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 Coleoptera and tolerant to glyphosate herbicide (Modified *cry3Aa2*, *mEPSPS*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604×GA21, OECD UI：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

The parent lines of this stack line, MIR604 and GA21, were developed by Syngenta Seeds, Inc. and Monsanto Company, respectively. This stack line is given the traits to be resistant to Coleoptera due to the transferred genes in the MIR604, and to be tolerant to glyphosate herbicide due to the transferred genes in the GA21. Therefore, information on the MIR604 and the GA21 is explained individually in the following statements. To create this evaluation document, we referred to the proprietary data of Syngenta Seeds, Inc., the published international publication for patent applications (Reference 1) and the Biological Diversity Risk Assessment Report (Annex 1) prepared based on the published references for the GA21, and the Biological Diversity Risk Assessment Report for the MIR604 (Annex 2).

#### (1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of the MIR604 are shown in Table 1. In addition, the composition of donor nucleic acid and the origins of component elements used for the development of the GA21 are shown in Table 2.

**Table 1 Origins and functions of the component elements of the donor nucleic acid used for the production of MIR604**

| Insect pest-resistant gene cassette | |
|---|---|
| Component elements | Origin and Function |
| *MTL* | A promoter derived from *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* | Replacement of codon is given to the nucleotide sequence of the *cry3Aa2* gene, which is derived from *Bacillus thuringiensis* subsp. *tenebrionis*, a typical gram-positive soil microorganism forming spores, to enhance its expression in maize. In addition, it is a modified gene to be replaced a part of its amino acid sequence to show activity against Corn Rootworm, and codes 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 *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 the *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. |

**Table 2 Origins and functions of the component elements of the donor nucleic acid used for the production of GA21**

| Herbicide resistant gene cassette | |
|---|---|
| Component elements | Origin and Function |
| Act promoter +intron | A promoter derived from 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 *Zea mays* (Reference 22); 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 24). |
| Backbone region (Not contained in 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 25), and the β-lactamase gene (*bla*) conferring the ampicillin tolerance derived from plasmid pBR322 of *Escherichia coli* (Reference 26); 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 27). |

2) Functions of component elements

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

Functions of component elements of the donor nucleic acid that was used for the development of the MIR604 are shown in Table 1. In addition, functions of component elements of the donor nucleic acid that was used for the development of the GA21 are shown in Table 2.

(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 Cry3Aa2 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 14). This mechanism of action also holds for the Cry3A protein.

The modified *cry3Aa2* gene has some amino acid sequences modified for its optimum expression in the recipient organism of maize and also for enhanced insecticidal efficiency against 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 Syngenta Seeds, Inc., the modified Cry3Aa2 protein showed insecticidal activity against four (4) kinds of insects of the order Coleoptera [Western Corn Rootworm (*Diabrotica virgifera virgifera*), Northern Corn Rootworm (*Diabrotica longicornis barberi*), Colorado Potato Beetle (*Leptinotarsa decemlineata*) and Banded Cucumber Beetle (*Diabrotica balteata*)]; however, it did not show insecticidal activity against other insects of the order Coleoptera such as Southern Corn Rootworm (*Diabrotica undecimpunctata*) and Cotton Boll Weevil (*Anthonomus grandis*). 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.

**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 14). The PMI protein exists widely in nature including digestive sustem 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.

**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 28). 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. It has been confirmed based on the homology search using the publicly available database (SWISS-PROT, FARRP, etc.) that the mEPSPS 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 Cry3Aa2 protein possesses any enzyme activity. 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 19). The mEPSPS protein is one of the enzymes that catalyze the shikimate pathway (Reference 20), and it is reported to react specifically with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 21).

Based on the above understanding, it is considered very unlikely that these proteins affect the metabolic system of the recipient organism, but they would function independently in the recipient organism.

## (2) Information concerning vectors

1) Name and origin

The plasmid pZM26 used for the production of the MIR604 was constructed based on the pUC19, etc. derived from *Escherichia coli*. In addition, the plasmid pDPG434 used for the production of the GA21 is derived from plasmids including pUC19, etc. from *Escherichia coli* (Reference 29).

