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

Name : Syngenta Seeds K.K.
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera, and tolerant to glufosinate and glyphosate herbicides (modified *cry1Ab*, modified *vip3A*, *pat*, *mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (Bt11×MIR162×GA21, OECD UI: SYN-BTØ11-1×SYN-IR162-4×MON-ØØØ21-9) [including the progeny lines isolated from the maize lines, Bt11, MIR162 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 possesses resistance to Lepidoptera, is tolerant to glufosinate and glyphosate herbicides, and is derived from three (3) 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 Bt11, MIR162 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 Bt11, MIR162 and GA21 are shown in Table 1 to Table 3 (p. 3 to p. 5), respectively.

Table 1 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 14).
IVS6-ADH1	An intron derived from the alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 15). Adh1-S intron was used to enhance the expression of target genes (modified *cry1Ab*) in plants (Reference 16).
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 17, Reference 18). 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 19).
IVS2-ADH1	An intron derived from the alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 15). Adh1-S intron was used to enhance the expression of the target gene (*pat*) in plants (Reference 16).
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 20).
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 17, Reference 18). 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 21, Reference 22). 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 22).

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

Table 2 Origins and functions of the component elements of the donor nucleic acid used for the production of MIR162

Component elements	Origin and function
Insect pest-resistant gene cassette	
ZmUbiInt	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (1,010bp). It provides constitutive expression of target gene in all the tissues of monocots (Reference 23).
Modified *vip3A*	A modified version of the native *vip3A* gene found in the *Bacillus thuringiensis* strain AB88, a gram-positive bacteria existing normally in soil (Reference 24), to accommodate the preferred codon usage in plants (Reference 25). The *vip3A* gene encodes the modified Vip3A protein which exhibits the insecticidal activity against Lepidopteran insect pests. In the modified Vip3A protein, the amino acid at position 284 in the amino acid sequence was substituted to glutamine from lysine. In addition, in the modified Vip3A protein expressed in MIR162, 129th methionine was substituted by isoleucine by mutation of forming transformant, as well as 284th amino acid substitution.
iPEPC9	Intron #9 sequence from the phosphoenolpyruvate carboxylase gene from *Z. mays*. Used to enhance the expression of target gene (Reference 26).
35S	Polyadenylation sequence derived from the cauliflower mosaic virus 35S RNA (Reference 27).
Selectable marker gene cassette	
ZmUbiInt	Same as described above
pmi	A *manA* gene derived from the K-12 strain of *Escherichia coli*, encoding phosphomannose isomerase (hereinafter referred to as the "PMI protein"); used as a selectable marker for transgenic plants for which genes are transferres (Reference 28).
NOS	Terminator sequence of the nopaline synthase gene of *Agrobacterium tumefaciens* (Reference 29). Its function is to terminate transcription of mRNA by polyadenylation (Reference 30).
Other regions (hereinafter referred to as "Backbone region")	
LB	T-DNA left border region (Reference 31) derived from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Reference 29).
spec	The streptomycin adenylyltransferase gene *aadA*, derived from the transposon Tn7 of *Escherichia coli* (*E. coli*) (Reference 32). Used as a vector selectable marker to confer resistance to erythromycin, streptomycin, and spectinomycin.
Cos	Sticky-end region of linear DNA of lambda phage which is necessary for transferring plasmid to *Escherichia coli* and self-replication of plasmid in *Escherichia coli* (Reference 33).
ColE1 ori	The replication origin that permits replication of plasmid in bacteria derived from *Escherichia coli* (*E. coli*) (Reference 35).
RB	T-DNA right border region (Reference 34) derived form *Agrobacterium tumefaciens* nopaline Ti-plasmid (Reference 29).

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

Table 3 Origins and functions of the component elements of the donor nucleic acid used for the production of GA21

Herbicide tolerant 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 36).
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 37).
mEPSPS	A gene obtained from mutation of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) maize gene (Reference 38); 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 17).
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 22), and the β-lactamase gene (*bla*) conferring the ampicillin tolerance derived from plasmid pBR322 of *Escherichia coli* (Reference 39); 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 35).

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 Bt11, MIR162 and GA21 are shown in Table 1 to Table 3 (p. 3 to p. 5)

(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 known to possess any allergen (except allergenicity as food)

[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 a 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 40). This mechanism of action also holds for the Cry1Ab protein and the Vip3A protein.

