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 Coleoptera (Modified *cry3Aa2*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604, OECD UI: SYN-IR604-5)
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

Table 1 shows the composition and the origins of component elements of the donor nucleic acid used to develop the maize resistant to Coleoptera (modified *cry3Aa2*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604, OECD UI: SYN-IR604-5) (hereinafter referred to as "this recombinant maize").

Table 1 Sizes, origins and functions of component elements of plasmid pZM26

Component elements	Size (Kbp)	Origin and Function
Insect pest-resistant gene cassette		
MTL promoter	2.56	A promoter derived from *metallothionein* gene of maize. Corn Rootworm eats and damages the roots of maize and then, *MTL* promoter provides root-preferential expression in corn, defining the start of transcription of target genes in the roots (mRNA synthesis).
Modified *cry3Aa2* gene	1.80	A modification is given to *cry3Aa2* gene, which is derived from *Bacillus thuringiensis* subsp. *tenebrionis*, a typical gram-positive soil microorganism forming spores, to enhance its expression. This gene produces the modified Cry3Aa2 protein given a trait of insecticidal activity against insects of the order Coleoptera.
Nos terminator	0.25	The terminator region of nopaline synthase gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA. Terminates transcription of mRNA and induces polyadenylation.
Selectable marker gene cassette		
ZmUbiInt promoter	1.99	A promoter derived from *polyubiquitin* gene of maize, to define the start of transcription (mRNA synthesis) of target genes through the tissue of monocotyledonous plants.
pmi gene	1.18	A gene derived from *E. coli*, which encodes PMI protein (Phosphomannose isomerase) and catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate.
Nos terminator	0.25	The terminator region of nopaline synthase gene of *Agrobacterium tumefaciens*, which contains a transcription terminator and a signal for polyadenylation of mRNA. Terminates transcription of mRNA and induces polyadenylation.

2) Functions of component elements

(a) Functions of target genes, expression-regulating regions, localization signals, selectable markers and other component elements of donor nucleic acid

Functions of component elements of donor nucleic acid that was used for the development of this recombinant maize are shown in Table 1.

(b) Functions of proteins produced by the expression of target gene and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity (except any allergenicity as food)

Modified Cry3Aa2 protein:

The modified Cry3Aa2 protein produced by the modified *cry3Aa2* gene is only partly digested in the digestive tract of larvae of Corn Rootworm and other insects of the order Coleoptera when consumed by them, and a specific peptide (core toxin) remains undigested. This peptide specifically binds to the specific receptors in the intestinal tract and acts on the mucosal layer of digestive tract of larvae, which leads to the inhibition of digestion process and results in the insecticidal activity.

The Cry3A protein is known to exhibit little to no insecticidal activity against any insect other than the order Coleoptera (Reference 6). In addition, the Cry3Aa2 protein, one of the proteins belonging to the Cry3A family, is known to specifically show an insecticidal activity against Corn Rootworm, which is one of the major pest insects of order Coleoptera to maize cultivation in the US (Reference 7).

The modified *cry3Aa2* gene has some base sequences modified for its optimum expression in the recipient organism of maize and also for enhanced insecticidal efficiency against Western Corn Rootworm (*Diabrotica virgifera virgifera*) and Northern Corn Rootworm (*Diabrotica longicornis barberi*). In order to enhance the insecticidal activity of the modified Cry3Aa2 protein against target pest insects, some amino acid sequences are substituted by cathepsin G protease recognition sequence (chymotrypsin-like serine protease), though the other amino acid sequences remain unchanged from those in the Cry3Aa2 protein.

