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

Name: Syngenta Seeds K.K.
Applicant: Hidero Ohtomo, President
Address: 401-2, Mukounodai, Takatsuhara, Tako-machi, Katori-gun, Chiba Prefecture

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize to produce thermostable α-amylase, resistant to Lepidoptera and Coleoptera, and tolerant to glufosinate and glyphosate herbicides (modified *amy797E*, modified *cry1Ab*, modified *cry3Aa2*, *pat*, *mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (3272×Bt11×MIR604×GA21, OECD UI:SYN-E3272-5×SYN-BTØ11-1×SYN-IR6Ø4-5×MON-ØØ Ø21-9) [including the progeny lines isolated from the maize lines, 3272, Bt11, MIR604 and GA21, that contain a combination of any of the transferred genes in the individual maize lines (except those already granted an approval regarding Type 1 Use Regulation)]
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 stack maize line produces a thermostable α-amylase, possesses resistance to Lepidoptera and Coleoptera, is tolerant to glufosinate and glyphosate herbicides, and is derived from four (4) recombinant maize parent lines. In addition, this stack maize line will be commercialized as a hybrid variety (F1) and the grain harvested from this stack maize line is composed of combinations of the transferred genes in the individual parent lines of this stack maize line due to the genetic segregation. Information concerning preparation, etc. of 3272, Bt11, MIR604 and GA21 are described below. Regarding GA21, Syngenta Seeds K.K.'s own data and international patent public data (Reference 1) were used as reference.

(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 production of 3272, Bt11, MIR604 and GA21 are shown in Table 1 to Table **3**4 (p.3 to p.6), respectively.

Table 1 Origins and functions of the component elements of the donor nucleic acid used for the production of 3272

Component elements	Origin and function
Modified *amy797E* gene expression cassette	
GZein promoter	Endosperm-specific promoter sequence derived from the 27 kDa storage protein (γ-zein) gene of Zea mays (Reference 14); used to express target genes specifically in the endosperm tissue of maize seeds.
Modified *amy797E* gene	The *α-amylase* gene is derived from the hyperthermophilic microorganisms of the archaeal order *Thermococcale* (Reference 15) and encodes a thermostable α-amylase protein (hereinafter referred to as the "modified AMY797E α-amylase"). The sequence of the modified *amy797E* gene has been codon optimized to maximize expression in maize (Reference 16). The modified *amy797E* gene has the maize γ-zein signal peptide added to the N-terminus. This sequence is intended to transport the target proteins into the lumen of endoplasmic reticulum (Reference 17). In addition, this gene has an endoplasmic reticulum retention signal added to the C-terminus (Reference 18). These additional sequences are considered to retain the encoded α-amylase in the endoplasmic reticulum of endosperm cells.
PEPC9 intron#9	An intron #9 sequence derived from the phosphoenolpyruvate carboxylase gene from Zea mays (Reference 19); used to enhance the expression of target genes.
35S terminator	Polyadenylation sequence derived from the cauliflower mosaic virus 35S RNA (Reference 20).
pmi gene expression cassette	
ZmUbiInt promoter	A promoter containing the first intron region derived from the polyubiquitin gene of Zea mays, providing constitutive expression of target genes throughout all tissues of monocotyledons (Reference 21).
pmi gene	The *manA* gene derived from *Escherichia coli*, encoding phosphomannose isomerase (hereinafter referred to as the "PMI protein") (Reference 22); used as a selectable marker for transgenic plants for which genes are transferred (Reference 23).
NOS terminator	Polyadenylation sequence of the nopaline synthase gene of *Agrobacterium tumefaciens* (Reference 24).
T-DNA backbone region	
LB	T-DNA left border sequence (LB) derived from *Agrobacterium tumefaciens*, required for integration of T-DNA region into the genome of plants (Reference 25).
spec	The streptomycin adenylyltransferase gene *aadA*, derived from *Escherichia coli* (*E. coli*). This gene is used as a bacterial selectable marker to confer resistance to erythromycin, streptomycin and spectinomycin (Reference 26).
VS1ori	The origin of replication consensus sequence derived from the *Pseudomonas*. Functions as the replication origin of plasmid in *Agrobacterium tumefaciens* (Reference 27).
ColE1ori	The origin of replication derived from *Escherichia coli* (*E. coli*) that permits replication of plasmid in *Escherichia coli* (*E. coli*) (Reference 28).
virG	VirGN54D derived from *Agrobacterium tumefaciens*, a required gene for efficient transformation of plants based on the *Agrobacterium* method (Reference 29).
repA	Replicon (minimum functional replication unit controlling DNA replication) region derived from *Pseudomonas* bacteria; a gene required for retention of vectors in *Agrobacterium* (Reference 30).
RB	T-DNA right border sequence (RB) derived from *Agrobacterium tumefaciens*, required for integration of T-DNA region into the genome of plants (Reference 31).

Table 2 Origins and functions of the component elements of the donor nucleic acid used for the production of Bt11

Gene cassette resistant to Lepidoptera	
Component elements	Origin and function
35S promoter	A promoter obtained as *Dde*I-*Dde*I fragment derived from cauliflower mosaic virus (CaMV) CM1841 strain. This promoter makes the target gene (modified *cry1Ab*) expressed in all the tissues constitutively (Reference 32).
IVS6-ADH1	An intron derived from the alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 33). Adh1-S intron was used to enhance the expression of target genes (modified *cry1Ab*) in plants (Reference 34).
Modified *cry1Ab*	A modified version of the full-length *cry1Ab* gene that encodes the Cry1Ab protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 strain, by partially deleting the C-terminal code region which is independent from the insecticidal activity of the Cry1Ab protein and modifying some nucleotide sequences to change the contents of GC and enhance its expression level in plants. This modification does not change any amino acid sequences of the core protein of the Cry1Ab protein.
NOS term	The 3' untranslated region of nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA (Reference 24, Reference 35). This sequence terminates transcription of target genes (modified *cry1Ab*).
Gene cassettes tolerant to glufosinate herbicide	
Component elements	Origin and function
35S promoter	A promoter obtained as *Alu*I-*Dde*I fragment derived from cauliflower mosaic virus (CaMV) Cabb-s strain. This promoter makes the target gene (*pat*) expressed in all the tissues constitutively (Reference 20).
IVS2-ADH1	An intron derived from the alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 33). Adh1-S intron was used to enhance the expression of the target gene (*pat*) in plants (Reference 34).
pat	The gene that encodes the PAT protein of *Streptomyces viridochromogenes*. The PAT protein, that confers glufosinate herbicide tolerance, was used as a selectable marker for modified plants at the time of transferring of genes. The *pat* gene has some nucleotide sequences modified to change the GC contents and enhance its expression level in plants. The amino acid sequence of the PAT protein expressed by the modification remains unchanged (Reference 36).
NOS term	The 3' untranslated region of nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA (Reference 24, Reference 35). This sequence terminates transcription of the target genes (*pat*).
Other regions	
Component elements	Origin and function
ColE1 ori	The replication origin derived from *Escherichia coli* plasmid pUC18 (Reference 37, Reference 38). Permits replication of plasmid in bacteria.
amp^R	Derived from *Escherichia coli*, it has the function to code β-lactamase and confer the tolerance to antibiotic ampicillin (Reference 38).