Modified Cry1Ab protein:
Regarding the insecticidal activity of the Cry1Ab protein which has the same amino acid sequence of the modified Cry1Ab protein and core protein, detailed experimental results are listed in the database operated by the Canadian Government (Reference 41), 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 Vip3A protein:
The modified Vip3A protein exhibits high insecticidal activity against Fall Armyworm (*Spodoptera frugiperda*), Corn Earworm (*Helicoverpa zea*), Black Cutworm (*Agrotis ipsilon*) and other order Lepidopteran insects which are pest insects for maize cultivation in the U.S. However, the modified Vip3A protein does not exhibit insecticidal activity against Lepidopteran insects including European Corn Borer (*Ostrinia nubilalis*) and *Danaus plexippus* to which the Cry1Ab protein shows insecticidal activity (Reference 42).

In Reference 42 (Lee, *et al*), it was reported that the Vip3A protein and the Cry1Ab protein would bind with the brush border membrane vesicles (BBMV) without competing to one another. In addition, it was confirmed that the Vip3A protein would not bind with amino peptidase-like molecule and cadherin-like molecule known as receptors of the Cry1Ab protein in the BBMV of *Manduca sexta,* a sensitive Lepidopteran insect (Reference 42). Consequently, it is suggested that the Vip3A protein provides the similar mechanism of action as the Cry protein, though the Vip3A protein differs from the Cry1Ab protein regarding the receptors involved (Reference 42).

Compared to the Vip3A protein of *Bacillus thuringiensis* AB88 strain, a gram-positive bacteria existing normally in soil, the modified Vip3A protein shows some differences; the 284th amino acid sequence (lysine) of the modified Vip3A protein is substituted to glutamine. In addition, in the modified Vip3A protein expressed in MIR162, 129th methionine is substituted by isoleucine by mutation of forming ransformant, as well as 284th amino acid substitution.

[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 is 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, an nonselective herbicide acting on stems and leaves, inhibit 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 43). 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 28). 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 using the publicly available database (SWISS-PROT, FARRP, etc.) that the modified Cry1Ab protein, modified Vip3A protein, PAT protein, mEPSPS protein and PMI protein do not share structurally related homologous sequences with any of the known allergens.

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

There is no report that the modified Cry1Ab protein and the modified Vip3A 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 44). 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 45), and it is reported to react specifically with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 46). 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 48). 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 the Bt11, MIR162 and GA21 are listed below.

Bt11:	pZO1502 constructed based on the pUC18 derived from *Escherichia coli* (*E. coli*)
MIR162:	pNOV1300 constructed based on the pSB12 (Reference 47)
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 the Bt11, MIR162 and GA21 are listed below. The nucleotide sequences of the component elements of these plasmids are disclosed.

Bt11:	pZO1502, 7,240 bp
MIR162:	pNOV1300, 14,405 bp
GA21:	pDPG434, 6,128bp (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 Bt11, MIR162 and GA21 refers to the following antibiotic resistant marker genes. However, these antibiotic resistant marker genes are not transferred in the recipient organism.

Bt11:	amp^R gene, ampicillin resistance
MIR162:	*spec* gene, resistance to streptomycin, erythromycin and spectinomycin
GA21:	amp^R gene, ampicillin resistance (Reference 1)

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

There is no report that the plasmids pZO1502 and pDPG434 used for the production of the Bt11 and GA21 contain any sequence showing infectivity. In addition, the cos (the sticky-end region derived from lambda phage which

is necessary for transferring plasmids to *Escherichia coli*) exists in the plasmid pNOV1300 used for production of MIR162, however, the recipient organism other than *Escherichia coli* of lambda phage is not known.

(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 Bt11, MIR162 and GA21 are as follows.

Bt11: A part obtained by cleaving the plasmid pZO1502 by the restriction enzyme *Not*I and removing the amp^R gene
MIR162: 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 tolerant 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.

Bt11: Electroporation method
MIR162: *Agrobacterium* method
GA21: Particle gun bombardment (Reference 1)

3) Processes of rearing of 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 Bt11, MIR162 and GA21.

