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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize tolerant to glyphosate and glufosinate herbicides (Modified *cp4 epsps*, *pat*, *Zea mays* subsp. *mays* (L.) Iltis) (NK603×T25, OECD UI: MON-ØØ6Ø3-6×ACS-ZMØØ3-2)
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

Maize tolerant to glyphosate and glufosinate herbicides (modified *cp4 epsps*, *pat*, *Zea mays* subsp. *mays* (L.) Iltis) (OECD UI: MON-ØØ6Ø3-6 ×ACS-ZMØØ3-2) (hereinafter referred to as "this stack line maize") is a cross progeny line developed by crossing the following two (2) recombinant maize lines, using the traditional breeding method. The two recombinant maize lines are: i) the maize tolerant to glyphosate herbicide (modified *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (NK603, OECD UI: MON-ØØ6Ø3-6) (hereinafter referred to as "NK603"), and ii) the maize tolerant to glufosinate herbicide (*pat*, *Zea mays* subsp. *mays* (L.) Iltis) (T25, OECD UI: ACS-ZMØØ3-2) (hereinafter referred to as "T25"). Therefore, this stack line maize possesses the characteristics of these two parent recombinant maize lines, NK603 and T25. Then, the information concerning preparation of NK603 and T25 are explained individually in the following sections.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of NK603 and T25 are shown individually in Figure 1 and Figure 2(pp.3-4), and Table 1 and Table 2 (pp.5-7).

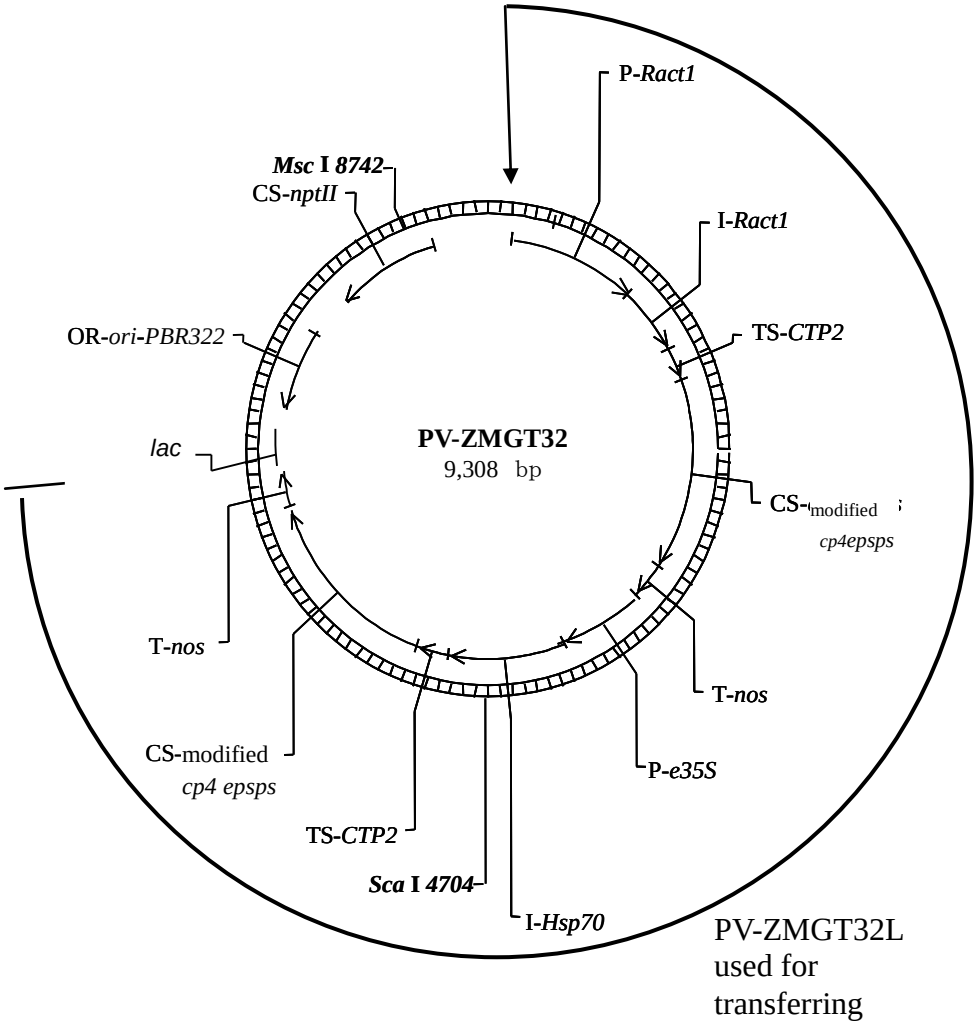

Figure 1 Map of the plasmid PV-ZMGT32 used for the development of NK603[1]