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Table 3 Origins and functions of the component elements of the donor nucleic acid used for the production of MIR604

Insect pest-resistant gene cassette	
Component elements	Origin and function
MTL	A promoter derived from the *metallothionein* gene of maize. Since Corn Rootworm, the target insect of the order Coleoptera, eats and damages the roots of maize, *MTL* promoter is used to define the start of transcription of target genes in the roots.
Modified *cry3Aa2*	A modified version of the *cry3Aa2* gene, which is derived from *Bacillus thuringiensis* subsp. *tenebrionis*, a typical gram-positive soil microorganism forming spores, by modifying some nucleotide sequences to change the contents of GC and enhance its expression level in plants and transferring cathepsin G protease recognition sequence to enhance the activity against Corn Rootworm. This gene encodes the modified Cry3Aa2 protein.
Nos	The terminator region of the nopaline synthase gene of *Agrobacterium tumefaciens*, which terminates transcription and induces polyadenylation.
Selectable marker gene cassette	
Component elements	Origin and function
ZmUbiInt	A promoter derived from the *polyubiquitin* gene of maize, to define the start of transcription of target genes in the entire plant body of monocotyledon.
pmi	The gene derived from *Escherichia coli* (*E. coli*.), which encodes the PMI protein (Phosphomannose isomerase). The PMI protein is an enzyme that has the capability of catalyzing the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. Transferring of this enzyme allows utilization of mannose as a carbon source. The *pmi* gene was used for selection of transformed cells.
Nos	The terminator region of the nopaline synthase gene of *Agrobacterium tumefaciens*, which terminates transcription and induces polyadenylation.
Other regions	
Component elements	Origin and function
Spec	The streptomycin adenylyltransferase gene *aadA*, derived from the transposon Tn7 of *Escherichia coli* (*E. coli*). This gene is used as a bacteria selectable marker to confer the resistance to erythromycin, streptomycin and spectinomycin.
VS1 ori	The replication origin consensus sequence derived from the plasmid pVS1 of *Pseudomonas* bacteria. Functions as the replication origin of plasmid in *Agrobacterium tumefaciens*.
ColE1 ori	The replication origin that permits replication of plasmid in bacteria.
LB	T-DNA left border region derived from *Agrobacterium tumefaciens* nopaline Ti-plasmid.
RB	T-DNA right border region derived from *Agrobacterium tumefaciens* nopaline Ti-plasmid.
VirG	A region involved in transfer of T-DNA, derived from *Agrobacterium tumefaciens*.
RepA	The pVS1 replication protein derived from *Pseudomonas* bacteria, taking on part of the responsibility for replication of pVS1 in the gram-positive bacteria living parasitically in plants.

Table 4 Origins and functions of the component elements of the donor nucleic acid used for the production of GA21

Herbicide resistant gene cassette	
Component elements	Origin and function
Act promoter ＋intron	A promoter derived from the rice actin 1 gene inducing the initiation of transcription of target gene throughout the entire plant body, including up to the first intron region which functions to enhance the efficiency of transcription (Reference 39).
sssu＋mssu (Hereinafter referred to as “OTP”)	The optimized transit peptide (OTP) sequences composed of the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) gene derived from chloroplast transit peptide sequence (sssu) from sunflowers and the *RuBisCo* gene derived chloroplast transit peptide sequence (mssu) from maize, functions to transport the mEPSPS protein expressed by the target gene *mEPSPS* gene to chloroplasts, where the protein takes action (Reference 40).
mEPSPS	A gene obtained from mutation of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) maize gene (Reference 41); It encodes the 5-enol-pyruvyl-shikimate-3-phosphate synthase (mEPSPS), the activity of which is not inhibited by the glyphosate herbicide, with the 102nd amino acid threonine in the wild-type EPSPS amino acid sequence modified to isoleucine, and the 106th proline modified to serine (Reference 1).
NOS	A polyadenylation sequence of the nopaline synthase (NOS) gene from *Agrobacterium tumefaciens*, terminating transcription (Reference 24).
Backbone region (Not contained in the GA21)	
Component elements	Origin and function
amp	Consists of the lac sequence, composed of partial coding sequence for lacI derived from bacteriophage M13, promoter plac and partial coding sequence for β-galactosidase or lacZ protein (Reference 38), and the β-lactamase gene (*bla*) conferring the ampicillin tolerance derived from plasmid pBR322 of *Escherichia coli* (Reference 42); selects and maintains the *Escherichia coli* which contains the constitutive plasmid by expression of β-lactamase.
ori-puc	The replication origin region derived from the plasmid pUC19 of *Escherichia coli*, conferring the autonomous replication potency of the plasmid in *Escherichia coli* (Reference 28).

2) Function of component elements

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

Functions of component elements of the donor nucleic acid that was used for the production of 3272, Bt11, MIR604 and GA21 are shown in Table 1 to Table 4 (p.3 to p.6)

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

[Thermostable α-amylase]

Modified AMY797E α-amylase:

The modified AMY797E α-amylase is an enzyme classified as an α-amylase (EC 3.2.1.1) that catalyzes the hydrolysis of starch into dextrins, maltose and glucose (Reference 43). Native α-amylases can be found in conventional maize seed and activity increases at the time of germination (Reference 44) in order to hydrolyze starch in the endosperm to support subsequent growth of the embryo.