Bt11: Glufosinate
MIR162: Mannose
GA21: Glyphosate (Reference 1)

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

For MIR162, 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 with use of Bt11 (maize resistant to Lepidoptera and tolerant to glufosinate), MIR162 (maize resistant to Lepidoptera) and GA21 (maize tolerant to glyphosate). The status of approvals and applications for approvals of Bt11, MIR162, and GA21 in Japan are listed in Table 4 (p. 12).

Table 4 The status of approval and application for approval of Bt11, MIR162 and GA21 in Japan

	Safety as food	Safety as feed	Environmental safety
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
MIR162	February, 2008: Pending application	February, 2008: Pending application	May, 2008: Pending application
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	September, 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 Bt11, MIR162 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 Bt11 and MIR162, 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 chromosome

-

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.

Bt11: Confirming the expression of proteins by ELISA method, the bioassay using pest insects of the order Lepidoptera, and glufosinate herbicide-spraying test
MIR162: Confirming the expression of proteins by ELISA method, the bioassay using pest insects of the order Lepidoptera
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 the Bt11, MIR162 and the 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 Bt11 and GA21, a method based on the quantitative PCR analysis is available from the European Commission. The detection sensitivity is as follows in terms of the ratio of concentration of genome DNA: 0.08% and over for Bt11 and 0.04 % and over for GA21 (Reference 49, Reference 50). In addition, as the detection method for MIR162, the result of Southern blotting analysis conducted as follows can be used; the genome DNA7.5 μg was cut by a restriction enzyme and the modified *vip3A* gene was used as a probe.

In order to detect and identify this stack maize line, one seed or plant body needs to be examined by the 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 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 MIR162: Resistance to Lepidoptera and being a selectable marker due to the modified Vip3A 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

It is considered that the modified Cry1Ab protein, the modified Vip3A 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. In addition, as mentioned in I-2-(1)-2)-(c) (p.8), it is considered that the modified Cry1Ab protein, the modified Vip3A protein, the PAT protein, the mEPSPS protein and the PMI protein 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 51 (Schrijver, *et al*) as requiring examinations on possible interaction. Therefore, it was considered unlikely that the proteins expressed in this stack maize line from individual parent lines would individually or mutually affect the metabolic pathway of recipient organisms.

In order to confirm that the proteins expressed in this stack maize line from the individual parent lines would not interact with each other, this stack maize line was tested as follows.

[Bioassay using insects of the order Lepidoptera]

Regarding resistance to Lepidoptera, the severity of insect damage was investigated using European Corn Borer and Black Cutworm, the target insect pests of the Cry1Ab protein and the modified Vip3A protein, respectively.

For the invesigation of the severity of insect damage by European Corn Borer, this stack maize line, Bt11, MIR162 and their non-recombinant control maize were cultivated in two (2) fields in the U.S. in 2006. 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 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 damage by European Corn Borer (Table 5, p.15). Therefore, it is considered that the resistance of this stack line maize to pest insect of the order Lepidoptera remains unchanged by crossing of parent lines.

Table 5 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		MIR162		Non-recombinant control maize	
		Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Stanton, Minnesota	First generation test: Severity of insect damage to leaves [1]	1.00 b [2]	0.00	1.00 b	0.00	2.83 a	0.23	2.97 a	0.45
	Second generation test: Length of eaten ear (cm)	0.00 b	0.00	0.10 b	0.17	2.87 a	1.08	3.87 a	1.08
	Second generation test: Length of trace of eaten stem (cm)	0.27 b	0.23	0.23 b	0.25	11.20 a	2.98	14.03 a	1.33
Bloomington, Illinois	First generation test: Severity of insect damage to leaves [1]	1.00 b	0.00	1.00 b	0.00	5.00 a	0.00	5.33 a	0.58
	Second generation test: Length of eaten ear (cm)	0.13 c	0.23	0.73 c	0.23	1.73 b	0.64	3.09 a	0.38
	Second generation test: Length of trace of eaten stem (cm)	0.10 c	0.17	0.10 c	0.10	6.07 b	0.80	9.23 a	2.72

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 52).
- 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).