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

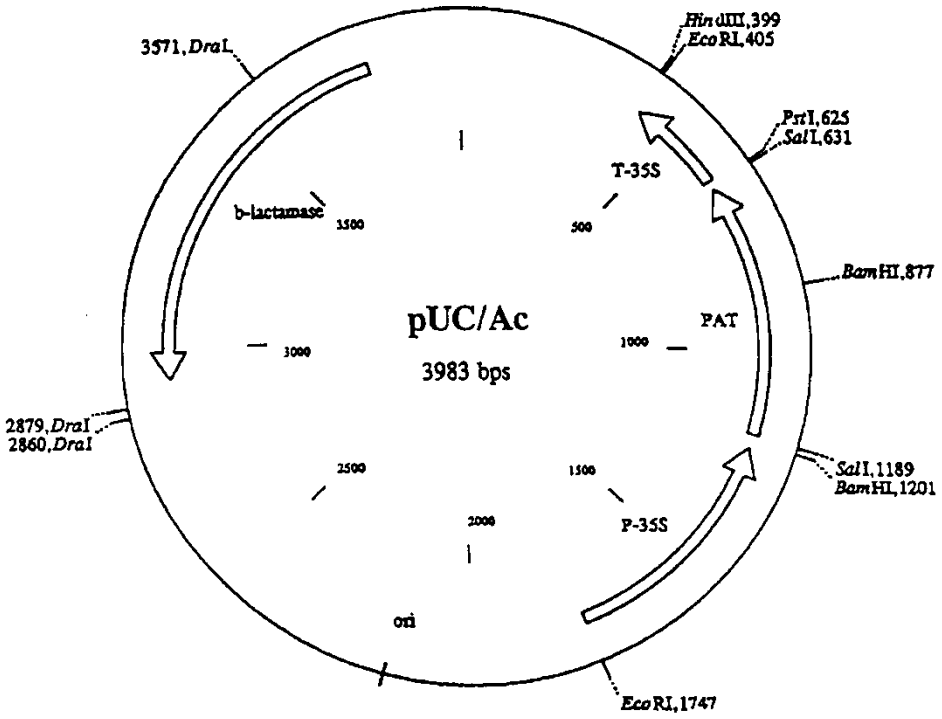

Figure 2 Map of the plasmid pUC/Ac used for the development of T25[2]

[2] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Bayer Crop Science K.K.

Table 1 Size, origins and functions of component elements of PV-ZMGT32 used for the development of NK603[3]

Component elements	Origin and function
Modified *cp4 epsps* gene cassette (1)	
P [*1]-*Ract1*	Promoter region of actin 1 gene derived from rice. It makes target genes expressed (Reference 16). Involved in the constant expression of the target gene in the entire tissue of plant body.
I [*2]-*Ract1*	Rice actin gene intron (Reference 17). Activates the expression of target gene.
TS* [3]-*CTP2*	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 18). Transfers target proteins from cytoplasm to chloroplast.
CS[*4]-modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 19; Reference 20). To enhance the expression in plants, the second amino acid from the N-terminal in the wild-type CP4 EPSPS protein is modified to leucine, instead of serine.
T [*5]-*nos*	3' untranscribed region of nopaline synthase (*nos*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 21).
Modified *cp4 epsps* gene cassette (2)	
P-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Reference 23) promoter and 9bp leader sequence, containing double enhancer regions (Reference 22). Involved in the constant expression of the target gene in the entire tissue of plant body.
I-*Hsp70*	Intron of heat shock protein gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants (Reference 24).
TS-*CTP2*	N-terminal chloroplast transit peptide sequence of EPSPS protein derived from the *Arabidopsis epsps* gene (Reference 18). Transfers target proteins from cytoplasm to chloroplast.
CS-modified *cp4 epsps*	5-enol-pyrovylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Reference 19; Reference 20). To enhance the expression in plants, the second amino acid from the N-terminal in the wild-type CP4 EPSPS protein is modified to leucine, instead of serine.
T-*nos*	3' untranscribed region of nopaline synthase (*nos*) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Reference 21).

Table 1 Size, origins and functions of component elements of PV-ZMGT32 used for the development of NK603[3] (continued)

Others (not existing in plant bodies)	
lac	Consists of partial coding sequence for *lacI* (Reference 25), *lac* promoter (Reference 26), and partial coding sequence for *lacZ*. Hydrolyzes lactose and expresses β-galactosidase used as a selective marker (Reference 27).
OR*[6]-*ori-PBR 322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Reference 28).
nptII	A gene derived from *E. coli* transposon Tn5 (Reference 29). Encodes phosphotransferase type II (NPT II) and confers resistance to neomycin and kanamycin. The region contains the partial *ble* gene derived from Tn5 (Reference 30), and is regulated by *nptII* promoter and β-lactamase termination sequence. Used as marker to select the transgenic plant during the gene transfer (Reference 31).