The modified *amy797E* gene uses the *GZein* promoter and contains a N-terminal maize γ-zein signal sequence and a C-terminal endoplasmic reticulum retention signal sequence (Reference 45). Consequently, it is considered that the modified AMY797E α-amylase expressed by the modified *amy797E* gene is retained in the endoplasmic reticulum of maize endosperm (Reference 17; Reference 18).

3272 was developed in order to produce ethanol efficiently from maize grain. Conventionally, in ethanol production from dry ground maize kernels, water is added to ground maize and is heated to dissolve the starch, which is then liquefied by addition of microbially produced thermostable α-amylase. In 3272, the thermostable modified AMY797E α-amylase derived from microorganisms from the archaeal order *Thermococcales* was expressed specifically in maize seed. By mixing 3272 seed with conventional seed during liquefaction of starch, it is expected that the manufacturing process would be simplified and costs reduced.

[The insecticidal protein]

The insecticidal protein (=Bt protein), isolated from the soil microorganism *Bacillus thuringiensis*, exhibits its insecticidal activity against limited species of insects. It is indicated that the Bt protein, when fed and digested by sensitive species of insects, becomes an active polypeptide (= core protein) through specific digestion of protein, which specifically binds to the specific receptors on the surface of midgut of insects, causing cytoclasis or cell-destruction and leading to destructed digestive tracts and death of the insects (Reference 46). This mechanism of action also holds for the Cry1Ab protein and the Cry3Aa2 protein.

Modified Cry1Ab protein:

Regarding the insecticidal activity of the Cry1Ab protein, detailed experimental results are listed in the database operated by the Canadian Government (Reference 47), showing that it exhibits insecticidal activity against European Corn Borer (*Ostrinia nubilalis*), Corn Earworm (*Helicoverpa zea*), Fall Armyworm (*Spodeptera frugiperda*) and other order Lepidopteran insects which are the major pest insects for maize cultivation. The Cry1Ab protein exhibits little to no insecticidal activity against any insects other than the order Lepidoptera.

Modified Cry3Aa2 protein:

The modified *cry3Aa2* gene has some nucleotide sequences modified to change the GC content for enhanced expression in the recipient organism of maize and also for enhanced insecticidal efficacy against Corn Rootworm, the target insect of order Coleoptera. In addition, in order to enhance insecticidal activity against Corn

Rootworm, the target insect of order Coleoptera, the nucleotide sequence was modified as follows; the 108th to 110th amino acid sequence (valine-serine-serine) of the Cry3Aa2 protein was changed to be four (4) amino acids (alanine-alanine-proline-phenylalanine), the cathepsin G protease recognition sequence. This modification causes the modified Cry3Aa2 protein to be cut at the C-terminal side of phenylalanine, the 4th amino acid of cathepsin G protease recognition sequence, and to become an active polypeptide (= core protein) in the midgut of Corn Rootworm. However, the amino acid sequences other than described above remain unchanged from those in the Cry3Aa2 protein derived from *Bacillus thuringiensis* subsp. *tenebrionis*.

The modified Cry3Aa2 protein showed insecticidal activity against four (4) kinds of insects of the order Coleoptera [Western Corn Rootworm (*Diabrotica virgifera virgifera*), Northern Corn Rootworm (*Diabrotica longicornis barberi*), Colorado Potato Beetle (*Leptinotarsa decemlineata*), and Banded Cucumber Beetle (*Diabrotica balteata*)]; however, it did not show any insecticidal activity against other insects of the order Coleoptera such as Southern Corn Rootworm (*Diabrotica undecimpunctata*) and Cotton Ball Weevil (*Anthonomus grandis*). The Cry3Aa2 protein exhibits little to no insecticidal activity against any insects other than the order Coleoptera.

[Herbicide tolerant protein]

PAT protein:

Glufosinate inhibits glutamine synthase in plants causing plants to die due to accumulation of ammonia in the cells. However, the expression of the PAT protein acetylates and inactivates glufosinate, so that glutamine synthase in not inhibited. Consequently, plants which express the PAT protein, exhibit tolerance to glufosinate herbicide. The PAT protein has been used as a selectable marker for Bt11.

mEPSPS protein:

The glyphosate herbicide, a nonselective herbicide acting on stems and leaves, inhibits the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis, and interrupts the aromatic amino acid biosynthesis, thereby causing plants to die (Reference 48). The mEPSPS protein encoded by the *mEPSPS* gene exhibits EPSPS activity even in the presence of glyphosate herbicide, enabling aromatic amino acid biosynthesis,thereby conferring tolerance to glyphosate herbicide.

[Selectable marker]

PMI protein:

The *pmi* gene is derived from *Escherichia coli*, and encodes the PMI protein (phosphomannose isomerase). The PMI protein catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. Generally, maize and many other plants cannot utilize mannose as a carbon source, though the cells containing the *pmi* gene can use mannose for their growth. For this reason, transferring the *pmi* gene into plant cells as a selecable marker together with the target gene and subsequent incubation in the mannose-containing medium, transformed cells, including not only the *pmi* gene but also the target gene, can be

selected (Reference 23). The PMI protein exists widely in nature, including the human digestive system and in fact, is present in soybean and other plants, though it has not been identified in maize.

It has been confirmed based on the homology search for amino acid sequences using the publicly available database (SWISS-PROT, FARRP, etc.) that the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein do not share structurally related homologous sequences with any of the known allergens. The modified AMY797E α-amylase and the Per a 3 allergen from American cockroach (*Periplaneta americana*) share an eight (8) amino acid homology. However, this sequence does not match the IgE binding epitope sequences of the Per a 3 allergen (Reference 49). Therefore, it is extremely unlikely that the modified AMY797E α-amylase would become a similar allergen. It was confirmed that the modified AMY797E α-amylase does not share structurally related homologous sequences with any other known allergens..