An investigation was made to identify the insecticidal activity of the modified Cry3Aa2 protein against different insect species, 6 species of the order Coleoptera (*Coleoptera*), 6 species of the order Lepidoptera (*Lepidoptera*), and one species of the order Diptera (*Diptera*). As a result of supplying the modified Cry3Aa2 protein in artificial feed to the first instar larvae of the individual species, the insecticidal activity was not identified against the order Lepidoptera and the order Diptera, though the insecticidal activity was identified against 4 of the 6 species of the order Coleoptera examined [Western Corn Rootworm (*Diabrotica virgifera virgifera*), Northern Corn Rootworm (*Diabrotica longicornis barberi*), Colorado Potato Beetle (*Leptinotarsa decemlineata*), and Banded Cucumber Beetle (*Diabrotica balteata*)] whereas

the insecticidal activity was not shown against Southern Corn Rootworm (*Diabrotica undecimpunctata*) and Cotton Boll Weevil (*Anthonomus grandis*) of the order Coleoptera.

As a result of an additional examination regarding possible effects of the modified Cry3Aa2 protein on non-target organisms such as bees, earthworms, fishes, birds and mammals in addition to pest insects of the order Coleoptera, no effect was observed.

Moreover, for human and other mammals, their living bodies are considered unsusceptible to the modified Cry3Aa2 protein based on the facts that they secrete strong acid gastric juice to digest the modified Cry3Aa2 protein and that they have no receptors for the peptide (core toxin) in the intestinal tract even if the peptide remains undigested.

For the information about allergenicity, an investigation was carried out with reference to the database (SWISS-PROT, FFARP, BLASTP, etc.) to check possible sequence homology between the modified Cry3Aa2 protein produced by the modified *cry3Aa2* gene and any known allergens and toxins. As a result, the modified Cry3Aa2 protein was found not to share sequence homology with any of the known allergenic proteins.

PMI protein:

The *pmi* gene is a gene derived from *E.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, the *pmi* gene was introduced into plant cells as a marker for the presence of the target gene. Upon subsequent incubation in the mannose-containing medium, it can be confirmed that the target gene and the *pmi* gene have both been introduced into the cells (Reference 8, Reference 10).

The PMI protein exists widely in nature and in fact, it is found present in soybean and other plants, though it has not been identified in maize.

The PMI protein produced by the *pmi* gene in plants was evaluated by the U.S. Environmental Protection Agency (EPA) as equivalent to the PMI protein produced by microorganisms and then, the PMI protein produced by the *pmi* gene is exempt from the scope of EPA regulations on residue limits even if it is produced from plants or microorganisms (May 14, 2005).

For information about allergenicity, an investigation was carried out with reference to the database (SWISS-PROT, FFARP, BLASTP, etc.) to check possible sequence homology between the PMI protein produced by the *pmi* gene and any known allergens and toxins. As a result, the PMI protein produced by the *pmi* gene was found not to share sequences homology with any of the known allergenic proteins.

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

Based on the fact that the modified Cry3Aa2 protein does not possess any enzyme activity and it works independently from the metabolic system of recipient organism, the modified Cry3Aa2 protein is unlikely to affect the metabolic pathway of recipient organism.

In addition, the PMI protein expressed by the *pmi* gene is a catalytic enzyme protein which catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate, it reacts specifically with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein (Reference 13).

(2) Information concerning vector

1) Name and origin

The vector used for the production of this recombinant maize is plasmid pZM26. This plasmid is produced from pUC19 derived from *Escherichia coli*.

2) Properties

The total number of base pairs of plasmid vector is 13,811bp.

The vector contains antibiotic resistant marker gene to select the microorganisms that contain transformed plasmid, which confer the resistances to streptomycin, erythromycin and spectinomycin. However, this gene is located outside the region of transferred gene and then not introduced into this recombinant maize.

(3) Method of preparing living modified organisms

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

Two (2) gene expression cassettes (insect pest-resistant gene cassette and selective marker gene cassette) were transferred into this recombinant maize.

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

Introduction of nucleic acid into recipient organism was conducted based on the *Agrobacterium* method.

3) Processes of rearing of living modified organisms

(a) Method of selecting the cell into which nucleic acid is transferred

After introduction of plasmid based on the *Agrobacterium* method and subsequent growth on a medium containing mannose as the only carbon source, only those individuals that express the PMI protein were selected.