[*1] P -promoter
[*2] I - intron
[*3] TS - targeting sequence
[*4] CS- coding sequence
[*5] T- transcript termination sequence
[*6] OR - origin of replication

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

Table 2 Size, origins and functions of component elements of pUC/Ac used for the development of T25[4]

Component elements	Origin and function
pat cassette	
P-35S	35S RNA promoter derived from Cauliflower Mosaic Virus. It expresses *pat* genes in plants constitutively (Reference 23).
pat	A gene derived from *Streptomyces viridochromogenes*, encodes PAT ptotein, and confers tolerance to glufosinate herbicide (Reference 32).
T-35S	35S RNA terminator derived from Cauliflower Mosaic Virus. Terminates transcription and induces polyadenylation (Reference 33).
Others (not existing in plant bodies)	
bla	A gene resistant to ampicillin derived from *E.coli*, and encoding β-lactamase only to bacteria (Reference 28).
ori-pUC	The replication origin of pUC18 (ColE1). Permits autonomous replication of plasmid (Reference 34).

[4] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Bayer Crop Science K.K.

2) Functions of component elements

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

Functions of individual component elements of donor nucleic acid used for the development of NK603 and T25 are shown in Table 1 (p.5) and Table 2 (p.7).

(b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergen (except allergenicity as food)

[Modified CP4 EPSPS protein]

The modified *cp4 epsps* gene expressed in NK603 is a gene isolated from the *Agrobacterium* CP4 strain, which encodes 5-enol-pyruvylshikimate-3-phosphate synthase (CP4 EPSPS) and expresses the modified CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The modified *cp4 epsps* gene has the nucleotide sequence in the wild-type *cp4 epsps* gene modified to enhance the expression level in plants without changing the functional activity of the wild-type CP4 EPSPS protein, with only a single modification introduced to the amino acid sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine. Two modified *cp4 epsps* gene cassettes were transferred to NK603 in order to enhance tolerance to the herbicide glyphosate.

Plants treated with glyphosate herbicide wither away and die, since the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19) is inhibited and then the synthesis of aromatic amino acids essential for synthesis of proteins fails. The modified *cp4 epsps* gene, the target gene of NK603, produces the modified CP4 EPSPS protein which has high tolerance to the glyphosate herbicide. The activity of the modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus the recombinant plants that express this protein have normal functions of shikimate synthesis pathway and can grow.

In order to investigate whether the modified CP4 EPSPS protein expressed in NK603 shares any functionally important amino acid sequences with known allergens, it was compared with the allergens in the database (GenBank, EMBL, PIR, RCSB PDB, SwissProt). As a result, they did not share structurally related sequences with any known allergens.

[PAT protein]

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. Glutamate synthase plays an important role in detoxification of the ammonia produced, however the glutamate synthase is inhibited if crops are sprayed with glufosinate herbicide, ammonia accumulates, and the crops wither and die.

The transferred *pat* gene produces the PAT protein (phosphinothricin acetyltransferase). The PAT protein acetylates glufosinate herbicide to transform it to N-acetylglufosinate, and inactivates inhibitory action to glutamate synthase of glufosinate herbicide. Thus, ammonia is not accumulated, and the recombinant plant sprayed with glufosinate herbicide would not die.

Since the wild-type *pat* gene obtained from *S. viridochromogenes* contains large amount of G:C (guanine:cytosine) not common in plants, the transferred *pat* gene is the modified wild-type *pat* gene sequence to provide codons more common in plants without changing the amino acid sequence of the PAT protein.

Homology search was conducted using EMBL database for *pat* gene sequence and SwissProt database for PAT protein amino acid sequence. As a result, they did not show significant homology with amino acid sequence other than PAT protein derived from various species, and did not share structually related sequences with known allergens.

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

The EPSPS, identical functionally to the modified CP4 EPSPS protein, is an enzyme protein that catalyzes the shikimate pathway for the biosynthesis of aromatic amino acid. However, it is not a rate-determining enzyme in the pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway (Reference 35; Reference 36). In addition, the EPSPS is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 37), and the only shikimate that is known to react with the EPSPS other than these offers the reactivity of only one-two millionth that of S3P (calculated based on the Reference 37); therefore, it is considered unlikely to react as the substrate of EPSPS in any living organisms. Therefore, it is considered unlikely that the modified CP4 EPSPS protein would affect the metabolic system of recipient organism.