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

It is considered that the modified AMY797E α-amylase would be retained locally in the endosperm's endoplasmic reticulum of maize kernels in 3272, though the substrate starch exists in the form of starch granules in the plastid in the maize kernels. Because the starch substrate exists in a different subcellular location within the cells, starch breakdown by modified AMY797E α-amylase would not occur without disruption of the cell. In practice, in compositional analysis of 3272 grain, the amount of starch was equivalent to that of the non-recombinant control maize. In addition, the modified AMY797E α-amylase exhibits very low enzyme activity at ordinary temperatures. As a result of observation of germination of 3272 and the non-recombinant control maize seedlings at temperatures of 10 to 40℃, there was no significant difference in the germination and the initial growth between 3272 and the non-recombinant control maize at all the temperatures examined. Based on the above, it is considered that modified AMY797E α-amylase does not affect the metabolic pathway of maize, the recipient organism.

There is no report that the modified Cry1Ab protein and the modified Cry3Aa2 protein possess any enzyme activity. Consequently, it is considered very unlikely that these proteins affect the metabolic pathway of maize of the recipient organism.

The PAT protein possesses very high substrate specificity to L-phosphinothricin (glufosinate herbicide) and dimethyl phosphinothricin, and there is no other protein or amino acid reported for the substrate of the PAT protein (Reference 50). Consequently, it is considered very unlikely that the PAT protein affects the metabolic pathway of maize of the recipient organism.

The mEPSPS protein is one of the enzymes that catalyze the shikimate pathway (Reference 51), and it is reported to react specifically with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 52). Consequently, it is considered very unlikely that the mEPSPS protein affects the metabolic pathway of maize of the recipient organism.

The PMI protein has the capability of catalyzing the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. The PMI protein reacts

specifically with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein (Reference 53). Consequently, it is considered very unlikely that the PMI protein affects the metabolic pathway of maize of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The plasmids used for the production of 3272, Bt11, MIR604 and GA21 are listed below.

3272: pNOV7013 constructed based on the pBluescript SK+derived from *Escherichia coli* (*E. coli*)
Bt11: pZO1502 constructed based on the pUC18 derived from *Escherichia coli* (*E. coli*)
MIR604: pZM26 constructed based on the pUC19 derived from *Escherichia coli* (*E. coli*)
GA21: pDPG434 constructed based on the pUC19 derived from *Escherichia coli* (*E. coli*)

2) Properties

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

The total number of base pairs of the plasmids used for the production of 3272, Bt11, MIR604 and GA21 are listed below, and the nucleotide sequences of these plasmids are disclosed.

3272: A total of 11,439 bp of the plasmid pNOV7013
Bt11: A total of 7,240 bp of the plasmid pZO1502
MIR604: A total of 13,811 bp of the plasmid pZM26
GA21: A total of 6,128bp of the plasmid pDPG434 (Reference 1)

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

The nucleotide sequence having specific functions included in plasmids and used for the production of 3272, Bt11, MIR604 and GA21 refers to the following antibiotic resistant marker genes. However, these antibiotic resistant marker genes are not transferred in the recipient organism.

3272: *spec* gene, resistance to streptomycin, erythromycin and spectinomycin
Bt11: amp^R gene, ampicillin resistance
MIR604: *spec* gene, resistance to streptomycin, erythromycin and spectinomycin
GA21: amp^R gene, ampicillin resistance (Reference 1)

(c) 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

There is no report that the plasmids pNOV7013, pZO1502, pZM26 and pDPG434 used for the production of 3272, Bt11, MIR604 and GA21 contain any sequence showing infectivity.

(3) Method of preparing living modified organisms

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

The nucleic acids transferred in the recipient organism of 3272, Bt11, MIR604 and GA21 are as follows.

3272: Two gene expression cassettes (the modified *amy797E* gene expression cassette and selectable marker gene cassette) between RB and LB of T-DNA region
Bt11: A part obtained by cleaving the plasmid pZO1502 by the restriction enzyme *Not*I and removing the amp^R gene
MIR604: Two gene expression cassettes (insect pest-resistant gene cassette and selectable marker gene cassette) between RB and LB of T-DNA region
GA21: A DNA fragment composed of the herbicide resistant gene cassette (Act promoter + intron/OTP/ *mEPSPS*/NOS) obtained by cleaving the plasmid pDPG434 by the restriction enzyme *Not*I (Reference 1)

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

The following methods were used to transfer the nucleic acid to the recipient organisms.

3272: *Agrobacterium* method
Bt11: Electroporation method
MIR604: *Agrobacterium* method
GA21: Particle gun bombardment (Reference 1)

3) Processes of rearing living modified organisms

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

Transformed cells were selected on the medium containing the substances listed below for individual recipient organisms of 3272, Bt11, MIR604 and GA21.

3272: Mannose
Bt11: Glufosinate
MIR604: Mannose

GA21: Glyphosate (Reference 1)

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

For 3272 and MIR604, after transferring of genes, the antibiotic Cefotaxime was added to the culture cell medium to remove any residual *Agrobacterium* used for the transformation. Then the PCR was carried out for regenerated plants, and the individual plants not containing the antibiotic-resistant marker gene in the backbone of plasmid were selected. Consequently, it is considered that there is no remaining *Agrobacterium.*

(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

This stack maize line was developed by cross-breeding between 3272 maize producing thermostable α-amylase; Bt11 maize resistant to Lepidoptera and tolerant to glufosinate herbicide; MIR604 maize resistant to Coleoptera; and GA21 maize tolerant to glyphosate herbicide. The status of approvals and applications for approvals of 3272, Bt11, MIR604 and GA21 in Japan are listed in Table 5 (p. 13).

Table 5 The status of approval and application for approval of 3272, Bt11, MIR604 and GA21 in Japan

	Safety as food	Safety as feed	Environmental safety
3272	December, 2007: Pending application	November, 2007: Pending application	October, 2006: Pending application
Bt11	March, 2001: Approved safety of use as food	March, 2003: Approved safety of use as feed	April, 2007: Approved for Type I Use Regulation
MIR604	August, 2007: Approved safety of use as food	August, 2007: Approved safety of use as feed	August, 2007: Approved for Type I Use Regulation
GA21	March, 2003: Approved safety of use as food	March, 2003: Approved safety of use as feed	November, 2005: Approved for Type I Use Regulation
This stack maize line	2009 Scheduled for application	2009 Scheduled for approval	July, 2009: Pending application

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

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

It was confirmed that the transferred genes of 3272, Bt11, MIR604 and GA21 exist on the chromosome.

