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 Coleoptera and tolerant to glufosinate herbicide and glyphosate herbicide (modified *cry1Ab*, modified *cry3Aa2*, *pat*, *mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (Bt11×MIR604×GA21, OECD UI: SYN-BTØ11-1×SYN-IR6Ø4-5×MON-ØØØ21-9)
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

(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 the Bt11, MIR604 and GA21 are shown in Table 1, Table 2 and Table 3, respectively.

Table 1 Origins and functions of the component elements of the donor nucleic acid used for the production of the 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 13).
IVS6-ADH1	Intron derived from alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 14). Adh1-S intron was used to enhance the expression of target gene (modified *cry1Ab*) in plants (Reference 15).
Modified *cry1Ab*	A modified version of the full-length *cry1Ab* gene that encodes 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 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 Cry1Ab protein.
NOS term	3' untranslated region of nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA (Reference 16, Reference 17). This sequence terminates transcription of target gene (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 18).
IVS2-ADH1	An intron derived from alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 14). Adh1-S intron was used to enhance the expression of target gene (*pat*) in plants (Reference 15).
pat	A gene that encodes the PAT protein of *Streptomyces viridochromogenes*. PAT protein, that confers glufosinate herbicide tolerance, was used as a selective 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 PAT protein expressed by the modification remains unchanged (Reference 19).
NOS term	3' untranslated region of nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA (Reference 16, Reference 17). This sequence terminates transcription of target gene (*pat*).
Other regions	
Component elements	Origin and function
ColE1 ori	The replication origin derived from *Escherichia coli* plasmid pUC18 (Reference 20, Reference 21). 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 21).

Table 2 Origins and functions of the component elements of the donor nucleic acid used for the production of the MIR604

Insect pest-resistant gene cassette	
Component elements	Origin and function
MTL	A promoter derived from *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 at the roots.
Modified *cry3Aa2*	A modified version of *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 nopaline synthase gene of *Agrobacterium tumefaciens*, and terminates transcription and induces polyadenylation.
Selective marker gene cassette	
Component elements	Origin and function
ZmUbiInt	A promoter derived from *polyubiquitin* gene of maize, to define the start of transcription of target genes in the entire plant body of monocotyledon.
pmi	A gene derived from *Escherichia coli* (*E. coli*.), which encodes 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 is used for selection of transformed cells.
Nos	The terminator region of nopaline synthase gene of *Agrobacterium tumefaciens*, and 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 selective 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 3 Origins and functions of the component elements of the donor nucleic acid used for the production of the GA21

Herbicide resistant gene cassette	
Component elements	Origin and function
Act promoter ＋intron	A promoter derived from 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 22).
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 chloroplast transit peptide sequence (sssu) from sunflowers and the *RuBisCo* gene derived chloroplast transit peptide sequence (mssu) from maize, functioning to transport the mEPSPS protein expressed by the target gene *mEPSPS* gene to chloroplasts, where the protein takes action (Reference 23).
mEPSPS	A gene obtained from mutation of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene of maize (Reference 24); 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 nopaline synthase (NOS) gene from *Agrobacterium tumefaciens*, terminating transcription (Reference 16).

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 21), and the β-lactamase gene (*bla*) conferring the ampicillin tolerance derived from plasmid pBR322 of *Escherichia coli* (Reference 25); 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 high-copy-number plasmid pUC19 of *Escherichia coli*, conferring the autonomous replication potency of plasmid in *Escherichia coli* (Reference 26).

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid used for the production of the Bt11, MIR604 and GA21 are shown in Table 1, Table 2 and Table 3, respectively.

(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 (=Bt protein), isolated from the soil microorganism *Bacillus thuringiensis*, exhibits its insecticidal activity against specific species of insects. It is known 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 27).

This mechanism of action is also attained similarly in the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki* and the Cry3Aa2 protein derived from *Bacillus thuringiensis* subsp. *tenebrionis*.

