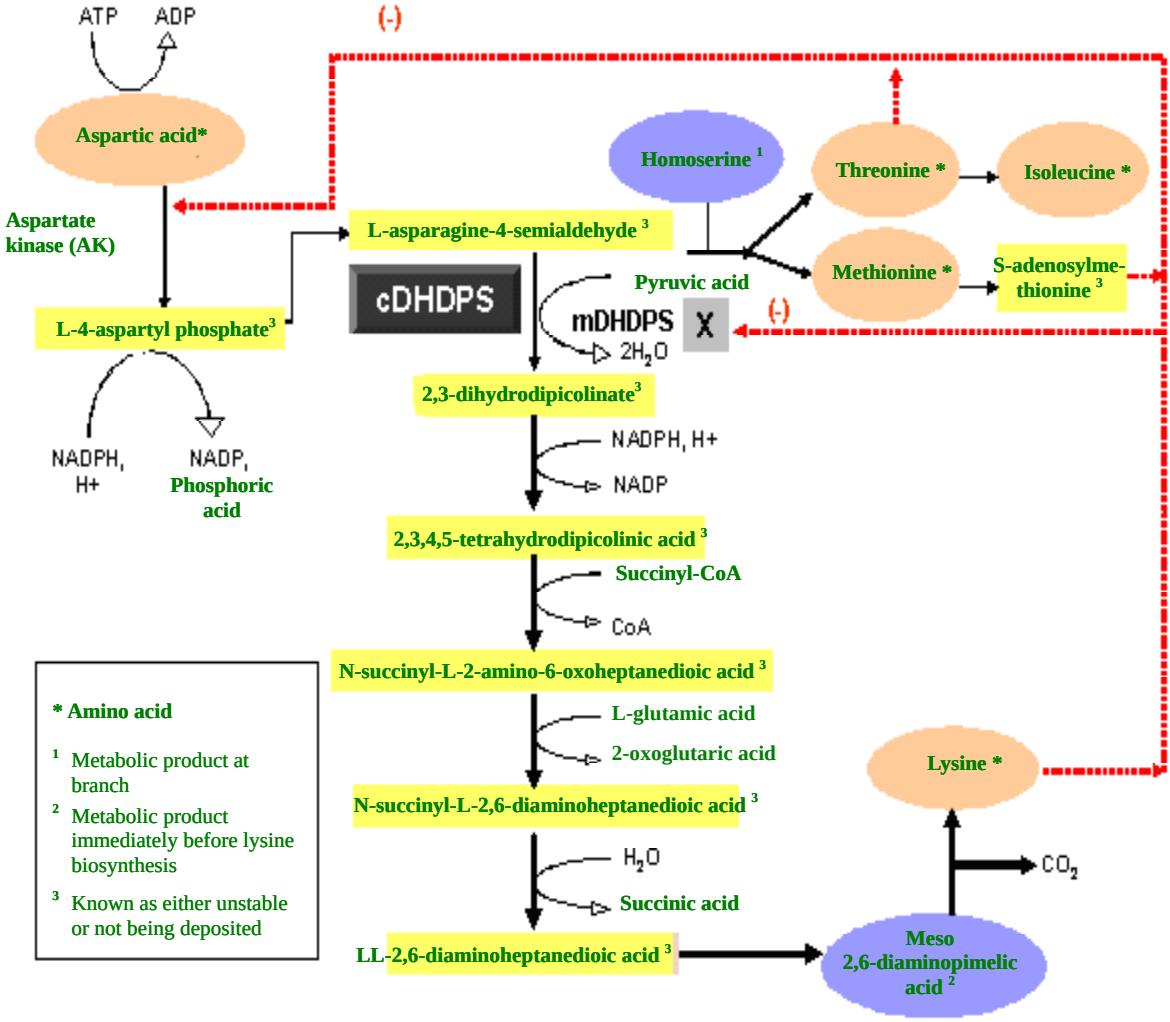

Figure 2 Lysine biosynthesis pathway[3]

(-); Lysine feedback inhibition

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.

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

(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 69). 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 29). 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 seen by the difference 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 30; Reference 31; 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 69, [2] Reference 29, [3] Reference 70, [4, 5] Reference 71

n/d – not determined

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

On the other hand, in the production of this recombinant maize, 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 this recombinant maize do not contain the *nptII* gene expression cassette (see I-2-(4)-ii)). Its gene product, neomycin phosphotransferase II (NPTII), utilizes ATP to phosphorylate and inactivate kanamycin, neomycin, paromomycin and other aminoglycoside derivative antibiotics.