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

In 3272, Bt11 and MIR604, as a result of Southern blotting analysis for the number of copies of the transferred gene, it was confirmed that one copy of each exists in the chromosome, and that the transferred genes are all inherited stably through multiple generations.

In GA21, as a result of Southern blotting analysis for the number of copies of the transferred gene, it was confirmed that transferred genes exist in the chromosome at one site, it consists of six (6) consecutive regions derived from the fragment of the transferred herbicide-tolerant gene cassette (Act promoter + intron/OTP/*mEPSPS*/NOS), and that the transferred genes are all stably inherited through multiple generations.

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

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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.

3272: Confirming the expression of proteins by ELISA method

Bt11: Confirming the expression of proteins by ELISA method, the bioassay using pest insects of the order Lepidoptera, and glufosinate herbicide-spraying test
MIR604: Confirming the expression of proteins by ELISA method, the bioassay using pest insects of the order Coleoptera
GA21: Glyphosate herbicide-spraying test

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

The transferred nucleic acid in 3272, Bt11, MIR604 and GA21 does not contain any sequence allowing transmission. Therefore, it is considered unlikely that the nucleic acid transferred to those plants could be transmitted to any other wild animals and wild plants.

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

For specific detection of the lines 3272, Bt11, MIR604 and GA21, a method based on the qualitative PCR analysis is available from the European Commission (Reference 54; Reference 55; Reference 56; Reference 57). Based on this method, the detection sensitivity was as follows in terms of the ratio of concentration of genome DNA.

3272: < 0.09% (Reference 54)
Bt11: 0.08% and over (Reference 55)
MIR604: < 0.09% (Reference 56)
GA21: 0.04% and over (Reference 57)

In order to detect and identify this stack maize line, one seed or plant body needs to be examined by the four (4) methods mentioned above, and this stack line can be confirmed when the results of all analyses are found positive.

(6) Difference from 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 maize line is given the traits as described below.

From 3272: Production of thermostable α-amylase and being a selectable marker due to the modified AMY797E α-amylase and the PMI protein respectively which are derived from the transferred genes
From Bt11: Resistance to Lepidoptera and tolerance to glufosinate herbicide due to the modified Cry1Ab protein and the PAT protein respectively which are derived from the transferred genes
From MIR604: Resistance to Coleoptera and being a selectable marker due to the modified Cry3Aa2 protein and the PMI protein respectively which are derived from the transferred genes

From GA21: Tolerance to glyphosate herbicide due to the mEPSPS protein derived from the transferred genes

As mentioned in I-2-(1)-2)-(c) (p.9 to p.10), it is considered that the modified AMY797E α-amylase, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein expressed in this stack maize line differ from each other in the action mechanism and thus function independently from each other. Consequently, these proteins are considered not to fall under the proteins referred to in Reference 58 (Schrijver, *et al*) as requiring examinations on possible interaction. In addition, it is considered that the modified AMY797E α-amylase, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein expressed in this stack maize line would not affect the metabolic pathway of their recipient organisms. 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 recipient organisms.

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

In order to confirm that the proteins expressed in this stack maize line from the individual parent lines would interact with each other, this stack maize line was tested as follows.

[Productivity of thermostable α-amylase]

Regarding the production of thermostable α-amylase, ELISA analysis was conducted on the samples collected from this stack maize line and 3272 cultivated in the U.S. fields (Bloomington, Illinois) in 2007, for determination of expression level of the modified AMY797E α-amylase in grain at maturation time of growth.

Experimental results confirm no significant difference was observed between this stack maize line and 3272 in expression level of the modified AMY797E α-amylase (Table 6, p. 15). Consequently, it was confirmed that the productivity of thermostable α-amylase of this stack maize line was not changed by crossing the parent lines.

Table 6 Expression level of the modified AMY797E α-amylase in grain of this stack maize line and 3272

Analyzed tissue[1]	This stack maize line		3272		P-value[2]
	Mean value (μg/g dry weight)	Standard deviation	Mean value (μg/g dry weight)	Standard deviation	
Grain	1322.68	188.33	1492.41	169.52	0.215

Investigation for expression level by ELISA method was conducted for 2 plant bodies and 5 repeats.

[1] It was confirmed that the expression level of the modified AMY797E α-amylase in the tissues other than grain (leaves, roots and pollens) shows lower value than the detection sensitivity in all tissues at the time of flowering.

[2] Statistical treatment by variance analysis was conducted, and less than 5% ($p<0.05$) in F test was determined as the significant value.

[Bioassay using insects of the order Lepidoptera]

Regarding resistance to Lepidoptera, this stack maize line, Bt11 and their non-recombinant maize lines were cultivated in two (2) fields in the U.S. in 2007 and the severity of insect damage by European Corn Borer, the target insect pest was investigated. European Corn Borer, the major target insect pest in maize cultivation in the U.S., could appear consecutively in two (2) generations. Therefore, in the first generation test, the first instar larvae of European Corn Borer (150 larvae/individual plant) were inoculated at the 6^{th} to 8^{th} leaf stage of maize, and on the 14^{th} day after inoculation, the severity of insect damage was observed visually. In the second generation test, the first instar larvae of European Corn Borer (200 larvae/individual plant) were inoculated at the time of flowering, and about the 45^{th} day after inoculation, length of trace of eaten cob, length of eaten ear, and length of trace of eaten stem were examined.

As a result, no significant difference between this stack maize line and Bt11 was observed in the severity of insect damage (Table 7, p.16). Therefore, it was confirmed that the resistance of this stack maize line to pest insects of the order Lepidoptera remains unchanged by crossing of parent lines.