Modified Cry1Ab protein:

As demonstrated by the results of study detailed in the Canadian Government Database (Reference 28) for the insecticidal activity of the Cry1Ab protein, the Cry1Ab protein exhibits the 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 cultivation of maize, though it exhibits no or least little 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 contents of GC for its enhanced expression in the recipient organism of maize and also for enhanced insecticidal efficiency against Corn Rootworm, the target insect of order Coleoptera. This modification causes the modified Cry3Aa2 protein 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*.

Based on the test result of the indoor bioassay conducted by the US Syngenta Seeds, Inc., the modified Cry3Aa2 protein showed insecticidal activity agasint 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*). On the other hand, the Cry3Aa2 protein exhibits no or least little insecticidal activity against any insects other than the

order Coleoptera.

PAT protein:

The glufosinate herbicide inhibits glutamine synthase in plants and then it causes plants to die due to the accumulated ammonia in the cells. However, the expression of the PAT protein acetylates and inactivates the glufosinate, which releases the glutamine synthase from inhibition. Consequently, the plants, which express the PAT protein, exhibit the tolerance to glufosinate herbicide and thus the PAT protein has been used as a selective marker for the Bt11.

mEPSPS protein:

The glyphosate herbicide is a nonselective herbicide acting on stems and leaves, which 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 29). The mEPSPS protein encoded by the *mEPSPS* gene exhibits the EPSPS activity even in the presence of glyphosate herbicide, and enables the aromatic amino acid biosynthesis in place of plant-intrinsic EPSPS and confers the tolerance to glyphosate herbicide.

PMI protein:

The *pmi* gene is a gene derived from *Escherichia coli*, which encodes the PMI protein (Phosphomannose isomerase), and the PMI protein has the capability of catalyzing 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, with transferring the *pmi* gene into plant cells as a selective marker together with the target gene and subsequent incubation in the mannose-containing medium, transformed cells, including not only *pmi* gene but also the target gene, can be selected (Reference 30). The PMI protein exists widely in nature including digestive system of human and in

fact, it is found 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 Cry3Aa2 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 modified Cry3Aa2 protein possess any enzyme activity. 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 31). The mEPSPS protein is one of the enzymes that catalyze the shikimate pathway (Reference 32), and it is reported to react specifically with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 33). The PMI protein has the capability of catalyzing the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. The other natural substrate of the PMI protein was not reported (Reference 34).

Based on the above understanding, it is considered very unlikely that these proteins affect the metabolic system of recipient organism.

(2) Information concerning vectors

1) Name and origin

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

Bt11: pZO1502 constructed based on the pUC18 derived from *Escherichia coli* (*E. coli*)

MIR604: pZM26 constructed based on the pUC19 derived from E. coli
GA21: pDPG434 constructed based on the pUC19 derived from 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, MIR604 and GA21 are listed below. The nucleotide sequences of the component elements of these plasmids have been disclosed.

Bt11: A total of 7,240bp of the plasmid pZO1502
MIR604: A total of 13,811bp 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 sequences having specific functions contained in the plasmids used for the production of the Bt11, MIR604 and GA21, and the functions are listed below.

Bt11 (pZO1502) and GA21 (pDPG434): amp^R gene and the resistance to ampicillin
MIR604 (pZM26): *spec* gene and the resistance to streptomycin, erythromycin and spectinomycin

However, these antibiotic resistant marker genes are not transferred in the Bt11, MIR604 and GA21.

(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 pZO1502, pZM26 and pDPG434 used for the production of the 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 acid transferred into the recipient organism of the Bt11 refers to the segment where the plasmid pZO1502 is cleaved by the restriction enzyme *Not*I and the amp^R gene is deleted.

To the MIR604, two gene expression cassettes (insect pest-resistant gene cassette and selective marker gene cassette) between RB and LB of T-DNA region were transferred.