For the invesigation of the severity of insect damage by Black Cutworm, this stack maize line, MIR162, Bt11 and their non-recombinant control maize were cultivated in two (2) greenhouses in the U.S. in 2008. The fourth instar larvae of Black Cutworm (5 larvae/10 plant bodies) were inoculated at the 1st to 2nd leaf stage of maize, and on the 17th to 19th day after inoculation, the severity of insect damage was observed visually.

As a result, no significant difference between this stack maize line and MIR612 was observed in the severity of insect damage (Table 6, 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 6 Investigation result of the severity of damage by Lepidoptera (Black Cutworm), based on bioassay of this stack maize line

Evaluation item		This stack maize line		MIR162		Bt11		Non-recombinant control maize	
		Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Stanton, Minnesota	Damaged plant body (%) [1]	10.0 b [3]	10.7	6.3 b	5.2	98.8 a	3.5	97.5 a	4.6
	Seveirty of damage [2]	1.9 b	0.4	1.8 b	0.2	6.7 a	0.3	6.5 a	0.4
Slater, Iowa	Damaged plant body (%) [1]	6.3 b	7.4	0.0 b	0.0	96.3 a	7.4	93.8 a	10.6
	Seveirty of damage [2]	2.1 b	0.3	1.8 b	0.3	6.8 a	0.7	6.9 a	0.7

Investigation for severity of insect damage was conducted for 10 plant bodies and 8 repeats.

1: Damaged plant body (%) shows the proportion of damaged plants (Their stems near soil were bitten during the investigation).

2: Severity of insect damage to leaves was evaluated on the following 9-step scales (Revised Reference 53 and Reference 54)

- 1 No feeding damage
- 2 Traces of minor insect damage (limited to 1 to 2 small spots)
- 3 There are three or more spots per leaf, or 1 to 2 leaves were bitten.
- 4-8 Depending on the degree of expansion of damaged area
- 9 Leaves are found seriously damaged (no recovery expected).

3: 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 2007, and the severity of injury by spraying of herbicide was investigated. At the 2nd leaf stage of maize (12th days after sowing), herbicide 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 7. p. 17). Therefore, it was confirmed that the tolerance of this stack maize line to herbicide glufosinate remains unchanged by crossing of parent lines.

Table 7 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 d [2]	0.0	0.0 d	0.0	98.8 b	0.2
1868	10.1 c	1.3	10.2 c	1.3	100.0 a	0.0
3736	17.8 b	2.3	18.0 b	2.0	100.0 a	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 visually 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 2007, and the severity of injury by spraying of herbicide was investigated. At the 2nd leaf stage (12th days after sowing), herbiside glyphosate (Product name: Touchdown Total™) was sprayed. The concentration reffers to 840g acid equivalent (a.e.)/ha (normal dosage), 3,360g a.e./ha(4-times higher dosage) and 6,720g a.e./ha (8-times higher dosage). On the 15th 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 8, 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 8 Investigation result of the severity of injury by spraying of herbicide glyphosate to this stack maize line

	Levels of herbicide injury (%) [1]					
Concentration of herbicide (g.a.e/ha)	This stack maize line		GA21		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
840	0.0 d [2]	0.0	0.0 d	0.0	99.5 a	0.5
3360	23.5 c	2.6	22.7 c	6.0	100.0 a	0.0
6720	43.2 b	6.3	37.5 b	11.6	100.0 a	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).

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 Bt11, MIR162 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 Bt11, MIR162 and GA21 and their non-recombinant control maize, examination was conducted for the items listed in Table 9 (p. 20). As a result, in all items examined except culm length of MIR162, no statistically difference was observed, or they showed comparable results (Table 2 to Table 11 in p.4 to p.8 of Annex 1; Table 1 to Table 14 in p.3 to p.7 of Annex 2; Table 1 to Table 21 in p.2 to p.6 of Annex 3). Regarding the culm length in which significant difference was observed, MIR162 showed 191.5 cm and the non-recombinant control maize showed 199.7 cm (Table 5, p.4 of Annex 2).

Table 9 Items examined for investigation of morphological and growth characteristics of Bt11, MIR162 and GA21

	Bt11	MIR162	GA21
Start of germination	-	○	○
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 (Total weight of plant)	-	-	○

○:Examined
-: Notexamined

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

Bt11, MIR162 and GA21 withered or died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control maize (Photo 5 in p.9 to p.10 and p.29 of Annex 1; p. 8 of Annex 2; p.8 of Annex 3).