Table 7 Investigation result of the severity of damage by Lepidoptera (European Corn Borer), based on bioassay of this stack maize line

Evaluation item		This stack maize line		Bt11		Non-recombinant control maize	
		Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Stanton, Minnesota	First generation test: Severity of insect damage to leaves[1]	1.0 a [2]	0.00	1.0 a	0.00	3.3 b	0.61
	Second generation test: Length of trace of eaten cob (cm)	0.0 a	0.00	0.1 a	0.10	3.2 b	0.57
	Second generation test: Length of eaten ear (cm)	0.1 a	0.08	0.0 a	0.00	0.9 b	0.31
	Second generation test: Length of trace of eaten stem (cm)	0.2 a	0.10	0.8 a	1.19	18.6 b	4.78
Bloomington, Illinois	First generation test: Severity of insect damage to leaves[1]	1.0 a	0.00	1.0 a	0.00	4.7 b	1.15
	Second generation test: Length of trace of eaten cob (cm)	0.2 a	0.17	0.2 a	0.12	1.9 b	0.23
	Second generation test: Length of eaten ear (cm)	0.3 a	0.29	0.4 a	0.10	1.7 b	0.40
	Second generation test: Length of trace of eaten stem (cm)	1.7 a	1.33	1.2 a	1.03	22.9 b	2.62

Investigation for severity of insect damage was conducted for 10 plant bodies and 3 repeats.

[1] Severity of insect damage to leaves was evaluated based on the following 9-step scales (Reference 59).

1 No feeding damage, or traces of minor insect damage (limited to 2 to 3 small spots)

2 Traces of feeding damage are all found 2 mm or less in size, and the number of damaged leaves is limited to one or two.

3 Small penetrated traces are observed on three or more leaves.

4 - 8 Depending on the degree of expansion of damaged area
9 Leaves are found seriously damaged, and the damage virtually extends to leaf vein.

[2] Evaluation items were individually subjected to statistical treatment, and for each evaluation item, different alphabetical letters indicate that a significant difference was observed between the relevant mean values based on the LSD after F test (p=0.05).

[Bioassay using insects of the order Coleoptera]

Regarding resistance to Coleoptera, this stack maize line, MIR604 and their non-recombinant maize lines were cultivated in two (2) fields in the U.S. in 2007 and the severity of insect damage of root by Western Corn Rootworm, the target insect pest was investigated. In the field in Minnesota, eggs of Western Corn Rootworm (1,500 eggs/individual plant) were inoculated at the 2nd to 3rd leaf stage of maize, and the severity of insect damage of root was observed visually at the time of silking. In the field of Illinois, where eggs of Western Corn Rootworm exist in soil, maize was cultivated to hatch eggs at the time of 2nd to 3rd leaf stage of maize, and the severity of insect damage of root was observed visually at the time of silking.

As a result of investigation, no statistically significant difference was observed between this stack maize line and MIR604 in the observed severity of insect damage to root (Table 8, p.17). Therefore, it was confirmed that the resistance of this stack maize line to pest insects of the order Coleoptera remains unchanged by crossing of parent lines.

Table 8 Investigation result of the severity of damage by Coleoptera (Western Corn Rootworm), based on bioassay of this stack maize line

Evaluation item	Seveity of insect damage to root[1]					
	This stack maize line		MIR604		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Stanton, Minnesota	0.44 a [2]	0.37	1.26 ab	0.72	2.21 b	0.13
Bloomington, Illinois	0.09 a	0.02	0.10 a	0.02	0.63 b	0.13

Investigation for severity of insect damage was conducted for 6 plant bodies and 3 repeats.

[1] Degree of root damage by Western Corn Rootworm were evaluated based on the 16 scales from 0.01 (no damage; or one or two minor damage on the surface) to 3.00 (three nodes of the root were all damaged) (Reference 60).

[2] Evaluation items were individually subjected to statistical treatment, and for each evaluation item, different alphabetical letters indicate that a significant difference was observed between the relevant mean values based on the LSD after F test (p=0.05).

[Bioassay using glufosinate herbicide]

Regarding tolerance to glufosinate, this stack maize line, Bt11 and the non-recombinant control maize were cultivated in a greenhouse in the U.S. in 2008, and the severity of injury by spraying of herbicide was investigated. At the 2nd leaf stage of maize (10 to 12th days after sowing), herbiside glufosinate (Product name: Liberty™) was sprayed. The concentration refers to 467g active ingredient (a.i.)/ha (normal

dosage), 1,868g a.i./ha (4-times higher dosage) and 3,736g a.i./ha (8-times higher dosage). On the 12th day after spraying herbicide glufosinate, the severity of injury was observed visually.

As a result, no significant difference between this stack maize line and Bt11 was observed in the severity of injury (Table 9. p.18). Therefore, it was confirmed that the tolerance of this stack maize line to herbicide glufosinate remains unchanged by crossing of parent lines.

Table 9 Investigation result of the severity of injury by spraying of herbicide glufosinate to this stack maize line

Concentration of herbicide (g a.i./ha)	Levels of herbicide injury (%)[1]					
	This stack maize line		Bt11		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
467	0.0 a[2]	0.0	0.0 a	0.0	68.0 d	2.8
1868	7.7 b	0.8	9.3 b	0.8	97.3 e	1.6
3736	16.0 c	2.2	15.8 c	2.8	100 f	0.0

Investigation for severity of herbicide injury was conducted for 10 plant bodies and 3 repeats.

[1] For each maize line, a non-sprayed plot was prepared. The level of herbicide injury in the non-sprayed plot is set as 0% (intact) for comparison. Then the levels of herbicide injury were evaluated based on the scale from 0% (intact) to 100% (complete death) in the herbicide sprayed plots.

[2] Different alphabetical letters indicate that a significant difference was observed between the relevant mean values (Student-Newman-Keuls test, p=0.05).

[Bioassay using glyphosate herbicide]

Regarding tolerance to glyphosate, this stack maize line, GA21 and the non-recombinant control maize were cultivated in a greenhouse in the U.S. in 2008, and the severity of injury by spraying of herbicide was investigated. At the 2nd leaf stage (10 to 12th days after sowing), herbiside glyphosate (Product name: Touchdown Total™) was sprayed. The concentration reffers to 840g acid equivalent (a.i.)/ha (normal dosage), 3,360g a.e./ha(4-times higher dosage) and 6,720g a.e./ha (8-times higher dosage). On the 19th day after spraying herbicide glufosinate, the severity of injury was observed visually.