(b) Presence or absence of any residual body cell of *Agrobacterium* when *Agrobacterium* method was used for transferring nucleic acid into the recipient organism

After introduction of genes, the antibiotic Cefotaxime was added to the culture cell medium to remove any residual *Agrobacterium*.

(c) Process of rearing and genealogical tree

After introduction of the plasmid based on the *Agrobacterium* method, the callus containing the *pmi* gene was selected. Then the recombinant plants were selected as the first generation of MIR604 (recombinant of the current generation) among those which were confirmed to express the modified Cry3Aa2 protein and which stably express the modified Cry3Aa2 protein even in the progeny, and they were crossed with an elite strain of dent type maize inbred which is medium maturing and cultivable over a wide range of areas.

In May 2006, an application for approval of the safety as food was submitted to the Ministry of Health, Labour and Welfare and an application for approval of the safety as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries.

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

Location of the copy of transferred nucleic acid

1) As a result of genetic segregation tests, it was confirmed that the transferred genes were stably inherited dominantly in accordance with the law of Mendelian inheritance. Consequently, the transferred nucleic acid is considered to exist on the chromosome.

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

As a result of Southern blotting analysis for existence of the transferred gene, it was confirmed that one copy of each of the modified *cry3Aa2* gene and the *pmi* gene exists and that the introduced genes are all inherited stably through at least three generations.

3) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

In 2002, field tests were carried out in Minnesota and Illinois in the US, to study the Corn Rootworm (CRW) resistance property. At the six-leaf stage of maize when CRW is most likely to appear, the first instar larvae of Western Corn Rootworm (*Diabrotica virgifera virgifera*) were released to the roots of maize to check the degree of damage to the maize roots. As a result, little damage to the roots of the recombinant plants was found, while the roots of the control plants were seriously damaged. Based on the findings, it was confirmed that the introduced target gene has conferred the resistance to the insects of the order

Coleoptera.

Regarding the stability of expression of the *pmi* gene, in the process of selection of the recombinant plants in which only the PMI protein expressing cells are selected through growth on the medium containing mannose as the only carbon source, stable expression of the PMI protein was confirmed.

(5) Difference from the recipient organism or the taxonomic species to which the recipient organism belong**s**

1) Details of physiological or ecological properties conferred as a result of the expression of copies of the introduced nucleic acid.

The recipient organism is given the following new properties: resistance to the insects of the order Coleoptera including Corn Rootworm (CRW), which is conferred by the modified Cry3Aa2 protein produced by expression of the modified *cry3Aa2* gene (Figure 1); and the PMI protein produced by expression of the *pmi* gene.

This recombinant maize	Non-recombinant control maize

Figure 1 Comparison of the degree of root damage caused by insects of the order Coleoptera to this recombinant maize and the non-recombinant control maize

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

For this recombinant maize, commercial varieties produced by crossing with dent type maize have been developed for use as feed or food processing. In 2005, at the National Institute of Livestock and Grassland Science (NILGS) of National Agriculture and Food Research Organization (NARO), an isolated field test was conducted using this recombinant maize and the non-recombinant control maize.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made regarding the uniformity of germination, germination rate, time of tassel exertion, time of silking, culm length, plant type, tiller number, height of ear, 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 above ground part at the time of harvesting between this recombinant maize and the non-recombinant control maize. As a result, in all characteristics evaluated, no difference was observed between this recombinant maize and the non-recombinant control maize.

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

For this recombinant maize and the non-recombinant control maize, cold-tolerance at the early stage of growth was evaluated. The evaluation was conducted for the first to the second leaf stage seedlings at a low temperature (5℃) representing the winter season. As a result, under the low-temperatures condition, this recombinant maize and the non-recombinant control maize exhibited interrupted growth. Based on the findings, it was judged that there is no difference in cold-tolerance between this recombinant maize and the non-recombinant control maize.