2) Properties

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

The total number of base pairs of the plasmid pZM26 used for the production of the MIR604 is 13,811bp. In addition, the total number of base pairs of the plasmid pDPG434 used for the production of the GA21 is 6,128bp (Reference 1). 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 pZM26 used for the production of the MIR604 contains the antibiotic resistant marker gene which is used as a selective marker for bacteria and thus, the plasmid possesses the resistance to streptomycin, erythromycin and spectinomycin. The plasmid pDPG434 used for the production of the GA21 contains the *amp* gene which is used as a selective marker for bacteria and thus, the plasmid possesses the resistance to ampicillin, though the antibiotic resistant marker gene is not transferred in the MIR604 and the 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 plasmid pDPG434 used for the production of the pZM26 and the GA21 used for the production of MIR604 contain any sequence showing infectivitiy.

## (3) Method of preparing living modified organisms

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

Two gene expression cassettes (insect pest-resistant gene cassette and selective marker gene cassette) between RB and LB of T-DNA region among the plasmid pZM26 used for production of the MIR604 were transferred to the MIR604. 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 MIR604, the *Agrobacterium* method was used. In addition, to transfer the nucleic acid to the recipient organism for creating the GA21, the particle gun bombardment was used (Reference 1).

3) Processes of rearing of living modified organisms

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

Regarding the MIR604, transformed cells were selected on the medium containing mannose. Regarding the GA21, transformed cells were selected on the medium containing glyphosate herbicide (Reference 1).

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

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.* Reagrding the GA21, this item is not applicable since *Agrobacterium* method was not used.

(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 developed by cross-breeding between the MIR604, maize resistant to Coleoptera, and the GA21, maize tolerant to glyphosate herbicide.

The status of approval and application for approval of the MIR604 and the GA21 in Japan are the following.

**MIR604:**

| | |
|---|---|
| May, 2005: | Type I Use (Cultivation, processing, 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, 2006: | Based on the “Procedure to Check the Safety of Food and Additives Produced by Recombinant-DNA Techniques”, |

| | |
|---|---|
| | the safety of use for food was approved by the Ministry of Health, Labour and Welfare. |
| May, 2006: | 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. |
| December, 2006: | Public comment was completed regarding 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" by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment. |

**GA21:**

| | |
|---|---|
| May, 1998: | 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. |
| December, 1998: | Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being imported to Japan and cultivated in Japan was certified by the Ministry of Agriculture, Forestry and Fisheries. |
| November, 1999: | 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). |
| December, 1999: | 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. |
| March, 2003: | 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. |
| 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. |
| November, 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.

**(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 MIR604 and the GA21, 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 MIR604, it was confirmed that one copy of transferred genes is present on the 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 Coleoptera in the MIR604 was 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 Coleoptera derived from the MIR604 and tolerance to glyphosate herbicide derived from the GA21, Western Corn Rootworm resistance test and glyphosate herbicide spraying test were carried out using this stack line, the parent line MIR604 or GA21, and the non-recombinant control maize.

As a result of the Western Corn Rootworm resistance test, no significant difference was observed in insect damage to root between this stack line and the MIR604 (Table 3). In addition, as a result of the glyphosate herbicide 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 dosages of herbicide (1x, 4x and 8x) (Table 4).

Based on the above results, it has been confirmed that this stack line is equivalent to the patent lines MIR604 and GA21 in the resistance to Coleoptera and tolerance

to glyphosate herbicide, and also that the traits given are stably expressed similarly as in the MIR604 and the GA21.

**Table 3 Levels of resistance to Coleoptera in this stack line (Measured in the fields in Ohio and Illinois in the U.S. in 2005)**

| Evaluation item | 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 field in Ohio [1, 3] | 0.09 a [4] | 0.04 | 0.05 a | 0.02 | 0.85 b | 0.94 |
| Degree of root damage: in the field in Illinois [2, 3] | 0.11 a | 0.04 | 0.09 a | 0.03 | 2.17 c | 0.14 |

1 In the fields in Ohio, the eggs of Western Corn Rootworm (*Diabrotica virgifera virgifera*) were incubated in the maize leaves (2nd to 3rd leaf stage), and degree of root damage at the time of silking were evaluated by visual inspection.

2 In the fields in Illinois, where the eggs of Western Corn Rootworm (*Diabrotica virgifera virgifera*) exist, maize samples were cultivated, and degree of root damage at the time of silking were evaluated by visual inspection.

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 16).

4 For each field, the same alphabetical letters indicate that there is no significant difference between the relevant mean values (P=0.05).