In addition, for transferring the nucleic acid to the recipient organism of the GA21, the DNA fragment composed of only the herbicide resistant gene cassette (Act promoter + intron/OTP/*mEPSPS*/NOS) obtained by cleaving the plasmid pDPG434 by the restriction enzyme *Not*I is used (Reference 1).

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

To transfer the nucleic acid to the recipient organism for creating the Bt11, MIR 604 and GA21, the following methods were used respectively.

Bt11: Electroporation method
MIR604: 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 the Bt11, MIR604 and GA21.

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

Regarding the Bt11 and GA21, this item is not applicable since *Agrobacterium* method was not used.

Regarding the MIR604, after transferring of genes, the antibiotic Cefotaxime was added to the culture cell medium to remove any residual *Agrobacterium* used for transformation and thus 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 with which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

This stack line was produced by cross-breeding between the lepidopteran-resistant and glufosinate-tolerant maize line Bt11, the coleopteran-resistant maize line MIR604, and the glyphosate-tolerant maize line GA21.

The status of approval and application for approval of the Bt11, MIR604, GA21 and their respective stack lines in Japan is listed below.

Status of approval and application for approval of the Bt11, MIR604, and GA21

Description of approval		Year and month of approval		
		Bt11	MIR604	GA21
Safety as food	Based on the “Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4”, the safety of use for food was approved by the Ministry of Health and Welfare (the Ministry of Health, Labour and Welfare, currently).	September, 1996		November, 1999
	The approval was obtained, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques” for the safety of use as food from the Ministry of Health, Labour and Welfare.	March, 2001	August, 2007	March, 2003
Safety as feed	Based on the “Guideline for the Safety Evaluation of Feed derived from Recombinant-DNA Plants, 6-(2)”, safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.	September, 1996		December, 1999
	Based on the “Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques”, the safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.	March, 2003	August, 2007	March, 2003
Environmental safety	Based on the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries", the compatibility to the guideline regarding recombinant being utilized in a simulated environment was certified by the Ministry of Agriculture, Forestry and Fisheries. (General: General use/ Cultivation: Use for cultivation)	General-May,1996 Cultivation-May, 2001		Cultivation-May, 1998
	Based on the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries", the compatibility to the guideline was certified by the Ministry of Agriculture, Forestry and Fisheries. (General: General use/ Cultivation: Use for cultivation)	General-October, 1996 Cultivation-June, 2002		Cultivation-December, 1998

	Type I Use (Cultivation, storage, transportation, disposal and acts incidental to them in isolated fields) in accordance with the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" was approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment.		May, 2005	
	Type I Use (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) in accordance with the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" was approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment.	April, 2007	August, 2007	November, 2005

Status of approval and application for approval of the stack lines using the Bt11, MIR604, and GA21

Description of approval		Year and month of approval			
		Bt11xMIR604	Bt11xGA21	MIR604xGA21	Bt11xMIR604xGA21
Safety as food	The approval was obtained, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques” for the safety of use as food from the Ministry of Health, Labour and Welfare.	November, 2007	November, 2007	November, 2007	November, 2007
Environmental safety	Type I Use (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them) in accordance with the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" was approved by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment.	Under examination	November, 2007	November, 2007	(This 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 (on the chromosome, in the cell organelle, or in the protoplasm)

Presence of the transferred genes on the chromosome was confirmed based on the following methods.

Bt11: Segregation analysis and sequence analysis
MIR604 and GA21: Southern blotting analysis and segregation analysis

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

In the Bt11 and MIR604, it was confirmed based on the Southern blotting analysis that one copy of transferred genes is present on the genome of chromosome and also that the transferred genes are inherited stably in multiple generations.

In addition, in the GA21, it was confirmed that the transferred genes are present on the chromosome at one site, which is composed of six (6) consecutive regions derived from the herbicide tolerant gene cassette (Act promoter+ intron/OTP/*mEPSPS*/NOS) fragments, and also that the transferred genes are stably inherited in multiple generations.