(2) Information concerning vector

i) Name and origin

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

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 this recombinant maize is 8,819 bp. The entire nucleotide sequence of this plasmid vector is provided in Annex 2.

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

This vector contains the *AMP* gene as a selective marker gene, which expresses β-lactamase to confer the ampicillin resistance from *E. coli* (Reference 25).

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 this vector 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 (Former designation: PV-ZMCTB331) constructed for the production of this recombinant maize 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 of PV-ZMPQ76 prepared by cleaving the DNA with restriction enzyme *Xho*I was used to transfer the gene cassette to maize is required for expression of *cordapA* and *nptII* (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 and prepared by the restriction enzyme *Xho* I 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 are nucleotide sequences recognized by Cre recombinase, the topoisomerase derived from the bacteriophage P1 (see Table 1).

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

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.

iii) Processes of rearing of living modified organisms

[Process of rearing of this recombinant maize]

(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 this recombinant maize 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, R0 was selected and raised, and crossed with the inbred line of conventional maize varieties [Confidential] to produce the [Confidential] generation. Then, in order to remove the *nptII* gene expression cassette region from the transferred genes, the [Confidential] generation (Figure 3) was crossed with another recombinant maize line (Cre event). This Cre event contains the *cre* gene

expression cassette that expresses Cre recombinase, the topoisomerase derived from bacteriophage P1.

Cre recombinase from the Cre event, in conjunction with the two *loxP* recognition sequences in the event containing the *cordapA* gene expression cassette to catalyze excision of the straight-chain DNA that spans between the middle of the two *loxP* sites. Cre recombinase recognizes the recombination site in the *lox*P nucleotide sequence so that the *nptII* gene cassette region is removed (Figure 5). 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 34; Reference 35; Reference 35; Reference 19). The subsequent maize inbreeding results in the individual plants that contain the *cordap*A gene expression cassette and do not contain the *cre* gene in the [Confidential] generation plant body. Southern blots and PCR methods were used to select for plants that lack the *cre* gene, and the *nptII* gene and contain the *cordapA* gene. 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, for the production of commercial lines of this recombinant maize in the [Confidential] and later generations, the *cordapA* gene is present while all individual plants lack the *cre* gene and *nptII* gene. (Figure 3).

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 3).

This application for approval 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 4, and the origins and functions of the component elements of PV-ZM003 are listed in Table 3. 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 36) and paromomycin was added to the culture medium to select against any untransformed individuals.

[Molecular analysis of Cre event] (Annex 3)

For analysis of the transferred gene to the Cre event, Southern blot 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). 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).

Furthermore, as a result of the Southern blot 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). 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). The Southern blotting analysis is detailed in Annex 3.

[Growth characteristics of Cre event] (Annex 5)

For the growth characteristics of Cre event (cre positive isoline of Annex 5), 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). 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).

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 this recombinant maize 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: The Ministry of Health, Labour and Welfare 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: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of the use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques".