Consequently, it is considered to be extremely unlikely that the production of the modified CP4 EPSPS protein, which is functionally parallel to plant EPSPS protein, has an effect in someway on the metabolic pathways of plants.

The PAT protein acetylates the *L*-phosphinothricin (classified into *L*-amino

acids), an active ingredient of glufosinate herbicide, though it does not transfer acetyl group to various amino acids, and it has little affinity for the glutamic acid, which it resembles structurally. The PAT protein does not show transfer reaction in vivo except for acetylating the *L*-phosphinothricin (Reference 38). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by PAT protein was never inhibited (Reference 39). Consequently, for its high substrate specificity to glufosinate, the PAT protein is considered unlikely to affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

The plasmid vectors used for the production of the parent lines are as follows.

NK603: PV-ZMGT32 assembled from plasmids including the vector pUC119 derived from *E. coli*
T25: pUC/Ac assembled from plasmids including the vector pUC 18 derived from *E. coli*

2) Properties

(a) The numbers of base pairs and nucleotide sequence of vector

The total number of base pairs in the plasmid vectors used for the production of parent lines is as follows.

NK603: PV-ZMGT32; 9,308 bp
T25: pUC/Ac; 3,983 bp

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

The PV-ZMGT32 used for the production of NK603 contains the kanamycin/neomycin resistant gene (*nptII* gene) derived form transposon Tn5, as a selective marker gene (Reference 29).

The pUC/Ac used for the production of T25 contains the ampicillin resistant gene, as a selective marker gene. In addition, the characteristics of all genes existing in the pUC/Ac have been disclosed, and they do not contain any known nucleotide sequence having harmful functions.

(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

The infectivity of PV-ZMGT32 and pUC/Ac is not known.

(3) Method of preparing living modified organisms

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

The component elements of the plasmid vectors transferred in the recipient organisms for development of NK603 and T25 are listed in Table 1 (p.5) and Table 2 (p.7). In addition, the location and section broken by restriction enzyme of the component elements of the donor nucleic acid in the vectors are shown in Figure 1 (p.3) and Figure 2 (p.4).

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

Transferring nucleic acid into the recipient organism was based on the following method.

NK603: Particle gun method
T25: Polyethylene glycol method

3) Processes of rearing of living modified organisms

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

Selection of transformed cells was made on the medium containing the followings.

NK603: Glyphosate herbicide
T25: Glufosinate herbicide

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

Presence or absence of remaining *Agrobacterium* was not confirmed for NK603, since a DNA fragment was transferred by the particle gun method.

Presence or absence of remaining *Agrobacterium* was not confirmed for T25, since a DNA fragment was transferred by the polyethylene glycol method.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in 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

For NK603, commercial cultivar of yellow dent corn line and a wide variety of cultivar were crossed with, then selective breeding was conducted.

For T25, commercial cultivar of yellow dent corn line and a wide variety of cultivar were crossed with, then selective breeding was conducted.

This stack line maize is the first cross variety of self-fertilized lines of NK603 and T25 (Figure 3, p.13).

The status of application for approval of NK603 and T25 in Japan is summerized below (Table 3, p12).

Table 3 Status of application for approval of NK603 and T25 in Japan

	Safety as food	Safety as feed	Environmental safety
NK603	March, 2001: Approved safety of use as food	March, 2003: Approved safety of use as feed	November, 2004: Approved for Type I Use Regulation
T25	March, 2001: Approved safety of use as food	March, 2003: Approved safety of use as feed	November, 2004: Approved for Type I Use Regulation
This stack line maize	July, 2009: In the course of application	August, 2009: In the course of notification	July, 2009: In the course of application

[Process of rearing of NK603×T25]

[Confidential: Not made available or disclosed to unauthorized person]

Figure 3 Process of rearing of NK603 line×T25 line

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

The methods for both NK603 and T25 described below have been confirmed when they were approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment of Japan (November 2004), concerning the use in accordance with the Type I Use Regulation for Living Modified Organism (provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms.

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

It was confirmed that the transferred genes in NK603 and T25 exist on the maize genome.

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

[NK603]

As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy of transferred gene region of the modified *cp4 epsps* gene cassette (1) and (2) exist in the maize genomic DNA of NK603 at one site. In addition, it was also confirmed as a result of Southern blotting analysis on multiple generations that the transferred genes are inherited stably in offspring.

For NK603, it was confirmed that 217bp fragment of ract1 promoter exists in the reverse direction near the 3'-terminal of the transferred gene by Southern blotting analysis and nucleotide sequence analysis of the 3'-terminal.