3) 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 of resistance to Lepidoptera and tolerance to glufosinate herbicide in the Bt11, and the stability of expression of resistance to Coleoptera in the MIR604 were confirmed based on the ELISA method and bioassay.

In addition, the stability of expression of tolerance to glyphosate herbicide in the GA21 was confirmed based on the bioassay.

In order to investigate the stability of expression in this stack line of the resistance to Lepidoptera and the tolerance to glufosinate herbicide derived from the Bt11, of the resistance to Coleoptera derived from the MIR604 and of the tolerance to glyphosate herbicide derived from the GA21, European Corn Borer resistance test, Western Corn Rootworm resistance test, glufosinate herbicide spraying test and glyphosate spraying test were carried out using this stack line, the parent lines Bt11, MIR604 and GA21, and the non-recombinant control maize, and the tests were carried out at a level of significance of 5%.

As a result of the European Corn Borer resistance test, no significant difference was observed in insect damage to leaves between this stack line and the Bt11 (Table 4). In addition, as a result of the Western Corn Rootworm resistance test, no significant difference was observed in insect damage to roots between this stack line and the MIR604 (Table 5). Furthermore, as a result of the glufosinate and glyphosate herbicides spraying tests, no significant difference was observed between this stack line and the parent lines in the severity of herbicide injury due to the different recommended dosages (1x) and 4-times higher (4x) and 8-times higher (8x) dosage (Table 6 and Table 7).

Based on the above results, it has been confirmed that this stack line is equivalent to the parent lines Bt11, MIR604 and GA21 in the resistance to Lepidoptera and Coleoptera and the tolerance to glufosinate herbicide and glyphosate herbicide, and also that the traits given are stably expressed similarly as in the Bt11, MIR604 and GA21.

Table 4 Levels of resistance to Lepidoptera (European Corn Borer) in this stack line

(Measured in the fields of the US Syngenta Seeds, Inc. in 2005)

Evaluation item		Bt11×MIR604×GA21		Bt11		Non-recombinant control maize	
		Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
In the fields in Minnesota [1]	First generation test: Severity of insect damage to leaves [2]	1.00 a [3]	0.00	1.00 a	0.00	6.20 b	0.70
	Second generation test: Length of trace of eaten cob (cm)	0.0 a	0.00	0.10 a	0.31	3.60 b	1.93
	Second generation test: Length of eaten ear (cm)	0.00 a	0.00	0.00 a	0.00	2.50 b	2.35
	Second generation test: Length of trace of eaten stem (cm)	0.40 a	0.82	0.40 a	0.82	19.60 b	10.22
In the fields in Illinois [1]	First generation test: Severity of insect damage to leaves [2]	1.00 a	0.00	1.00 a	0.00	6.50 b	0.71
	Second generation test: Length of trace of eaten cob (cm)	1.50 a	1.55	1.94 a	1.39	5.75 b	2.29
	Second generation test: Length of eaten ear (cm)	0.00 a	0.00	0.06 a	0.25	3.63 b	1.50

	Second generation test: Length of trace of eaten stem (cm)	0.69 a	1.54	2.00 a	2.42	13.50 b	4.41

1: Evaluation was conducted in the growing period (First generation test) and the maturation period (Second generation test) of maize since European corn borer (*Ostrinia nubilalis* Hübner), the major target insect pest in maize cultivation in the US, could appear consecutively in two generations.

2: Severity of insect damage to leaves was evaluated based on the following 9-step scales (Reference 35).

- 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 (4=extension of damaged trace of 1.3 cm or less, 8=about a half of all the leaves found damaged)
- 9 Leaves are found seriously damaged, and the damage virtually extends to leaf vein.

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 test (p=0.05).