**Table 4 Tolerance to glyphosate herbicide in this stack line (Measured in the greenhouse of Syngenta Seeds, Inc. in 2006)**

| Concentration of herbicide sprayed[1] | Levels of herbicide injury (%) [2] | | | | | |
|---|---|---|---|---|---|---|
| | MIR604×GA21 | | GA21 | | Non-recombinant control maize | |
| | Mean value | Standard deviation | Mean value | Standard deviation | Mean value | Standard deviation |
| No spraying | 0 d [3] | 0.0 | 0 d | 0.0 | 0 d | 0.0 |
| 1× | 0 d | 0.0 | 0 d | 0.0 | 100 a | 0.0 |
| 4× | 29.7 c | 2.6 | 27.7 c | 5.5 | 100 a | 0.0 |
| 8× | 34.6 b | 2.6 | 36.2 b | 1.3 | 100 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 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).

3 The same alphabetical letters indicate that there is no significant difference between the relevant mean values (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 MIR604 and the GA21 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 MIR604, a method based on the qualitative PCR analysis has been developed (Annex 2). For specific detection of the GA21, a method based on the qualitative PCR analysis is available from the European Commission. Based on this method, the detection sensitivity was found 0.1% for the GA21 in terms of the ratio of weight between GA21 and the control kernel (Reference 17).

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 to be resistant to Coleoptera and to be selective marker due to the modified Cry3Aa2 protein and the PMI protein respectively which are derived from the transferred genes in the MIR604, and also to be tolerant to glyphosate herbicide due to the mEPSPS protein derived from the transferred genes in the GA21.

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

This stack line is given 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 MIR604 and GA21. In addition, the modified Cry3Aa2 protein and the PMI protein derived from the MIR604 and the mEPSPS protein derived from the GA21 are considered to function independently from the characteristics of the individual proteins and then, it is considered very unlikely that these proteins would interact with each other in this stack line and could affect the metabolic pathway of the recipient organism.

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 MIR604 and GA21 based on the isolated field tests conducted in Japan (Annex 1, Annex 2).

(a) Morphological and growth characteristics

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, ear grain color, 100-kernel weight, ear grain shape, and fresh weight of aerial parts after harvesting. As a result, in all characteristics evaluated, no significant difference nor difference was observed between the MIR604 and the non-recombinant control maize. In addition, comparison was conducted between the GA21 and the non-recombinant control maize regarding the uniformity of germination, germination rate, time of tasseling, time of silking, culm length, height of ear, plant shape, maturation time, fresh plant weight at harvesting time, tiller number, number of ears, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, ear grain shape, and ear grain color. As a result, in all characteristics evaluated, no significant difference nor difference was observed between the GA21 and the non-recombinant control maize.

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

The MIR604 and the 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).

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 that the MIR604 died after maturation similarly as the non-recombinant control maize. In addition, it was actually observed in the isolated field tests that the GA21 died after maturation similarly as the non-recombinant control maize.

(d) Fertility and size of the pollen

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 MIR604, the GA21 and the non-recombinant control maize.

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 MIR604 and the 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 MIR604 and the GA21 and the non-recombinant control maize. Dormancy has not been examined, though the possibility is considered low that the dormancy of the MIR604 and the 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 MIR604 and the 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 MIR604 and the GA21, and as a result, they indicated no significant differences between the MIR604 and the 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 "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." Results of the review are listed below.

This stack line maize was produced by crossing 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 I Use described in the application for this stack line.

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. On the other hand, 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. It is therefore considered unlikely that traits conferred by the modified *cry3Aa2* gene and *mEPSPS* gene would interact with each other.

It has been confirmed that this stack line maize expresses resistance to Coleoptera and tolerance to glyphosate herbicides, by Western Corn Rootworm resistance test and herbicide spraying tests, respectively.

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 Coleoptera due to the modified Cry3Aa2 protein encoded by the modified *cry3Aa2* gene from the MIR604, and also to be tolerant to glyphosate, due to the mEPSPS protein encoded by the *mEPSPS* gene from the GA21. However, it is not generally considered that the insect damage by Coleopteran insects is the major cause making the difficult to grow, and the herbicide glyphosate is sprayed and exerts pressure for selection in the natural environment in Japan. Therefore, it is unlikely that these characteristics enhance the competitiveness of this recombinant maize and therefore this stack line maize would be more competitive than its parent lines.

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**

This stack line maize has both the modified Cry3Aa2 protein productivity derived from the MIR604 and the mEPSPS protein productivity derived from the GA21. The modified Cry3Aa2 protein possesses the insecticidal activity against the insects of order Coleoptera. However, the mEPSPS protein confers tolerance to glyphosate herbicide, though they are confirmed not to be harmful substances to animals and plants. In addition, it is considered unlikely that the modified Cry3Aa2 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.

Therefore, 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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