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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera and Coleoptera and tolerant to glufosinate herbicide (modified *cry1Ab*, modified *cry3Aa2*, *pat*, *Zea mays* subsp. *mays* (L.) Iltis) (Bt11×MIR604,OECD UI:SYN-BTØ11-1×SYN-IR6Ø4-5)
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 and MIR604 are shown in Table 1 and Table 2, 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 12).
IVS6-ADH1	An intron derived from alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 13). Adh1-S intron was used to enhance the expression of target gene (modified *cry1Ab*) in plants (Reference 14).
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 15, Reference 16). 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 17).
IVS2-ADH1	An intron derived from alcohol dehydrogenase 1S (Adh1-S) gene of maize (Reference 13). Adh1-S intron was used to enhance the expression of target gene (*pat*) in plants (Reference 14).
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 18).
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 15, Reference 16). 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 19, Reference 20). 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 20).

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 replacing amino acid sequence to enhance the activity against Corn Rootworm. This gene encodes the modified Cry3Aa2 protein. This modification causes the modified Cry3Aa2 protein to become an active polypeptide (= core protein) in the midgut of Corn Rootworm.
Nos	The terminator region of nopaline synthase gene of *Agrobacterium tumefaciens*, which 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 *E. coli*, which encodes PMI protein (Phosphomannose isomerase) and used for the selection of the transformed cell. 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, so its cultured cell can not propagate in the medium containing mannose. However, the cell to express the PMI protein due to the transferring *pmi* gene can use mannose for their growth. Therefore, the selection of the transformed cell is available.
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.

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 and the MIR604 are shown in Table 1 and Table 2, 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)

Modified Cry1Ab protein:

The insecticidal protein (=Bt protein), isolated from the soil microorganism *Bacillus thuringiensis*, exhibits its insecticidal activity against limited 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 21). This mechanism of action is also attained similarly in the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*. As demonstrated by the results of study detailed in the Canadian Government Database (Reference 22) 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. It has been confirmed based on the homology search using the publicly available database (SWISS-PROT, FARRP, etc.) that the modified Cry1Ab protein does not share structurally related homologous sequences with any of the known allergens.

Modified Cry3Aa2 protein:

There are some types of insecticidal proteins (=Bt proteins), isolated from the soil microorganism *Bacillus thuringiensis*, and each of them exhibits insecticidal activity against limited 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 21). This mechanism of action is also attained similarly in the 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. It has been confirmed based on the homology search using the publicly available database (SWISS-PROT, FARRP, etc.) that the modified Cry3Aa2 protein does not share structurally related homologous sequences with any of the known allergens.

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. It has been confirmed based on the homology search using the publicly available database (SWISS-PROT, FARRP, etc.) that the PAT protein does not share structurally related homologous sequences with any of the known allergens.

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 the *pmi* gene but also the target gene, can be selected (Reference 23). 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 PMI protein does 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 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 24). In addition, 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 25).

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 plasmid pZO1502 used for the production of the Bt11 was constructed based on the pUC18 derived from *Escherichia coli.* The plasmid pZM26 used for the production of the MIR604 was constructed based on the pUC19 derived from *Escherichia coli*.

2) Properties

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

The total number of base pairs of the plasmid pZO1502 used for the production of the Bt11 is 7,240bp. The total number of base pairs of the plasmid pZM26 used for the production of the MIR604 is 13,811bp. The nucleotide sequences of the component elements of these plasmids have been disclosed.

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

The plasmid pZO1502 used for the production of the Bt11 possesses the amp^R gene to show the ampicillin resistance as a bacteria selective marker. The plasmid pZM26 used for the production of the MIR604 possesses the *spec* gene to show the resistance to streptomycin, erythromycin and spectinomycin. However, these antibiotic resistant marker genes are not transferred in the Bt11 and the MIR604.

(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 and pZM26 used for the production of the Bt11 and the MIR604 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. The nucleic acid transferred into the recipient organism of the MIR604 are two gene expression cassettes (insect pest-resistant gene cassette and selective marker gene cassette) between RB and LB of T-DNA region.