Table 5 Levels of resistance to Coleoptera (Western Corn Rootworm) in this stack line

(Measured in the fields of the US Syngenta Seeds, Inc. in 2006)

Evaluation item	Bt11×MIR604×GA21		MIR604		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Degree of root damage: in the fields in Minnesota [1, 3]	0.02 a [4]	0.01	0.01 a	0.01	0.86 b	0.47
Degree of root damage: in the field A in Illinois [2, 3]	0.21 a	0.14	0.20 a	0.12	2.44 b	0.28
Degree of root damage: in the field B in Illinois [2, 3]	0.09 a	0.05	0.13 a	0.07	1.57 b	0.49

1: In the fields in Minnesota, the eggs of the Western Corn Rootworm (*Diabrotica virgifera virgifera*) were inoculated to maize in the 2nd to 3rd leaf stage and then, severity of insect damage to the roots was evaluated by visual inspection at the time of silking,.

2: In the fields A and B in Illinois, where the eggs of Western Corn Rootworm exist in the soil, maize samples were cultivated and then, severity of insect damage to the roots was evaluated by visual inspection at the time of silking.

3: 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 36).

4: 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 test (p=0.05).

Table 6 Tolerance to glufosinate herbicide in this stack line

(Measured in the greenhouse of the US Syngenta Seeds, Inc. in 2006)

Concentration of herbicide sprayed[1]	Levels of herbicide injury (%) [2]					
	Bt11×MIR604×GA21		Bt11		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
1×	0.0 f [3]	0.2	0.1 f	0.4	88.8 b	9.4
4×	16.3 e	4.1	16.7 de	3.7	100.0 a	0.0
8×	18.8 cd	4.1	19.3 c	4.7	100.0 a	0.0

1: Individual maize samples cultivated in a greenhouse were sprayed with herbicide containing the glyphosate as an active ingredient at a recommended dosage (1×) and 4-time higher (4×) and 8-time higher (8×) dosages than the recommended dosage and then, observed for levels of herbicide injury 16 days after herbicide spraying.

2: Levels of herbicide injury were evaluated by visual inspection based on the scale from 0% (intact) to 100% (complete death) for the herbicide sprayed plots compared to the plots without spraying of herbicide provided for individual maize lines where the level of herbicide injury was defined at 0% (intact).

3: Different alphabetical letters indicate that a significant difference was observed between the relevant mean values based on the LSD test (p=0.05).

Table 7 Tolerance to glyphosate herbicide in this stack line

(Measured in the greenhouse of the US Syngenta Seeds, Inc. in 2006)

Concentration of herbicide sprayed[1]	Levels of herbicide injury (%) [2]					
	Bt11×MIR604×GA21		GA21		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
1×	0.0 d [3]	0.0	0.0 d	0.0	100.0 a	0.0
4×	23.2 c	5.8	23.5 c	7.3	100.0 a	0.0
8×	35.8 b	7.2	36.3 b	9.6	100.0 a	0.0

1: Individual maize samples (2nd leaf stage, 11 days after sowing) cultivated in a greenhouse were sprayed with herbicide containing the glyphosate as an active ingredient at a recommended dosage (1×) and 4-time higher (4×) and 8-time higher (8×) dosages than the recommended dosage and then, observed for levels of herbicide injury 19 days after herbicide spraying.

2: Levels of herbicide injury were evaluated by visual inspection based on the scale from 0% (intact) to 100% (complete death) for the herbicide sprayed plots compared to the plots without spraying of herbicide provided for individual maize lines where the level of herbicide injury was defined at 0% (intact).

3: Different alphabetical letters indicate that a significant difference was observed between the relevant mean values based on the LSD test (p=0.05).

4) 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, 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 Bt11, MIR604 and GA21, a method based on the quantitative PCR analysis is available from the European Commission. Based on this method, the detection sensitivity was found 0.1% for the Bt11 and GA21 and more than 0.045% for the MIR604 in terms of the ratio of concentration of genome DNA (Reference 37, Reference 38, and Reference 39).