(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 fertility (maturity of the pollen due to staining), shape and size of the pollen between Bt11, MIR162 and GA21, and their non-recombinant control maize (Photo 3 to Photo 4 in p.8 to p.9 and p.27 to p.28 of Annex 1; Table 15 and Table 16 in p.7 of Annex 2; p.7 of Annex 3).

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

Regarding seed production in Bt11, MIR162 and GA21, and their non-recombinant control maize, no significant difference was observed in characteristics of seed production (Table 6 to Table 10 in p.6 to p.8 of Annex 1; Table 9 and Table 12 to Table 14 in p.5 to p.6 of Annex 2; Table 17 to Table 22 in p.5 to p.7 of Annex 3).

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 the Bt11, MIR162 and the GA21, similarly as the non-recombinant control maize, the ears were found covered with husk at harvest time.

Regarding germination rate, germination tests were carried out for the seeds for sowing and harvested seeds of Bt11, MIR162 and GA21 and their non-recombinant control maize. They showed comparable results (Table 2 in p.4 and p.9 of Annex 1; Table 2 and Table 17 in p.3 and p.8 of Annex 2; Table 2 and Table 23 in p.2 to p.3 and p.7 to p.8 of Annex 3). Regarding dormacy, it is considered unlikely that the seeds of Bt11, MIR162 and GA21 show different dormacy from that of their non-recombinant control maize, since the germination rate of seeds for sowing and harvested seeds was as high as the non-recombinant control maize.

(f) Crossability

Crossability test was not performed for the parent lines Bt11, MIR162 and GA21 since there is no report that any wild relatives that can be crossed with maize are 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 Bt11, MIR162 and GA21, no statistically significant difference from their non-recombinant control maize was observed (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 1; Table 18 to Table 24 in p.8 to p.10 of Annex 2; Table 24 to Table 35 in p.8 to p.11 of Annex 3).

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 was produced by crossing maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Bt11), maize resistant to Lepidoptera (MIR162), and maize tolerant to glyphosate herbicide (GA21). The Committee on Assessment of Adverse Effect on Biological Diversity judged that each of these parent lines would not result in Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stack line.

It is considered that the modified Cry1Ab protein, the modified Vip3A protein, the PAT protein, the mEPSPS protein and the PMI protein expressed in this stack maize line do not affect the matabolic pathway of their recipient organisms. In addition, it is considered that these proteins differ from each other in the action mechanism and thus function independently from each other. 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 bioassay conducted, it is considered that this stack maize line shows comparable levels to individual parent lines regarding resistance to Lepidoptera and tolerance to glufosinate and glyphosate herbicides. 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. Consequently, 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 Bt11, MIR162 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 statistically significant difference was observed in Bt11 and GA21 and their non-recombinant control maize. In fact, significant difference was found in the culm length of MIR162 (191.5 cm for MIR162 and 199.7 cm for the non-recombinant control maize), however, the differnce was small. Therefore, it is considered that this differnce of the culm length does not increase the competitiveness of this stack maize line.

This stack maize line is given traits to be resistant to Lepidoptera. However, it is not generally considered that the insect damage by Lepidopteran 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 exist 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 investigation on whether the modified Cry1Ab protein, modified Vip3A protein, PAT protein, mEPSPS protein and PMI protein expressed in this stack maize line share functionally important amino acid sequences with known allergens, it was confirmed that they do not share structually related homologous sequences with any of the known allergens examined.

The modified Cry1Ab protein, modified Vip3A protein, PAT protein, mEPSPS protein and PMI protein were considered unlikely to act on the metabolic pathway of their recipinent organism due to their characteristics. Therefore, it is considered unlikely that these proteins would cause production of harmful substances in the parent lines, Bt11, MIR162 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 insects were identified and examined as wild organisms that could be affected by the modified Cry1Ab protein and the modified Vip3A protein. It was concluded that the possibility of the identified Lepidopteran 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 insects that could eat directly this stack maize line or eat pollens dispersed from this stack maize line by eating with dietary plants, would live within a 50 meter-radius locally of a maize field. Therefore, it was concluded that the possibility is very low of the Lepidopteran insects to be affected by eating directly this stack maize line or to be affected by dispersed pollens 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 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 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.