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

To transfer the nucleic acid to the recipient organism for producing the Bt11, the Electroporation method was used. In addition, to transfer the nucleic acid to the recipient organism for producing the MIR604, the *Agrobacterium* method was used.

3) Processes of rearing of living modified organisms

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

Regarding the Bt11, transformed cells were selected on the medium containing glufosinate. In addition, regarding the MIR604, transformed cells were selected on the medium containing mannose.

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

Reagrding the Bt11, 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*, 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 and the Coleopteran-resistant maize line MIR604.

The status of approval and application for approval of the Bt11 and the

MIR604 in Japan is listed below.

Bt11:

May, 1996: 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 (isolated field test for import).

September, 1996: 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: Based on the “Guideline for the Safety Evaluation of Feed derived from Recombinant-DNA plants, 6-(2)”, the compatibility to the guideline regarding use for feed was certified by the Ministry of Agriculture, Forestry and Fisheries.

October, 1996: Based on the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries", the compatibility to the guideline regarding recombinant being imported was certified by the Ministry of Agriculture, Forestry and Fisheries.

March, 2001: The approval was obtained, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques” from the Ministry of Health, Labour and Welfare.

May, 2001: 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 (isolated field test for cultivation).

June, 2002: Based on the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries", the compatibility to the guideline regarding recombinant being cultivated was certified by the Ministry of Agriculture, Forestry and Fisheries.

March, 2003: 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.

April, 2007: Type 1 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.

MIR604:

May, 2005: Type 1 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.

August, 2007: The approval was obtained, in accordance with “Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques” from the Ministry of Health, Labour and Welfare.

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.

Type 1 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.

(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)

In the Bt11, it was confirmed based on the segregation analysis and the sequence analysis that the transferred genes are present on the chromosome. In addition, in the MIR604, it was confirmed based on the Southern blotting analysis and the segregation analysis that the transferred genes are present on the chromosome.

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

In the Bt11 and the MIR604, it was confirmed 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.

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 order to investigate the stability of expression in this stack line of resistance to Lepidoptera and tolerance to glufosinate herbicide derived from the Bt11 and of

resistance to Coleoptera derived from the MIR604, European Corn Borer resistance test, Western Corn Rootworm resistance test, and glufosinate herbicide spraying test were carried out using this stack line, the parent lines Bt11 and MIR604, 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 and insecticidal activity between this stack line and the Bt11 (Table 3, p13). In addition, as a result of the Western Corn Rootworm resistance test, no significant difference was observed in insect damage to roots and insecticidal activity between this stack line and the MIR604 (Table 4, p15). Furthermore, as a result of the glufosinate herbicides spraying tests, no significant difference was observed between this stack line and the Bt11 in the severity of herbicide injury (Table 5, p15).

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

Table 3 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		Bt11		Non-recombinant control maize	
		Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
Degree of damage: In the fields in Minnesota [1]	First generation test: Severity of insect damage to leaves [2]	1.0 a [3]	0.0	1.0 a	0.0	5.9 b	1.0
	Second generation test: Length of trace of eaten cob (cm)	0.1 a	0.2	0.0 a	0.0	1.8 a	1.6
	Second generation test: Length of eaten ear (cm)	0.1 a	0.3	0.0 a	0.0	4.1 b	1.6
	Second generation test: Length of trace of eaten stem (cm)	0.3 a	0.9	0.4 a	1.8	19.8 b	11.3
Degree of damage: In the fields in Illinois [1]	First generation test: Severity of insect damage to leaves [2]	1.0 a	0.0	1.0 a	0.0	6.5 b	0.7
	Second generation test: Length of trace of eaten cob (cm)	0.6 a	1.2	1.0 a	1.6	3.6 b	1.5

	Second generation test: Length of eaten ear (cm)	4.1 a	2.7	3.4 a	2.5	6.6 b	1.4
	Second generation test: Length of trace of eaten stem (cm)	1.3 a	2.0	0.8 a	1.4	13.8 b	6.7

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 26).