Confidential: Not made available or disclosed to unauthorized persons

Figure 3 Process of rearing of high lysine maize line LY038

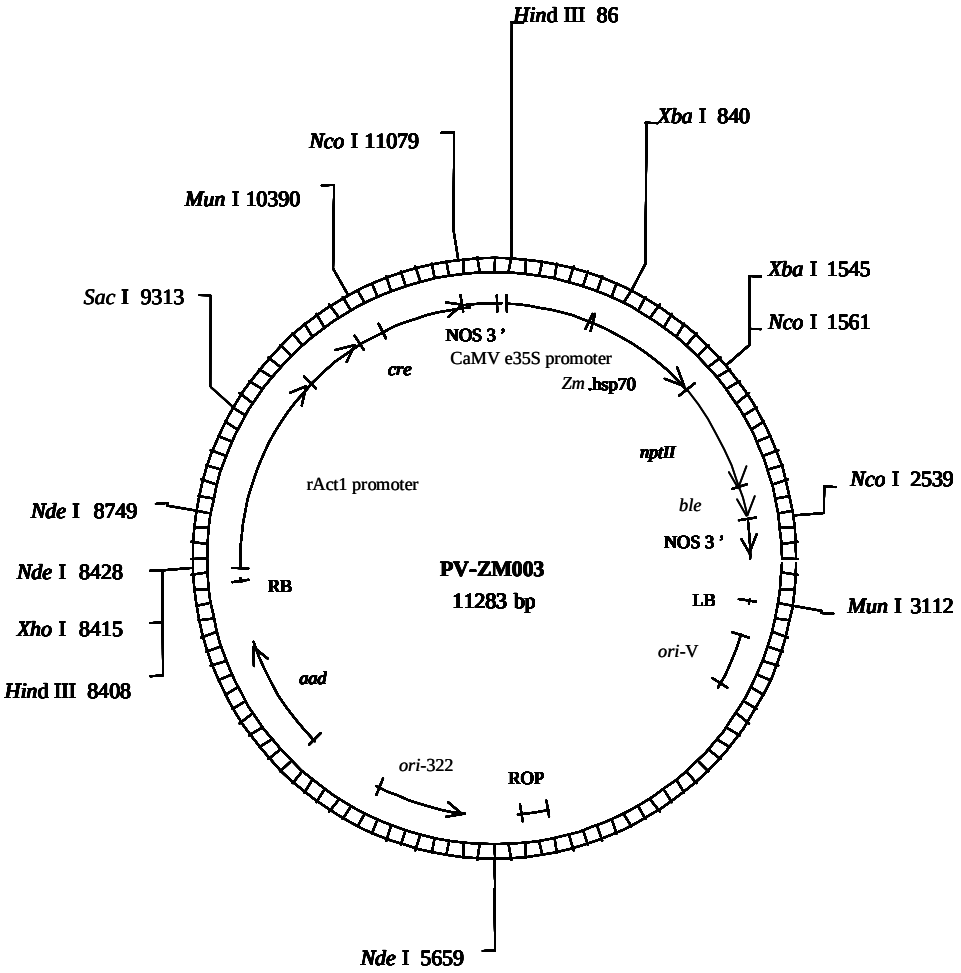

Figure 4 PV-ZM003 used for the production of Cre event[5]

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 this recombinant maize 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 PV-ZM003 backbone seqeunces (Figure 14 in p53 to Figure 18 in p57 of Annex 4, Figure 14 of Annex 3).

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

Table 3 Origins and functions of the component elements of plasmid PV-ZM003 used for the production of Cre event[6]

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 37).
cre	The coding region for Cre recombinase (rec3) gene, the topoisomerase of bacteriophage P1 (Reference 38). The recombinase recognizes two *lox*P sites (Table 2) to remove the DNA region present between the sites (Reference 39).
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 40). 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 72).
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 41). 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 42). 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 43).
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 44). (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 45).

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

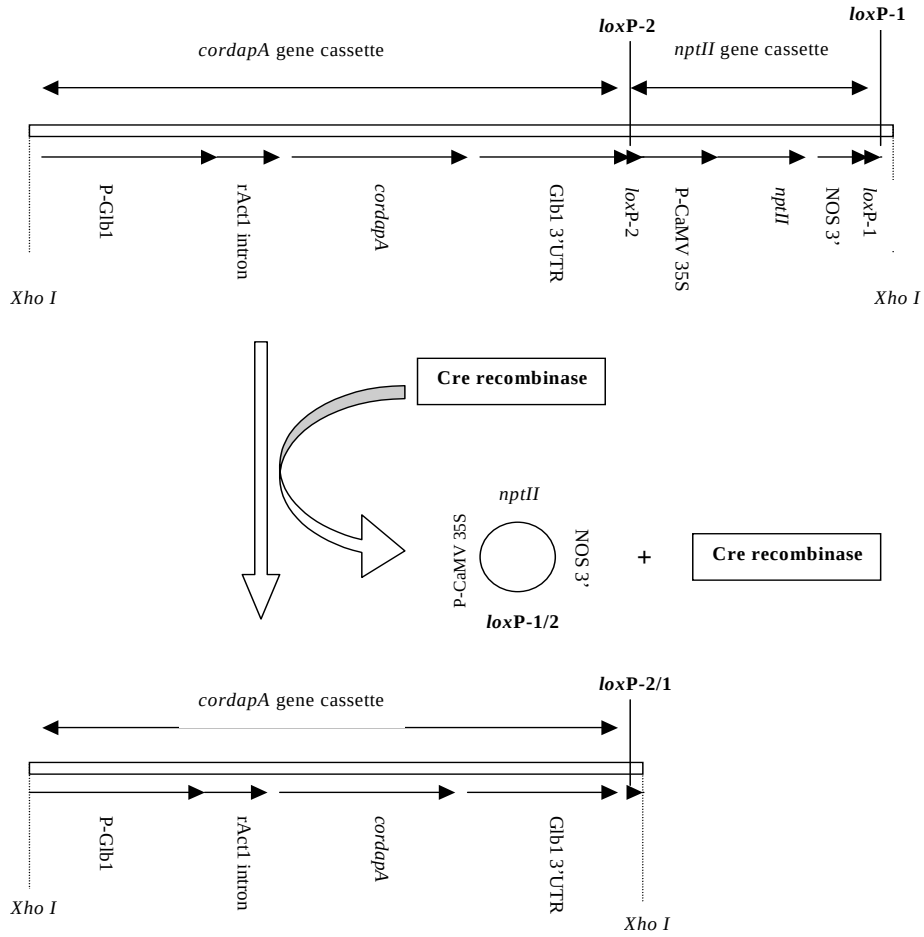

Figure 5 Schematic diagram for removal of *nptII* gene expression cassette by Cre-*lox*P[7]