Regarding 217bp fragment which is ract1 promoter near the 3'-terminal of the transferred gene in NK603, the strand-specific RT-PCR was conducted and as a result, transcription product was found that was considered to start from either ract1 promoter of the transferred gene or E35S promoter and to read through NOS 3' terminator. However only the modified CP4 EPSPS protein is confirmed in NK603, and it is considered that the stop codon is kept in upstream of the terminator of the transcription product which reads through the terminator in the NK603 transferred gene. Therefore, it is concluded that this read-through would not affect the safety assessment. Consequently, NK603 was approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment of Japan in November 2004, concerning the use in accordance with the Type I Use Regulation for Living Modified Organism (provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) based on the Law concerning the Conservation and Sustainable Use of Biological

Diversity through Regulations on the Use of Living Modified Organisms.

In addition, in the transferred gene of NK603, each of the 456th base and the 641st base from 5'-terminal of coding region in the modified *cp4 epsps* gene induced by the E35S promoter was changed from thymine (T) to cytosine (C) compared to the base in plasmid for expression of plant. It was proved that the change of the 456th base is not connected with the change of amino acid, but in the modified CP4 EPSPS protein which is expressed by the E35S promoter by the change of the 641st base, leucine changes to proline in the 214th amino acid from N-terminal in the original CP4 EPSPS protein (hereinafter this protein is referred to as "L214P").

Regarding L214P, the following are considered: 1) Proline which is the 214th amino acid from N-terminal is not included in the seven amino acids essential for activating the EPSPS protein family; 2) This change of the amino acid does not affect the active side of the EPSPS protein and three-dimensional structure; and 3) As the traits of enzyme activity and immune response of the L214P protein and the modified CP4 EPSPS protein are substantially comparable, the structure and function of the L214P protein and the modified CP4 EPSPS protein are substantially comparable.

In order to investigate whether the L214P shares functionally important amino acid sequences with known contact allergens, it was compared with contact allergens in the database. As a result, the L214P did not share structurally related homologous sequences with any of the known allergens examined.

The change of bases described above was observed in multiple generations, and the stability of the inheritance through multiple generations was confirmed.

[T25]

As a result of Southern blotting analysis of transferred genes, it was confirmed that one copy of pUC/Ac exists in the maize genome DNA of T25 at one site. In addition, it was also confirmed as a result of Southern blotting analysis on multiple generations that the transferred genes are inherited stably in offspring.

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

This item is not applicable because of one copy for both of NK603 and T25.

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

The stability of expression was identified as follows.

NK603: During the rearing process, glyphosate herbicide was sprayed, and it was

confirmed that tolerance to glyphosate herbicide was conferred in multiple generations.

T25: During the rearing process, glufosinate herbicide was sprayed, and it was confirmed that tolerance to glufosinate herbicide was conferred in multiple generations.

5) 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 the plasmid PV-ZMGT32 used for the production of NK603, the region of recipient organism, which allows autonomous replication, is limited to gram-negative bacteria such as *E. coli*. Therefore, there is no possibility that the plasmids might be transmitted to any wild animals and wild plants under natural environment.

In addition, the vector used for transformation of T25 does not contain any sequence allowing transmission and thus, transmission of this item is absence.

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

Specific method for detection and identification of NK603 and T25 is available by using the DNA sequences of the transferred genes and the nearby regions of the plant genome as primers.

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

(6) Difference between the modified organism and the recipient organism or the species to which the recipient organism belongs

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

This stack line maize is given the following traits derived from individual parent lines.

NK603: Tolerance to herbicide glyphosate due to the modified CP4 EPSPS protein which is derived from the transferred genes
T25: Tolerance to herbicide glufosinate due to the PAT protein which is derived from the transferred genes

The EPSPS, identical functionally to the modified CP4 EPSPS protein expressed in NK603 line, is not any rate-determining enzyme in the shikimate pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration

of aromatic amino acids, the end products of this pathway (Reference 35; Reference 36). In addition, EPSPS is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 37), and the only shikimate that is known to react with EPSPS other than these offers the reactivity of only one-two millionth that of S3P (calculated based on the Reference 37); therefore, it is considered unlikely to react as the substrate of EPSPS in any living organisms. Consequently, it is considered to be extremely unlikely that the production of the modified CP4 EPSPS protein has an effect on the metabolic pathways of plants.

The PAT protein expressed in T25 acetylates the *L*-phosphinothricin (classified into *L*-amino acid), an active ingredient of glufosinate herbicide, though it does not transfer acetyl group to various amino acids, and it has little affinity for the glutamic acid, which is especially resembling structually. The PAT protein does not show transfer reaction in vivo except for acetylating the *L*-phosphinothricin (Reference 38). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by PAT protein was never inhibited (Reference 39). Consequently, for its high substrate specificity to glufosinate, the PAT protein is considered unlikely to affect the metabolic pathways of plants.