In order to detect and identify this stack line, one seed or plant body needs to be examined by the three 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 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 MIR604: Resistance to Coleoptera and being a selective 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

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

This stack line is given the Lepidoptera resistance and glufosinate herbicide tolerance derived from the Bt11, the Coleoptera resistance derived from the MIR604 and the glyphosate herbicide tolerance derived from the GA21, though these traits have been confirmed not to be significantly different from those in the parent lines Bt11, MIR604 and GA21. In addition, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein, the mEPSPS protein, and the PMI protein are considered very unlikely to interact with each other in this stack line and affect the metabolic pathway of the recipient organism from the characteristic of the individual proteins.

Based on the above understanding, regarding the physiological or ecological difference between this stack line and the taxonomic species of maize to which the recipient organism belongs, evaluation was conducted on the parent lines Bt11, MIR604 and GA21 based on the isolated field tests conducted in Japan.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between the Bt11, MIR604 and GA21 and the non-recombinant control maize regarding the items listed below. As a result, in all the items examined, no significant difference nor difference was observed between the Bt11, MIR604 and GA21 and the non-recombinant control maize.

For the Bt11, MIR604 and GA21, examination was conducted regarding the

germination rate, uniformity of germination, time of tassel exertion, time of silking, maturation time, plant type, tiller number, number of ears, number of productive ears, grain color, grain shape, culm length, height of ear, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, and fresh weight at the time of harvesting. In addition, examination was also conducted regarding the time of flower initiation, time of flower completion, and flowering period for the Bt11, and the time of flower initiation and time of flower completion for the GA21.

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

The Bt11, MIR604 and GA21 withered otherwise died similarly as the non-recombinant control maize due to the cold treatment at the early stage of growth.

(c) Wintering ability of the matured plant

Maize is a summer type annual plant, and after ripening the matured plant body usually withers and dies out. Maize does not contain any tissue or organ other than seeds, which can regenerate the plant body, and it is considered to fail to survive when exposed to sub-zero temperatures for 6 to 8 hours, though depending on maize growing stage and cultivation environment (Reference 3).

It was actually observed in the isolated field tests that the Bt11 and the GA21 died after maturation similarly as the non-recombinant control maize. There is no report that the matured plants of the MIR604 used in foreign countries have overwintered, and it was observed in the isolated field tests in the US that the MIR604 died after maturation similarly as the non-recombinant control maize.

(d) Fertility and size of the pollen

As a result of the observation under a microscope with pollen stained with a neutral red solution, no difference was observed in the fertility (maturity of the pollen due to staining), shape and size of the pollen between the Bt11 and the

non-recombinant control maize. In addition, as a result of the observation with pollen stained with an acetocarmine solution, no difference was observed in the fertility (maturity of the pollen due to staining), shape and size of the pollen between the MIR604, the GA21 and the non-recombinant control maize.

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

Regarding seed production, no significant difference was observed between the Bt11, MIR604 and GA21 and the non-recombinant control maize in the ear length, ear diameter, row number per ear, grain number per row, and 100-kernel weight and thus, no significant difference was observed regarding seed production.

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

The germination rate was found equivalent for both the sowing seeds and harvested seeds from the Bt11, MIR604 and GA21 and the non-recombinant control maize. Dormancy has not been examined, though the possibility is considered low that the dormancy of the Bt11, MIR604 and GA21 is significantly different from that of the non-recombinant control maize, since no difference was observed in the germination rate of sowing seeds sown under different temperature conditions and harvested seeds between the parent lines and the non-recombinant control maize.

(f) Crossability

Crossability test was not performed for the parent lines Bt11, MIR604 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

A plow-in test, a succeeding crop test and a soil microflora test were carried out for the Bt11, MIR604 and GA21, and as a result, they indicated no significant differences between the Bt11, MIR604 and GA21, and the non-recombinant control maize in all items.

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 "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." Results of the review are listed below.

This stack line maize was produced by crossing maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Bt11), maize resistant to Coleoptera (MIR604), 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.