- 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 4 Levels of resistance to Coleoptera (Western Corn Rootworm) in this stack line

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

Evaluation item		Bt11×MIR604		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]	Hybrid 1	0.06 a [4]	0.03	0.22 a	0.18	1.95 b	0.35
	Hybrid 2	0.26 a	0.25	0.22 a	0.27	2.15 b	0.46
Degree of root damage: in the field in Illinois [2, 3]	Hybrid 1	0.17 a	0.10	0.12 a	0.07	2.08 b	0.36
	Hybrid 2	0.28 a	0.20	0.10 a	0.06	2.58 b	0.44

1: In the fields in Minnesota, the eggs (1,500-2,000 eggs /plant) 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 in Illinois, where the eggs of Western Corn Rootworm (*Diabrotica virgifera virgifera*) exist, 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 27).

4: In each field, 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 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		Bt11		Non-recombinant control maize	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
1×	0.1 e [3]	0.3	0.0 e	0.2	84.9 b	5.4
4×	12.2 d	3.4	11.3 d	3.5	99.0 a	2.3
8×	18.7 c	2.6	20.2 c	3.8	99.8 a	0.6

1: Individual maize samples (3rd leaf stage, 11 days after sowing) cultivated in a greenhouse were sprayed with herbicide containing the glufosinate 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 11 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).

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 and the MIR604 does not contain any sequence allowing transmission. Therefore, it is considered unlikely that the nucleic acid transferred to the both 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, 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 in terms of the ratio of concentration of genome DNA (Reference 28). In addition, for specific detection of the lines MIR604, a method based on the PCR analysis has been developed.

In order to detect and identify this stack line, one seed or plant body needs to be examined by the two methods mentioned above, and this stack line can be confirmed when the results of the both 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 follows: Resistance to Lepidoptera due to the modified Cry1Ab protein derived from the transferred gene to the Bt11; resistance to Coleoptera due to the modified Cry3Aa2 protein derived from the transferred

gene to the MIR604; tolerance to glufosinate herbicide due to the PAT protein derived from the transferred gene to the Bt11; and being a selective marker due to the PMI protein derived from the transferred gene to the MIR604.

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 and the Coleoptera resistance derived from the MIR604, though these traits have been confirmed not to be significantly different from those in the parent lines Bt11 and MIR604. In addition, the modified Cry1Ab protein, the modified Cry3Aa2 protein, the PAT 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 and MIR604 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 and the non-recombinant control maize regarding the germination rate, uniformity of germination, time of tassel exertion, time of silking, time of flower initiation, time of flower completion, flowering period, 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 after harvesting. As a result, in all the items examined, no significant difference nor difference was observed between the Bt11 and the non-recombinant control maize.

For the morphological and growth characteristics, comparison was made between the MIR604 and the non-recombinant control maize regarding the uniformity of germination, germination rate, time of tasseling, time of silking, culm length, plant shape, tiller number, height of ears, maturation time, 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, and fresh weight of aerial parts after harvesting. As a result, in all the items examined, no significant difference nor difference was observed between the MIR604 and the non-recombinant control maize.

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

The Bt11 and the MIR604 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 2).

It was actually observed in the isolated field tests that the Bt11 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 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 and the MIR604 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 2). Also in the Bt11 and the MIR604, 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 and the MIR604 and the non-recombinant control maize. Dormancy has not been examined, though the possibility is considered low that the dormancy of the Bt11 and the MIR604 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 and MIR604 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 and the MIR604, and as a result, they indicated no significant differences between the Bt11 and the MIR604, 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) and maize resistant to Coleoptera (MIR604). 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. 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 and the *cry3Aa2* 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 herbicide 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 herbicide.

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 glufosinate herbicide due to the PAT protein which are encoded by the *pat* gene from the Bt11. 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 glufosinate herbicide is sprayed and the glufosinate herbicide exerts 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 and the modified Cry3Aa2 protein productivity derived from the MIR604. The modified Cry1Ab protein and the modified Cry3Aa2 protein possess the insecticidal activity against the insects of order Lepidoptera and Coleoptera, respectively. However, the PAT protein confers tolerance to glufosinate herbicide, 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, and the PAT 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.

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex List

Annex 1: Isolated field test report for the Bt11

Annex 2: Isolated field test report for the MIR604

Confidential: Not made available or disclosed to unauthorized person