Therefore, it is considered unlikely that the proteins expressed (NK603×T25) from the individual parent lines in this stack line maize would interact with each other and additionally affect the metabolic pathways of plants.

Based on the above understanding, it is considered unlikely that the proteins expressed in this stack line maize from the individual parent lines would interact with each other.

In order to confirm that the proteins expressed in this stack line maize from the individual parent lines do not interact with each other, regarding tolerance to herbicides of this stack line maize, the bioassay based on the glyphosate (Product name: Roundup WeatherMAX) and glufosinate (Product name: Liberty) spraying tests was carried out in the U. S. Monsanto company.

[Bioassay using glyphosate herbicide]

Regarding tolerance to glyphosate herbicide, five (5) individuals each of this stack line maize, NK603 and the non-recombinant control maize were cultivated in pots in a greenhouse (1 individual/plot×5 repeats), and at the 2nd to 4th leaf stage, herbicide glyphosate (Product name: Roundup WeatherMAX) was sprayed. On the 10th day after spraying herbiside glyphosate, the sensitivity of injury by spraying of herbicide glyphosate to plant bodies was evaluated based on 11-step scales from 0 (no injury) to 10 (nearly the entire planr body withered and died due to the injury). The concentration of glyphosate sprayed of 27.0 kg acid equivalent/ha refers to 32-times higher dosage than the normal dosage 0.84kg acid equivalent/ha. The sensitivity of injury in the table refers to mean value ± standard error.

As a result of investigation, regarding the sensitivity of injury by spraying of herbicide in all dosage, no statistically significant difference was observed between this stack line maize and NK603 (*t*-test, significant leve 5%) (Table 4 and Table 5, p.18 and p.19). Therefore, it was confirmed that the tolerance of this stack maize line to glyphosate herbicide remains unchanged by crossing of parent lines.

Table 4 Investigational result of the severity of injury by spraying glyphosate herbicide to this stack maize line (mean value ± standard error of the severity of injury by the herbicide to plant body [1]) (n=5 replicates)[5]

Samples tested	Concentration			
	0.84kg acid equivalent/ha[2]		27.0 kg acid equivalent /ha[3]	
This stack maize line	0.0 ± 0.0	A[4]	2.0 ± 0.3	A
NK603	0.0 ± 0.0	A	2.2 ± 0.2	A
Non-recombinant control maize	9.8 ± 0.2	B	10.0 ± 0.0	B

1 0: No injury, 1 to 9: Approx. 10 to 90% of a leaf area turned white, yellow and/or decayed, 10: 100% of a leaf area died due to the injury.

2 Normal dosage

3 32-times higher dosage than the normal dosage

4 In a given line, different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (*t*-test, significant level 5%).

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

Table 5 Investigational result (P-value) of the severity of injury by spraying glyphosate herbicide to this stack maize line (n=5 replicates)[6]

Test samples for comparison	Concentration	
	0.84kg acid equivalent /ha[1]	27.0 kg acid equivalent /ha[2]
This stack line maize versus NK603	1.0000	0.4643
This stack line maize versus non-recombinant control maize	<0.0001	<0.0001
NK603 versus non-recombinant control maize	<0.0001	<0.0001

1 Normal dosage

2 32-times higher dosage than the normal dosage

[Bioassay using the glufosinate herbicide]

Regarding tolerance to glufosinate herbicide, five (5) individuals each of this stack line maize, T25 and the non-recombinant control maize were cultivated in pots in a greenhouse (1 individual/plot × 5 repeats), and at the 2nd to 4th leaf stage, herbicide glufosinate (Product name: Liberty) was sprayed. On the 10th day after spraying herbicide glufosinate, the severity of injury by spraying of herbicide glufosinate to plant bodies was evaluated based on 11-step scales from 0 (no injury) to 10 (nearly the entire plant body withered and died due to the injury). The concentration of glufosinate sprayed of 17.0 kg active ingredient/ha refers to 32-times higher dosage than the normal dosage of 0.54 kg active ingredient/ha. The severity of injury in the table refers to mean value ± standard error.

As a result of investigation, regarding the severity of injury by spraying of herbicide in all dosage, no statistically significant difference was observed between this stack line maize and T25 (t-test, significance level 5%)(Table 6 and Table 7, p.20). Therefore, it was confirmed that the tolerance of this stack line maize to glufosinate herbicide remains unchanged by crossing of parent lines.