A 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 resulting *cordapA* gene expression cassette, the excised ring-shaped *nptII* gene cassette or the *cre* gene expression cassette. These individual plantss were screened by PCR and then Southern blotting for the presence of each of these gene expression cassettes: *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 the final selected recombinant maize plant was the *cordapA* gene expression cassette and two half portionsa single recombined *lox*P locus. But this selected recombinant maize plant lacked the *nptII* gene cassette and *cre* gene cassette. This has been further confirmed by extensive GLP Southern blot analysis.

The mechanism of recombination by the Cre-*lox*P system has been well described in the scientific literature (Reference 39; Reference 46; Reference 34) 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 repeat sequences (Reference 47). The Cre recombinase

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

recognizes 13 bp inverted repeat 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 removedrecombined into as a circular 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.

(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 genes transferred to this recombinant maize are inherited in subsequent generations in agreement with Mendel's laws; therefore the transferred genes exist on the chromosome (Annex 7).

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 this recombinant maize, it was confirmed that one copy of an intact *cordapA* gene expression cassette is transferred into the genome of this recombinant maize at one site (see Annex 1 and Annex 4). 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 4, and the origins and functions of component elements of PV-ZM003 are listed in Table 3) used for crossing in the [Confidential] generation and other regions (see Annex 1, Annex 4 and Annex 3). Moreover, it was demonstrated that the transferred genes are stably inherited in multiple generations (Figure 19 and Figure 20 of Annex 4). 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). 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).

The map of transferred genes to this recombinant maize is shown in Figure 6.

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

This item does not apply due to one copy.

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

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 this recombinant maize. 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).

Based on the above results, it was demonstrated that the *cordapA* gene transferred to this recombinant maize is stably inherited in subsequent progeny and the cDHDPS protein is expressed in subsequent progeny.

In addition, the expression level of cDHDPS protein in this recombinant maize was measured in 2002 based on the ELISA method using the samples collected from 5 field sites in the US. As a result, the mean expression level of cDHDPS protein was found to be 24, 0.25, 0.14, and 0.43 μg/g fresh weight respectively in grain, stem and leaf, root, and pollen (Table 4). In addition, the expression level of cDHDPS protein in the leaves collected at different stages of growth (OSL-1 = V2-V4 growth stage, OSL-2 = V6-V7 growth stage, OSL-3 = V11-V12 growth stage, and OSL-4 = V13-V18 growth stage) was found equal to or below the limit of detection. This demonstrates that the expression of *cordapA* gene is enhanced in grain.

Table 4 Expression level of cDHDPS protein in this recombinant maize [a][8]

Sample analyzed	cDHDPS Mean value (μg/g fresh weight) (Standard deviation)	Range [b] (μg/g fresh weight)	LOQ / LOD [c] (μg/g fresh weight)
Grain	24 (9.1)	13 – 43	0.044 / 0.021
Leaf and stem	0.25 (0.21)	0.034 – 0.79	0.0025 / 0.00056
Root	0.14 (0.23)	0.011 – 0.62	0.0050 / 0.0050
Pollen	0.43 (0.14)	0.27 – 0.67	0.025 / 0.0052
OSL-1[d]	<LOD	–	0.038 / 0.013
OSL-2[d]	<LOD	–	0.038 / 0.013
OSL-3[d]	<LOD	–	0.038 / 0.013
OSL-4[d]	<LOD	–	0.038 / 0.013

a Measurement was made based on the ELISA method for the samples collected from 5 fields in the US. In the samples taken from the control Null type maize, no cDHDPS protein was detected.

b Minimum and maximum values are shown

c LOQ = Limit of quantitation, LOD = Limit of detection

d Expanded leaves were taken as samples at individual growth stages, OSL-1 = V2-V4 growth stage (2nd leaf stage to 4th leaf stage), OSL-2 = V6-V7 growth stage (6th leaf stage to 7th leaf stage), OSL-3 = V11-V12 growth stage (11th leaf stage to 12th leaf stage), and OSL-4 = V13-V18 growth stage (13th leaf stage to

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

18th leaf stage). For definition of growth stages, reference was made to Reference 48.