As a result, no significant difference between this stack maize line and GA21 was observed in the severity of herbicide injury (Table 10, p.18). Therefore, it was confirmed that the tolerance of this stack maize line to herbicide glyphosate remains unchanged by crossing of parent lines.

Table 10 Investigation result of the severity of injury by spraying of herbicide glyphosate to this stack maize line

Concentration of herbicide (g a.e./ha)	Levels of herbicide injury (%) [1]					
	This stack maize line		GA21		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
840	2.7 a[2]	1.5	2.7 a	1.5	95.8 d	1.2

3360	16.2 b	2.0	13.7 b	4.3	100 e	0.0
6720	20.3 c	1.3	19.3 c	0.8	100 e	0.0

Investigation for severity of herbicide injury was conducted for 10 plant bodies and 3 repeats.

[1] For each maize line, a non-sprayed plot was prepared. The level of herbicide injury in the non-sprayed plot is set as 0% (intact) for comparison. Then the levels of herbicide injury were evaluated based on the scale from 0% (intact) to 100% (complete death) in the herbicide sprayed plots.

[2] Different alphabetical letters indicate that a significant difference was observed between the relevant mean values (Student-Newman-Keuls test, p=0.05).

(All the rights pertinent to the information in the table above and the responsibility for the content rest upon Syngenta Seeds K.K.)

Based on the above results, it was concluded that the individual proteins expressed in the relevant parental lines do not interact with each other and that the traits obtained from the transferred genes remain unchanged in this stack maize line.

Consequently, regarding the differences in physiological or ecological characteristics between this stack maize line and the non-recombinant control maize, the taxonomic species to which the recipient organism belongs, evaluation was conducted based on the results of individual examinations on the parent lines 3272, Bt11, MIR604 and GA21.

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

(a) Morphological and growth characteristics

For the morphological and growth characteristics of 3272, Bt11, MIR604 and GA21 and their non-recombinant control maize, examination was conducted for the items listed in Table 11 (p.20). As a result, in all characteristics examined, no statistical difference was observed, or they showed comparable results (Table 1 to Table 4 in p.3 to p.8 of Annex 1; Table 2 to Table 11 in p. 4 to p.8 of Annex 2; Table 2 to Table 5 in p.3 to p.8 of Annex3; Table 1 to Table 21 in p.2 to p.6 of Annex 4).

Table 11 Items examined for investigation of morphological and growth characteristics of 3272, Bt11, MIR604 and GA21

	3272	Bt11	MIR604	GA21
Uniformity of germination	○	○	○	○
Germination rate	○	○	○	○
Time of tasseling	○	○	○	○
Time of silking	○	○	○	○
Time of flower initiation	—	○	—	—
Time of flower completion	—	○	—	—
Flowering period	—	○	—	—
Culm length	○	○	○	○
Plant shape	○	○	○	○
Tiller number	○	○	○	○
Height of ear	○	○	○	○
Maturation time	○	○	○	○
Number of ears (Total number of ears)	○	○	○	○
Number of productive ears	○	○	○	○
Ear length	○	○	○	○
Ear diameter	○	○	○	○
Row number per ear	○	○	○	○
Grain number per row	○	○	○	○
Grain color	○	○	○	○
100-kernel weight	○	○	○	○
Grain shape	○	○	○	○
Fresh weight of above-ground parts at the harvest time	○	○	○	—
Plant weight at the harvest time	—	—	—	○

○: Examined
-: Notexamined

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

3272, Bt11, MIR604 and GA21 withered or died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control maize (p.11 of Annex 1; Photo 5 in p.9 to p.10 and p.29 of Annex 2; Figure 6 in p.10 to p.11 of Annex 3; p.8 of Annex 4).

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

Maize is a summer type annual plant, and after ripening the matured plant body usually withers and dies out. In fact, there is no report that, after maturity, maize has further propagated by vegetative parts, set seeds again, or produced seeds. Actually, at the end of isolated field tests, withering had begun and death after ripening was observed.

(d) Fertility and size of the pollen

As a result of the observation under a microscope with stained pollen, no difference was observed in the fertility (maturity of the pollen due to staining), shape and size of the pollen between 3272, Bt11, MIR604 and GA21 and their non-recombinant control maize (Figure 6 and Table 5 in p.9 to p.10 of Annex1; Photo 3 to Pnoto 4 in p. 8 to p. 9 and p.27 to p.28 of Annex 2; Figure 5 and Table 6 in p.9 to p.10 of Annex 3; p.7 of Annex 4).

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

Regarding seed production in 3272, Bt11, MIR604 and GA21 and their non-recombinant control maize, no significant difference was observed regarding characteristics of seed production (Table 3 to Table 5 in p.6 to p.10 of Annex 1; Table 6 to Table 10 in p.6 to p.8 of Annex 2; .Table 4 to Table 6 in p.6 to p.10 of Annex 3; Table 17 to Table 22 in p.5 to p.7 of Annex 4).

Regarding shedding habit of the seed, maize seed never shed spontaneously, since they adhere to ears and the ears are covered with husks (Reference 3). Also in 3272, Bt11, MIR604 and GA21, similarly as the non-recombinant control maize, the ears were found covered with husks at harvest time.

Regarding germination rate, a germination test was carried out on the sowing and harvested seed from 3272, Bt11, MIR604 and GA21 and their non-recombinant control maize. As a result, no difference was observed between them (Figure 7, Table 1 and Table 5 in p3 to p.4 and p.9 to p.10 of Annex 1; Table 2 in p.4 and p.9 of Annex 2; Table 2 and Table 6 in p.3 to p.4 and p.9 to p.10 of Annex 3; Table 2 and Table 23 in p.2 to p.3 and p.7 to p.8 of Annex 4). Regarding dormancy, it was found that the germination rate of sowing and harvested seed from 3272, Bt11, MIR604 and GA21 and their non-recombinant control maize showed comparable results. Therefore, it is considered unlikely that the dormancy of the 3272, Bt11, MIR604 and GA21 seed is significantly different than their non-recombinant control maize.