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

Table 6 Investigational result of the severity of injury by spraying glufosinate herbicide to this stack maize line (mean value ± standard error of the severity of injury by the herbicide to plant body [1]) (n=5 replicates) [7]

Samples tested	Concentration			
	0.54kg active ingredient /ha[2]		17.0 kg active ingredient /ha[3]	
This stack line maize	0.0 ± 0.0	A[4]	2.8 ± 0.2	A
T25	0.4 ± 0.4	A	3.0 ± 0.3	A
Non-recombinant control maize	5.0 ± 0.0	B	9.4 ± 0.2	B

1 0: No injury, 1 to 9: Approx. 10 to 90% of a leaf area turned white, yellow and/or decayed, 10: 100% of a leaf area died due to the injury.

2 Normal dosage

3 32-times higher dosage than the normal dosage

4 In a given line, different alphabetical letters indicate that a statistically significant difference was observed between the relevant mean values (*t*-test, significant leve 5%).

Table 7 Investigational result (P-value) of the severity of injury by spraying glufosinate herbicide to this stack maize line (n=5 replicates)[8]

Test samples for comparison	Concentration	
	0.54kg active ingredient /ha[1]	17.0 kg active ingredient /ha[2]
This stack line maize versus T25	0.2149	0.4301
This stack line maize versus non-recombinant control maize	<0.0001	<0.0001
T25 versus non-recombinant control maize	<0.0001	<0.0001

1 Normal dosage

2 32-times higher dosage than the normal dosage

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

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

Based on the above result, it was concluded that the individual proteins expressed in this stackline maize would not interact with each other and that the traits newly obtained from the transferred genes remain unchanged in this stack line maize.

Therefore, with respect to the physiological or ecological characteristics, difference between this stack line maize and the taxonomic species to which the recipient organism belongs, evaluation was made based on the results of investigations conducted for NK603 and T25 individually.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present[9].

For NK603, two (2) kinds of hybrid varieties, NK603-A and NK603-B were tested[10]

[9] All the rights pertinent to the information of NK603 in (a) to (g) of this item and the responsibility for the content rest upon Monsanto Japan Limited, and all the rights pertinent to the information of T25 and the responsibility for the content rest upon Bayer Crop Science K.K.

[10] NK603-A and NK603-B is the same event.

(a) Morphological and growth characteristics

For the morphological and growth characteristics of NK603 and T25 and their non-recombinant control maize, examination was conducted for the items listed in Table 8 (p.22).

As a result, a statistically significant difference was observed in NK603-B of NK603 in 100-kernel weight.

Table 8 Investigation results of morphological and growth characteristics of NK603 and T25

	NK603-A	NK603-B	T25
Uniformity of germination	○	○	○
Germination rate	○	○	○
Time of tasseling	○	○	○
Time of silking	○	○	○
Culm length	○	○	○
Plant type	○	○	○
Tiller number	○	○	○
Height of ear	○	○	○
Maturation time	○	○	○
Number of ears	○	○	○
Number of productive ears	○	○	○
Row number per ear	○	○	○
Grain number per row	○	○	○
100-kernel weight	○	○*	○
Fresh weight of above-ground parts at the harvest time (fresh weight)	○	○	○
Ear length	○	○	○
Ear diameter	○	○	○
Grain color	○	○	○
Grain shape	○	○	○

○: Examined

-: Not examined

* There is a statistically significant difference (Table 3-2 in p.17 of Annex 1).

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

NK603 withered or died due to the low temperature treatment at the early stage of growth similarly to the non-recombinant maize, and no difference was observed between them (Table 3-4 in p.24 of Annex 1).

T25 and the non-recombinant control maize were treated by low temperature (4 ℃)

in the incubator for observation of their growth. As a result, it was observed that the growth of all plants was inhibited. In addition, all young plants left in the isolated field in winter withered and died, and no difference was observed between T25 and the non-recombinant control maize. (p. 15 of Annex 2).

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

Maize is a summer type annual plant, and after ripening it usually dies out in winter, and it does not re-grow and propagate vegetatively, or produce seeds.

Actually, in NK603, at the end of isolated field tests, start of withering and death after ripening was observed.

Actually, T25, left in the isolated field at the end of isolated field tests after ripening, withered and died naturally due to the cold temperature in winter.

(d) Fertility and size of the pollen

NK603 and T25 exhibited high fertility of the pollen similarly to their non-recombinant control maize, and no difference was observed also regarding the shape and size of pollen (Table 3-3 and photos in pp.21-22 of Annex 1; pp.13-14 of Annex 2).

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

Regarding seed production, comparison was conducted between NK603 and their non-recombinant control maize for the characteristics referring to the production of seeds listed in I-2-(6)-2)-(a). As a result, a statistically significant difference was observed in NK603-B of NK603 in 100-kernel weight (Table 3-2 in p.17 of Annex 1).