(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, the region of the recipient organism which allows autonomous replication is limited to gram-negative bacterium such as *E. coli*. Consequently, there is no possibility that the plasmid might be transmitted to any wild animals and wild plants.

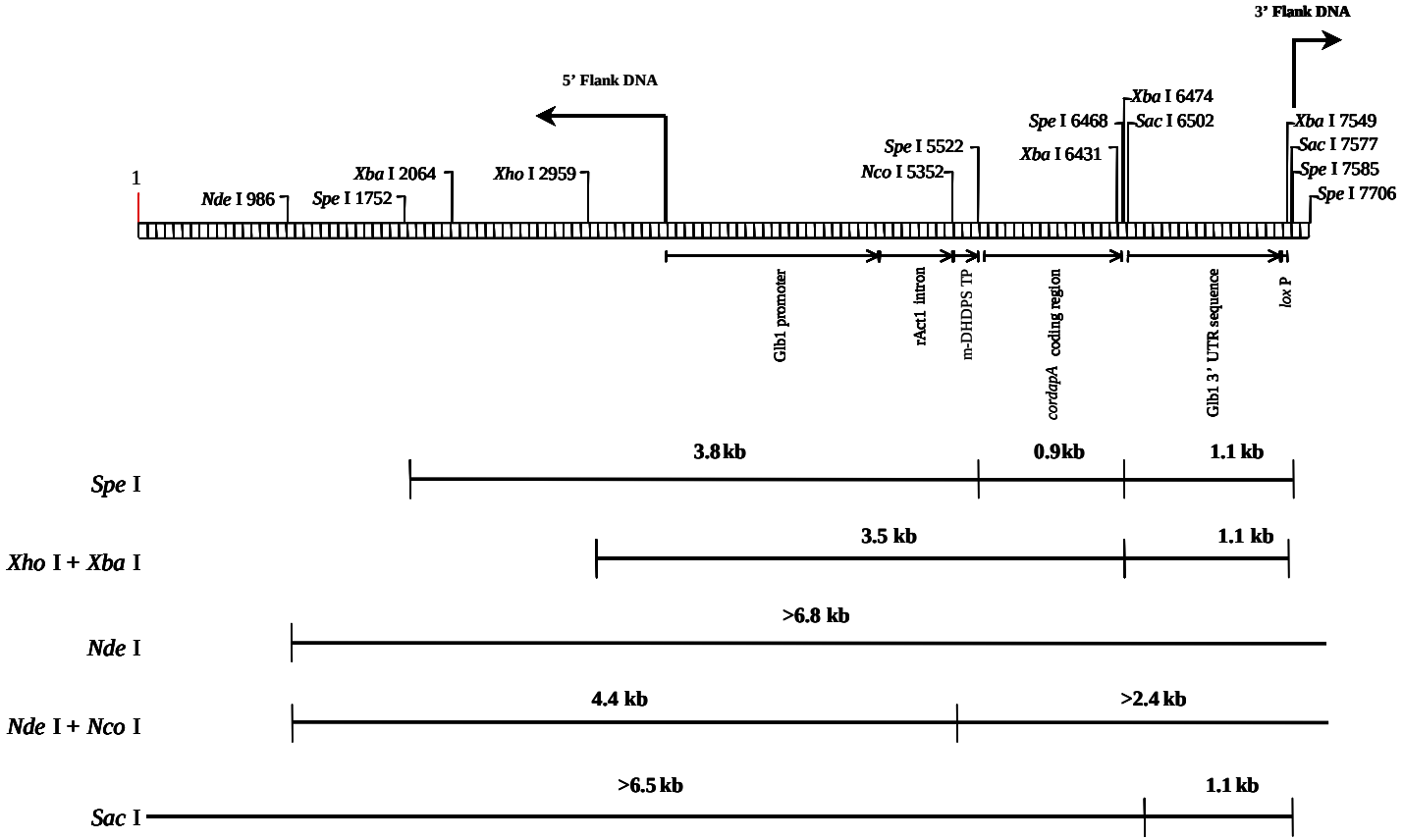

Figure 6 Map of transferred genes to this recombinant maize based on the Southern blotting analysis (Top)[9]

Bottom: Length of DNA fragment including the transferred genes, obtained by treatment with individual restriction enzymes (see Annex 1)

[9] (35.35 x 3,000(g) × 0.558)/70 = 845 µg = 0.845 mg/1kg body weight, 330/0.845 = 391

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

For the detection and identification of this recombinant maize, the transferred genes and the DNA sequences in the flanking plant genome are used as primers. This method makes it possible to specifically detect this recombinant maize (see Annex 4).