The modified Cry1Ab protein encoded by the modified *cry1Ab* gene (Lepidoptera resistant gene) derived from the Bt11 possesses the insecticidal activity against the insects of the order Lepidoptera but it is considered not to have any enzyme activity. In addition, the PAT protein (phosphinothricin acetyltransferase) encoded by the *pat* gene (glufosinate tolerant gene) derived from the Bt11 is the enzyme that possesses high substrate specificity. On the other hand, the modified Cry3Aa2 protein encoded by the modified *cry3Aa2* gene (Coleoptera resistant gene) derived from the MIR604 possesses the insecticidal activity against the insects of the order Coleoptera but it is considered not to have any enzyme activity. Furthermore, the mEPSPS protein (5-enol-pyruvyl-shikimate-3-phosphate synthase) encoded by the *mEPSPS* gene (glyphosate herbicide tolerant gene) derived from the GA21 is also the enzyme that possesses high substrate specificity. In addition, the modified Cry1Ab protein and the

modified Cry3Aa2 protein exhibit the insecticidal activity against the specific insects of the order Lepidoptera and the order Coleoptera, respectively, and the both Cry proteins do not overlap each other in their insecticidal spectra; therefore, it is considered unlikely that these Cry proteins interact with each other. It is therefore considered unlikely that traits conferred by the modified *cry1Ab* gene, the *pat* gene, the *cry3Aa2* gene and the *mEPSPS* gene would interact with each other.

It has been confirmed that this stack line maize possesses the equivalent levels of resistance to Lepidoptera and Coleoptera and tolerance to glufosinate and glyphosate herbicides as the parent lines possess, as a result of European corn borer and Western corn rootworm resistance tests regarding the resistance to Lepidoptera and Coleoptera, and herbicide spraying tests regarding the tolerance to glufosinate and glyphosate herbicides.

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

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

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis) has been long used in Japan, though there is no report that it has become self-seeding in a natural environment in Japan.

This stack line maize is given traits to be resistant to Lepidoptera and Coleoptera due to the modified Cry1Ab protein and the modified Cry3Aa2 protein which are encoded by the modified *cry1Ab* gene from the Bt11 and the modified *cry3Aa2* gene from the MIR604, respectively, and to be tolerant to herbicides glufosinate and glyphosate due to the PAT protein and the mEPSPS protein which are encoded by the *pat* gene from the Bt11 and the *mEPSPS* gene from the GA21, respectively. However, it is not generally considered that the insect damage by Lepidopteran and Coleopteran insects is the major cause making the maize difficult to grow in the natural environment in Japan, and the herbicides glufosinate and glyphosate are sprayed and the herbicides glufosinate and glyphosate exert pressure for selection.

Consequently, it is considered that these characteristics do not increase the

competitiveness and thus this stack line maize is not predominant over the parent lines in the competitiveness.

Based on the above understanding, it was judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

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

This stack line maize has the modified Cry1Ab protein and the PAT protein productivity derived from the Bt11, the modified Cry3Aa2 protein productivity derived from the MIR604, and the mEPSPS protein productivity derived from the GA21. The modified Cry1Ab protein and the modified Cry3Aa2 protein possess the insecticidal activity against the insects of order Lepidoptera and Coleoptera. However, the PAT protein and the mEPSPS protein confer tolerance to glufosinate herbicide and tolerance to glyphosate herbicide, respectively, though they are confirmed not to be harmful substances to animals and plants. In addition, it is considered unlikely that the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT protein and the mEPSPS protein would interact with each other. As a result, even though this stack line maize contains these proteins in conjunction, it is unlikely that the productivity of harmful substances will be greater in this stack line maize than its parent lines.

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

(3) Crossability

In the Japanese natural environment, there are no wild plants which can cross with maize. Therefore, it was judged that there are no specific wild plants that are

possibly affected by this recombinant maize, and that the use of such maize poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by applicant is valid.

2. Conclusion based on the Biological Diversity Risk Assessment Report

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

Bibliography

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Annex

Confidential: Not made available or disclosed to unauthorized person