In addition, the possibility that seeds spilled into the fields during harvesting could germinate and survive over the winter was investigated. The seeds used in the field tests were raised to the third leaf stage and then transferred to the open field condition (nighttime minimum temperature of -5°C). As a result, all of this recombinant maize and the non-recombinant control maize died completely.

Based on the above results, regarding the cold-tolerance, no difference was observed between this recombinant maize and the non-recombinant control maize.

(c) Wintering ability of the matured plant

Maize is a summer type annual plant, and after grain maturity it usually dies out. In fact, there is no report that, after maturity, maize has further propagated by vegetative parts or set seeds again and produced seeds.

(d) Fertility and size of the pollen

To examine the fertility (viability) and size of the pollens, pollen grains were stained with Acetocarmine solution and observed under a microscope. As a result, regarding the fertility and size of the pollen grains, it was considered that there is no difference between this recombinant maize and the non-recombinant control maize.

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

Regarding the production of the seed, comparison was conducted for kernel yield. As a result, regarding the number of productive ears, row number per ear, grain number per row, and 100-kernel weight, no difference was observed between this recombinant maize and the non-recombinant control maize. Consequently, also regarding the production of seeds, it was judged that there is no difference between this recombinant maize and the non-recombinant control maize.

Regarding the shedding habit, the ears of both this recombinant maize and the non-recombinant control maize were covered with bracts at the time of harvesting and thus, it was judged that the difference of shedding habit between them was not observed under the natural condition.

Regarding the dormancy, the seeds of both this recombinant maize and the non-recombinant control maize harvested in the field tests exhibited higher germination rate and no difference was observed between them and thus, it was judged that dormancy of the seeds is extremely low.

Regarding the germination rate of harvested seeds, no difference was observed between this recombinant maize and the non-recombinant maize and thus, it was judged that there is no difference in germination rate of seeds between them.

(f) Crossability

Crossability tests were not performed for this recombinant maize, since no wild relatives that can be crossed grow in Japan.

(g) Productivity of harmful substances

Regarding the productivity of harmful substances under the natural environment in Japan, possible effects of plowing-in, effects on succeeding cropping and effects on soil microorganisms were examined.

As the plow-in test, dried powder of leaves and stems at the time of harvesting was mixed with Andosol (kuroboku soil) and packed in 1/5000a Wagner pots, to which the seeds of radish were sown per pot to examine the germination rate. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize. In addition, as a result of measurement of plant height, fresh weight and dry weight of radishes after germination, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

As the succeeding crop test, soil was collected from individual experimental plots and mixed with each other then packed in 1/5000a Wagner pots, to which the seeds of radish were sown per pot to examine the germination rate. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize. In addition, as a result of measurement of plant height, fresh weight and dry weight of radishes after

germination, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

At the time of sowing and harvesting of this recombinant maize and the non-recombinant control maize, soil was sampled from isolated fields to measure the number of colonies of filamentous fungi, bacteria and actinomyces for the microorganisms in soil based on the dilution plate technique. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

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 applied 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.

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

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis), to which the recipient organism belongs, has been used, including for cultivation, etc., in Japan, though there is no report that it has become volunteerin Japan.

This recombinant maize is given a trait to be resistant to insects of the order Coleoptera by a transferred modified *cry3Aa2*. However, it is considered that the insect damage by Coleoptera is not the major factor restricting maize growth under the natural environment in Japan.

In addition, the transferred *pmi* gene expresses the PMI protein which uses mannose as a carbon source, though it is considered that this trait does not increase the competitiveness.

Moreover, as a result of examination of the characteristics relating to the competitiveness of this recombinant maize in the isolated field in Japan, no significant difference from the non-recombinant control maize was observed.

Based on the above understanding, no wild animals and plants can be identified as having some effects attributable to competitiveness and thus, 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

1) Specification of any wild animals and plants which may be subjected to the effects attributable to the productivity of harmful substances

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

This recombinant maize produces phosphomannose isomerase (PMI protein) which catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate due to the transferred *pmi* gene, though the reaction is specific and there is no other natural substrate known with which the PMI protein reacts.