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

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

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 7). As the control plant, Null-type maize obtained by segregation in the process of development of this recombinant maize 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 this recombinant maize 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 5 and Table 6). 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 5).

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). 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). Therefore the level of amino acids other than lysine in this recombinant maize was judged to fall within the variable ranges for the conventional maize varieties.

Based on the above results, the following was confirmed: In this recombinant maize, 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 this recombinant maize, a feeding study, review of the literature and acute toxicity test were conducted and the results are summarized below.

[Feeding test] (Annex 8)

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 this recombinant maize 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 this recombinant maize (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).

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). 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 (%) (Table 1 and Table 3 of Annex 8).

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 this recombinant maize.

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 this recombinant maize 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 7). In addition, it is confirmed that saccharopine is present in asparagus (4 μg/g FW), lettuce (4 μg/g FW) (Reference 49), mushroom (102

μg/g) (Reference 50), broccoli (122 μg/g DW)*, cauliflower (97 μg/g DW)* and other plants.

Regarding saccharopine, it is considered that saccharopine is produced in the body of human or livestock consuming typical lysine-containing foods or feeds. Additionally, as Fellow *et al*. point out, 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 51). As a result of the comparison between the amount of saccharopine taken as feed, which is increased in this recombinant maize, 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 this recombinant maize (Table 7). 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 this recombinant maize 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 52; Reference 53), 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 this recombinant maize is ensured since animals or livestock rapidly metabolize the saccharopine even if they take in this recombinant maize 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 7). 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 54; Reference 55), 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 8) based on the report (Reference 56). This suggests that swine are regularly exposed to the

* Monsanto in-house data

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 composed entirely of the recombinant maize, 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, 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 56). 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 7). 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 57).

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 body weight to rats (Reference 58). This dose was compared to the amount of pipecolic acid possibly consumed by livestock when ingesting this recombinant maize. Assuming that adult swine (body weight ranging from 70 to 115 kg) ingests approx. 3 kg of formula feed per day (Reference 59), of which maize accounts for 55.8% (Reference 60) which is all taken from this recombinant maize, the amount of pipecolic acid that swine would ingest per day from the grain of this recombinant maize is determined to be approx. 0.845 mg[10]. 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 this recombinant maize 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.

[10] (818.42 × 3000(g) × 0.558)/70 =19,571 μg = 19.6 mg/1kg body weight, 2000/19.6= 102

[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).

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 this recombinant maize per day.

The maximum amount of saccharopine observed in the grain of this recombinant maize harvested in the US field tests was 818.42 µg/g dwt (Table 5). Adult swine (body weight of 70 kg) consume approx. 3 kg formula feed per day (Reference 59). Assuming that the maize content of formula feed is 55.8% (Reference 60) and all of the maize in the feed is from this recombinant maize, the amount of saccharopine the swine consume per day from the grain of this recombinant maize is found to be approx 19.6 mg per 1 kg weight body[11]. 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 this recombinant maize 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).

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 this recombinant maize per day.

The maximum amount of α-aminoadipic acid observed in the grain of this recombinant maize harvested in the US field tests was 89.32 µg/g dwt (Table 5). Adult swine (body weight of 70 kg) consume approx. 3 kg formula feed per day (Reference 59). Assuming that the maize content of formula feed is 55.8% (Reference 60) and all of the maize in the feed is from the recombinant

[11] (89.32 × 3000(g) × 0.558)/70 = 2,136 µg = 2.136 mg/1kg body weight, 2000/2.136 = 936

maize, the amount of α-aminoadipic acid the swine consumes per day from the grain of this recombinant maize is approx 2.1 mg per 1 kg weight body[12] 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 this recombinant maize 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.