(f) Crossability

A crossability test was not performed for the parent lines 3272, Bt11, MIR604 and GA21 since there is no report of any wild relatives that can be crossed with maize growing voluntarily in Japan.

(g) Productivity of harmful substances

As a result of plow-in tests, succeeding crop tests and soil microflora tests conducted for 3272, Bt11, MIR604 and GA21, no statistically significant difference from their non-recombinant control maize was observed. (Table 6 to Table 8 in p.12 to p.14 of Annex 1; Table 13 to Table 15 and Table 25 to Table 27 in p.10 to p.14 and p.32 to p.34 of Annex 2; Table 7 to Table 9 in p.12 to p.15 of Annex 3; Table 24 to Table 35 in p.8 to p.11 of Annex 4).

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 maize line is a cross progeny line developed by crossing maize to produce thermostable α-amylase (3272), maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Bt11), maize resistant to Coleoptera (MIR604), and maize tolerant to glyphosate herbicides (GA21) using the traditional crossbreeding 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 maize line.

The modified AMY797E α-amylase, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein differ from each other in the action mechanism and function independently from each other and thus, these proteins are considered not to fall under the proteins referred to by Schrijver *et al.* (2007) as requiring examinations on possible interaction. In addition, they are considered not to affect the metabolic pathway of recipient organisms. 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 actual examinations, the expression level of thermostable α-amylase, resistance to Lepidoptera and Coleoptera, and tolerance to glufosinate and glyphosate herbicides expressed in this stack maize line were found to be similar levels as offered by the individual parent lines. Consequently, it is considered unlikely that the proteins expressed in this stack maize line from individual parent lines would interact with each other in the plant body of this stack maize line.

In addition, based on the above-mentioned findings that in this stack maize line produced by crossing of all of the parent lines, the proteins expressed from individual parent lines do not interact with each other, it is considered that also in the progeny lines of this stack maize line isolated from the parent lines, that contain a combination of any of the transferred genes in the individual parent lines of this stack maize line, no interaction would occur among the expressed proteins and no changes in the obtained traits would occur.

Based on the above understanding, it is considered unlikely that notable changes in traits have occurred in this stack maize line, 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 3272, Bt11, MIR604 and GA21, the parent lines of this stack maize line, 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, no significant difference or biologically relevant difference from their non-recombinant control maize was observed in 3272, Bt11, MIR604 and GA21.

This stack maize line is given traits to produce modified AMY797E α-amylase expressed in 3272. The α-amylase is an enzyme that catalyzes the hydrolysis of starch, and relates to germination. The modified AMY797E α-amylase is considered to be retained in the endoplasmic reticulum of endosperm tissue in maize grain,, whereas the substrate starch is located in a different subcellular location in the cell, the plastid as starch granules As a result of compositional analysis, the starch level in grain showed comparable results in 3272 and the non-recombinant control maize. In addition, the modified AMY797E α-amylase possesses thermostability, and the enzyme activity of the modified AMY797E α-amylase is substantially suppressed at ordinary temperatures. As a result of observation of germination and initial growth of 3272 and the non-recombinant control maize at temperatures of 10 to 40, no significant difference was observed in all conditions. Consequently, it is extremely unlikely that the modified AMY797E α-amylase expressed in this stack maize line affects metabolism and germination characteristics under natural conditions. Based on the above understanding, it is considered that the given trait of production of modified AMY797E α-amylase will not cause this stack maize line to become competitive in the natural environment in Japan.

This stack maize line is given traits to be resistant to Lepidoptera and Coleoptera. However, it is not generally considered that the insect damage by Lepidopteran and Coleopteran insects are a major cause in making maize difficult to grow in the natural environment in Japan. In addition Corn Rootworm is not reported to live in Japan. Consequently, it is considered that this trait does not increase the competitiveness of this stack maize line.

This stack maize line is given traits to be tolerant to glufosinate and glyphosate herbicides, however, it is unlikely that glufosinate and glyphosate herbicides are sprayed in the natural environment in Japan. Consequently, it is considered that this trait does not increase the competitiveness of this stack maize line.

In addition, in this stack maize line, the PMI protein is expressed in which mannose can be a carbon source. However, carbon sources other than mannose exists in the natural environment in Japan. Therefore, it is considered unlikely that this trait enhances the competitiveness of this stack maize line.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and the progeny lines of stack maize line isolated from the parent lines of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

(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.

As a result of the structural homology search of amino acid sequences with known allergens, it is considered extremely unlikely that the modified AMY797E α-amylase, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein possess allergenicity.

The modified AMY797E α-amylase, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein and the PMI protein were considered unlikely to act on the metabolic pathway of their recipient organism. Therefore, it is considered unlikely that these proteins would cause production of harmful substances in the parent lines, 3272, Bt11, MIR604 and GA21.

In practice, as a result of succeeding crop tests, plow-in tests and soil microflora tests conducted to examine the ability of the parent lines of this stack maize line 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 maize was observed in all tests. Therefore, it is considered unlikely that this stack maize line possesses productivity of unintended harmful substances.

The Lepidopteran and Coleopteran insects were identified and examined as wild organisms that could be affected by the modified Cry1Ab protein and the modified Cry3Aa2 protein, respectively. It was concluded that the possibility of the identified Lepidopteran and Coleopteran insects to eat pollen on any level is extremely low in cases where they are at least 10 meters away from a maize field, and is almost non-existent in cases where they are at least 50 meters away from a maize field. In addition, it is considered unlikely that the Lepidopteran and Coleopteran insects, in a natural ecosystem, normally inhabit within a 50 meter-radius locally of a maize field. Thus, it is judged extremely unlikely that the Lepidopteran and Coleopteran insects would be affected by this stack maize line in population. In addition, for the Lepidopteran and Coleopteran insects that could eat this stack maize line directly, they are considered unlikely to live locally around the cultivation fields of this stack maize line, and thus, it is judged extremely unlikely that the Lepidopteran and Coleopteran insects would be affected by directly eating this stack maize line in population.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack maize line and the progeny lines of stack maize line isolated from the parent lines of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances.

(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 stack maize line, 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 line and the progeny lines of stack maize line isolated from the parent lines of this stack maize line, that contain a combination of any of the transferred genes in the individual parent lines, in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.