Consequently, it is not considered that the PMI protein could affect the metabolic pathway of recipient organism and produce any harmful substances.

In addition, as a result of examination on the production of harmful substances of this recombinant maize (the effects of the secretion from roots on other plants, the effects of the secretion from roots on the microorganisms in soil, and the effects of the possession in the plant body on other plants after dying) in the isolated field, no significant difference from the non-recombinant maize was observed. On the other hand, since this recombinant maize produces the modified Cry3Aa2 protein that possesses the insecticidal activity against a limited number of insects of the order Coleoptera, the insects of the order Coleoptera living in Japan are specified as possibly affected wild animals/plants.

2) Evaluation of the specific contents of the effects

Western Corn Rootworm that exhibited the highest sensitivity to the modified Cry3Aa2 protein was provided an artificial agar-based diet containing the modified Cry3Aa2 protein at the concentration of 1.4μg/mL for 144 hours. As a result, about a half of the individuals examined became dead.

3) Evaluation of susceptibility to the effects

Possible route of exposure of the modified Cry3Aa2 protein to larvae of insects of the order Coleoptera other than insect pests for farming includes:

a. Direct ingestion of this recombinant maize,
b. Ingestion of plant body plowed back in soil or protein eluted from it together with plant body plowed back in soil, and/or
c. Ingestion of pollens dispersed from this recombinant maize together with feed plants.

However, the main habitat of the species of insects of the order Coleoptera other than those that should be controlled is not in the cultivation field of maize or the surroundings. Therefore, it was considered very unlikely that the insects of the order Coleoptera could be affected at the level of individual population through the routes "a" and "b" listed above.

The expression level of the modified Cry3Aa2 protein in the pollens of this recombinant maize is less than the limit (0.01 μg/g) of detection by the ELISA method used for the examination. Based on the comparison with the lethal concentration for Western Corn Rootworm which shows the highest sensitivity to the Cry3Aa2 protein as described in 2) above, it is considered unlikely that the pollens of this recombinant maize are accumulated under the natural condition to such an extent that they can affect the insects of the order Coleoptera and thus, it was considered extremely low that the insects of the order Coleoptera are subjected to the effects through the route "c".

Based on the above understanding, the modified Cry3Aa2 protein possessed by this recombinant maize is considered not to be detrimental to maintenance of these Coleopteran species or groups of individuals living in Japan.

In addition, as a result of examination of the number of individuals of arthropod collected into pitfall traps in the isolated fields in Japan, no difference was observed between this recombinant maize and the non-recombinant control maize.

4) Judgment of existence of Adverse Effect on Biological Diversity

From the above discussion, it was judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is valid.

(3) Crossability

In Japan, the growth of wild relatives (teosinte) that can be crossed with maize in natural environment has not been reported. Based on the above understanding, no wild species that can be affected by this recombinant maize is specified, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability is valid.

2. Conclusion based on the Biological Diversity Risk Assessment Reports

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 is valid.

Annex List

Annex 1: A List of Members of Control Board for Adverse Effect on Biological Diversity

Annex 2: Procedures for Forming the Control Board for Adverse Effect on Biological Diversity

Annex 3: Safety Assessment of Maize resistant to Coleoptera (Modified *cry3Aa2*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604)

Annex 4-1: Map of isolated field and the surroundings

Annex 4-2: Layout of isolated fields

Annex 4-3: Layout of isolated field test plots

Annex 5: A Summary of Safety Assessment Tests in the US on Maize resistant to Coleoptera (Modified *cry3Aa2*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604)

Annex 5: Attachment: Laboratory Soil Degradation of Modified Cry3A protein (MCRY3A-0102)

Annex 6: Identity of genes transferred to maize resistant to Coleoptera MIR604 (Modified *cry3Aa2*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR604, OECD UI: SYN-IR604-5) used in the tests