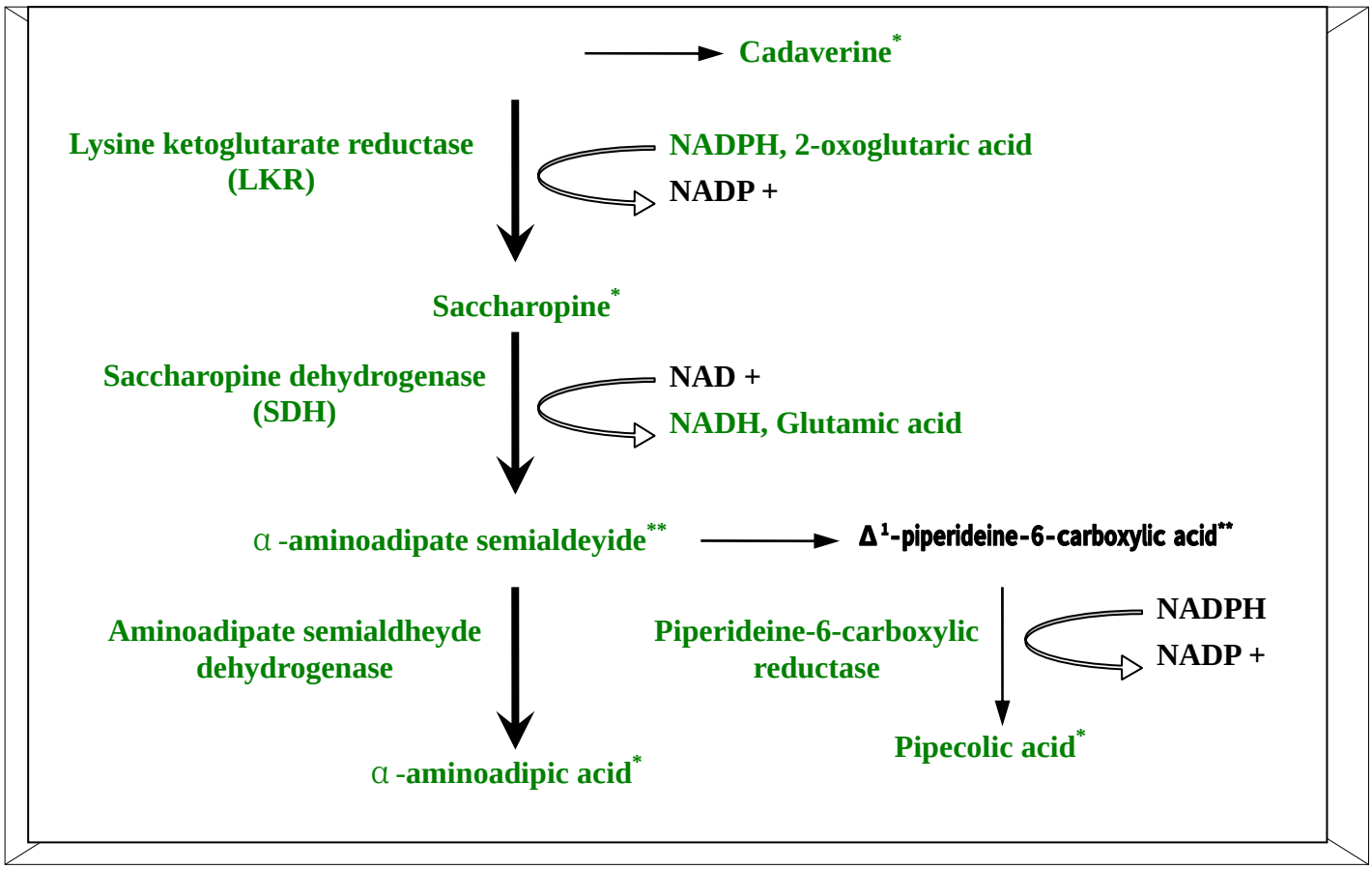

Figure 7 Metabolic pathway of lysine catabolism (Reference 61)[13]

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

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

Table 5 Analytical results on free lysine, lysine and related catabolites in grain[14]

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]

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 6 Analytical results on α-aminoadipic acid in grain of this recombinant maize[15, (1)]

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)

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

(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 weight

Table 7 Metabolism of saccharopine in human and other animals[16]

	Metabolism amount of saccharopine by SDH (g /whole liver/day)	Estimated daily intake of saccharopine from this recombinant maize (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 62

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 59), of which maize accounts for 55.8% (Reference 63), which is all taken in from this recombinant maize.
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 64), of which maize accounts for 40.1% (Reference 63), which is all taken in from this recombinant maize.
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 65, 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.

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

Table 8 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 this recombinant maize[17]

(Calculated based on the data from experiments in which young swine weighing 10.5 kg ingests 1,057 g basal diet per day (Reference 56).)

	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	—	—		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 66).
(2) Reference 56
(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 of α-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 this recombinant maize) = 21,772 μg = 21.77 mg

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

ii) [18]Differences between the recombinant plant and the taxonomic species to which the recipient organism belongs

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 belong to this recombinant maize, 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 this recombinant maize with two different non-recombinant maize inbred lines respectively (see Figure 3). 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 this recombinant maize with two different non-recombinant maize inbred lines respectively (see Figure 3). It has been confirmed by the Southern blotting analysis and the 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).