Regarding row number per ear, grain number per row and 100-kernel weight, comparison was made between T25 and the non-recombinant control maize. As a result, they showed the equivalent values. Consequently, a statistically significant difference was not confirmed regarding seed prodution (p.12 of Annex 2).

Regarding shedding habit of the seed, shedding habits of the seed are considered extremely low, since the ears of all NK603, T25 and their non-recombinant control maize were covered with bracts at the time of harvesting. Therefore, shedding habit test was not performed.

Regarding germination rate to identfy the dormacy of seeds, germination test was carried out for the seeds harvested from NK603, T25 and their non-recombinant control maize. As a result, no statistically significant difference was observed, and they showed high germination rate of 98% or more. No

dormacy of seeds was confirmed (Table 3-4 in p.24 of Annex 1; p.14 of Annex 2).

(f) Crossability

A crossability test of the parent lines NK603 and T25 was not performed, since no wild relatives that can be crossed grow in Japan.

(g) Productivity of harmful substances

For maize, the secretion of any harmful substances from roots, which affect the surrounding plants or microorganisms in soil, has not been reported. In addition, the production of any allelochemicals, which affect other plants after they die, has not been reported.

As a result of succeeding crop tests, soil microflora tests, and plow-in tests conducted for NK603 and T25, no statistically significant difference was observed in all of their non-recombinant control maize (Table 3-5 to Table 3-7 in p.26-p.28 of Annex 1; p.17 of Annex 2).

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 is a cross progeny line developed by crossing the NK603 and T25 using the traditional breeding method. These parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied for Type I Use same as this stack line maize.

It is known that the modified CP4 EPSPS protein and the PAT protein differ from each other in the action mechanism and function independently from each other. In addition, they have high substrate specificity and thus, they are considered not to affect the metabolic pathway of plants. Therefore, it was considered unlikely that the proteins expressed in this stack maize line from individual parent lines would additionally affect the metabolic pathway of plants.

As a result of actually conducted bioassays, the tolerance to glyphosate herbicide and the tolerance to glufosinate herbicide expressed in this stack line maize were found in the

similar levels as offered by the individual parent lines. Consequently, it is considered low that the proteins expressed in this stack line maize from individual parent lines would interact with each other in the plant body of this stack line maize.

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

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

(1) Competitiveness

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

In order to investigate the characteristics regarding competitiveness of NK603 and T25, the parent lines of this stack line maize, morphological and growth characteristics, cold-tolerance at the early stage of growth, wintering ability of the matured plant, fertility and size of the pollen, production, shedding habit, dormancy and germination rate of the seed were examined. As a result, for T25, no significant difference or difference from the non-recombinant control maize was observed. On the other hand, for NK603, in a variety out of two hybrid varieties tested, a statistically significant difference from the non-recombinant control maize was found in 100-kernel weight. However, there was no statistically significant difference from the non-recombinant control maize in all the items examined but 100-kernel weight, and observed statistically significant difference was limited to only one of the two hybrid varieties tested. Therefore, it is considered unlikely that this difference could cause this recombinant maize to become dominant in competition.

Since the modified CP4 EPSPS protein and the PAT protein expressed in this stack line maize possess high substrate specificity, they are considered to function independently. In addition, this stack line maize possesses the tolerance to glyphosate herbicide and the tolerance to glufosinate herbicide, however, it is not generally considered that, in the natural environment less expected to suffer spraying of glyphosate and glufosinate, the tolerances to glyphosate and glufosinate would increase the competitiveness.

Based on the above understanding, it was judged that the conclusion by the applicant that this stack line maize would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

There has been no report that maize, the species to which the recipient organism

belongs, produces any harmful substances that could affect wild animals and wild plants.

It has been confirmed that the modified CP4 EPSPS protein and the PAT protein expressed in this stack line maize have no sequence that is structurally homologous with any known allergens.

In addition, since the modified CP4 EPSPS protein and the PAT protein possess high substrate specificity, it was considered unlikely that this stack line maize would act on the metabolic system of the recipient organism and produce any harmful substances. In practice, as a result of succeeding crop tests, soil microflora tests and plow-in tests conducted to examine the ability of NK603 and T25 to produce any harmful substances (the substances secreted from the roots which can affect other plants and microorganisms in soil, the substances existing in the plant body which can affect other plants after dying), no statistically significant difference from the non-recombinant control plots was observed in all tests.

Based on the above understanding, it was judged that the conclusion by the applicant that this stack line maize would pose no risk of Adverse Effect on Biological Diversity that is 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.

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 stack maize in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.