(a) Morphological and growth characteristics

For the morphological and growth characteristics, 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 at the harvest time). For the recombinant maize LY038-A, a statistically significant difference from the control Null-type maize Cont-38A was observed in culm 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). For the characteristics evaluated in which a difference was observed between the respective combinations of the recombinant maize and the 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 the 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).

(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).

[18] 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.

(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. Field studies with the recombinant maize compared to the control Null-type maize and other conventional maize hybrids showed no differences with all tested maize hybrids for overwintering characteristics. 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), and no difference was observed in the shape and size of the pollen (see Annex 6).

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

Regarding the production of seeds, 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). 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). 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).

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

Regarding the germination rate of harvested seeds, 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 4 of Annex 6). The both recombinant maize exhibited a high germination rate of 95% or more, and no dormancy of the seeds was observed.

(f) Crossability

A crossability test was not performed for this recombinant maize, since no wild relatives that can be crossed grow in Japan.

(g) Productivity of harmful substances

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).

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).

Moreover, the *cordapA* gene transferred to this recombinant maize 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 this recombinant maize and the control Null-type maize was conducted. As a result, no statistically significant difference was observed between this recombinant maize 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).

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).

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 the recombinant maize (see Table 7 of Annex 6). 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). 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 the recombinant maize 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).

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

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

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

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis), to which the recipient organism belongs, has been used, including for cultivation, etc., in Japan, though there is no report that it has become self-seeding in Japan.

In the isolated field tests in Japan, examination has been conducted on the morphological and growth characteristics of this recombinant maize for 19 items using the two lines (LY038-A and LY038-B). As a result, in the culm length, height of ear, ear diameter and row number per ear for the LY038-A, and in the row number per ear, 100-kernel weight and the number of grains formed per ear for the LY038-B, a significant difference from their respective control plants was observed. However, in the other items, no significant difference was observed. For the items in which a significant difference was observed regarding the lines concerned, their mean values were found to fall within the variable ranges for the maize used as control plants in the previous isolated field tests,and were considered not to exceed the variable ranges for the conventionally used maize. Therefore, though it has been confirmed that in this recombinant maize, the content of free lysine is enhanced specifically in the grain due to the dihydrodipicolinate synthase, the product of the transferred *cordapA* gene, and also the contents of saccharopine and α-aminoadipic acid, the downstream catabolites after biosynthesis of lysine, are increased, these differences are considered unlikely to cause this recombinant maize to become competitive.

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

(2) Productivity of harmful substances

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

This recombinant maize expresses dihydrodipicolinate synthase, however a bioinformatic homology search based on the amino acid sequence of this enzyme showed no homology with any known allergens or toxins.

In this recombinant maize, enhanced lysine content and also enhanced contents of saccharopine, α-aminoadipic acid and pipecolic acid, the secondary metabolites, have been observed compared to the control plants. Regarding the secondary metabolites:

(a) The broiler feeding test did not reveal any result suggesting possible toxic effects.

(b) Absence of toxic effect was suggested for the saccharopine and α-aminoadipic acid by the acute toxicity test on mice and for the pipecolic acid by the data on acute toxicity on rats in the literature.

As a result, these secondary metabolites are considered unlikely to have any toxic effect on wild animals.

In the isolated field tests in Japan, succeeding crop tests, soil microflora tests and plow-in tests have been conducted to investigate productivity of harmful substances of this recombinant maize (including secretion from roots to affect the other plants, secretion from roots to affect microorganisms in soil, and the possession in the plant body to affect the other plants after dying) using the two lines of this recombinant maize (LY038-A and LY038-B).

As a result, in the succeeding crop test using the LY038-B, only in the dry weight of the items examined, germination rate, fresh weight and dry weight of the test plant radish, a significant difference from the control plant was observed. However, in this test, the germination rate of radish was low and then, an additional succeeding crop test was conducted and as a result, no significant difference was observed in all the items examined including dry weight. In addition, in the soil microflora tests and plow-in tests, no significant difference was observed regarding both lines. Therefore, it is considered unlikely that the productivity of harmful substances is enhanced in this recombinant maize.

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

(3) Crossability

In the Japanese natural environment, there are no wild 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.

(4) Others

Due to the enhanced lysine content in the grain of this recombinant maize, a possibility is considered that the growth of wild animals would be accelerated when the wild animals ingest the grain constantly. In actuality, however, such an opportunity is extremely limited and thus, possible effect on the growth of specific wild animals is considered unlikely.

Based on the above understanding, it was judged that the conclusion by the applicant that this recombinant maize poses no indirect risk of Adverse Effect on Biological Diversity is reasonable.

2. Conclusion based on the Biological Diversity Risk Assessment Report

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

[Bibliography]