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	Thermostable α-amylase producing maize (Modified *amy797E*, *Zea mays* subsp. *mays* (L.) Iltis) (3272, OECD UI: SYN-E3272-5)
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 thermostable α-amylase producing maize (modified *amy797E*, *Zea mays* subsp. *mays* (L.) Iltis) (3272, OECD UI: SYN-E3272-5) (hereinafter referred to as "this recombinant maize") are shown in Table 1.

Table 1 Sizes, origins and functions of individual component elements of pNOV7013 used for the production of this recombinant maize

Component elements	Size (bp)	Origin and function
Modified *amy797E* gene expression cassette		
GZein promoter	677	Endosperm-specific promoter sequence derived from the 27 kDa storage protein (γ-zein) gene of Zea mays (Reference 13); used to express target genes specifically in the endosperm tissue of maize seeds.
Modified *amy797E* gene	1,383	*α-amylase* gene derived from the hyperthermophilic microorganisms of the archaeal order *Thermococcale* (Reference 14), encoding thermostable α-amylase protein (hereinafter referred to as the "modified AMY797E α-amylase"). The modified *amy797E* gene has the nucleotide sequences replaced by the codons suited for expression in maize (Reference 15). In addition, the modified *amy797E* gene has the maize γ-zein signal peptide added to the N-terminus. This sequence is intended to transport the target proteins into the lumen of endoplasmic reticulum (Reference 16). On the other hand, this gene has the endoplasmic reticulum retention signal added to the C-terminus (Reference 17). These additional sequences are considered to retain the encoded α-amylase in the endoplasmic reticulum of endosperm cells.

PEPC9 intron#9	108	An intron #9 sequence derived from the phosphoenolpyruvate carboxylase gene from Zea mays (Reference 18); used to enhance the expression of target genes.
35S terminator	70	Polyadenylation sequence derived from the cauliflower mosaic virus 35S RNA (Reference 19).
pmi gene expression cassette		
ZmUbiInt promoter	1,993	A promoter containing the first intron region derived from the polyubiquitin gene of Zea mays, providing constitutive expression of target genes in the entire tissue of monocotyledon (Reference 20).
pmi gene	1,176	*manA* gene derived from *Escherichia coli*, encoding phosphomannose isomerase (hereinafter referred to as the "PMI protein") (Reference 21); used as a selective marker for transgenic plants for which genes are transferred (Reference 22).
NOS terminator	253	Polyadenylation sequence of the nopaline synthase gene of *Agrobacterium tumefaciens* (Reference 23).
Other regions		
LB	25	T-DNA left border sequence derived from the *Agrobacterium tumefaciens* (LB), required for integration of T-DNA region into the genome of plants (Reference 24).
spec	789	*aadA* gene encoding the streptomycin adenylyltransferase, derived from *Escherichia coli*, a bacterial selective marker gene to confer the resistance to erythromycin, streptomycin and spectinomycin (Reference 25).
VS1ori	405	The replication origin consensus sequence derived from the *Pseudomonas* bacteria. The replication-starting region in the *Agrobacterium tumefaciens* (Reference 26).
ColE1ori	807	The replication origin region of plasmid in *Escherichia coli*, derived from *Escherichia coli* (Reference 27).
virG	726	VirGN54D derived from the *Agrobacterium tumefaciens*, a required gene for efficient transformation of plants based on the *Agrobacterium* method (Reference 28).
repA	1,074	Replicon (minimum functional replication unit controlling DNA replication) region derived from the *Pseudomonas* bacteria; a gene required for retention of vectors in *Agrobacterium* (Reference 29).
RB	25	T-DNA right border sequence derived from the *Agrobacterium tumefaciens* (RB), required for integration of T-DNA region into the genome of plants (Reference 30).

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 component elements of the donor nucleic acid used for the production of this recombinant maize are shown in Table 1.

(b) Functions of proteins produced by the expression of target genes and selective 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)

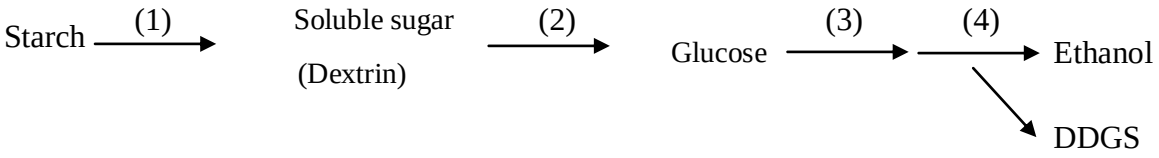
Modified AMY797E α-amylase

The modified AMY797E α-amylase expressed by the modified *amy797E* gene is an enzyme classified as α-amylase (EC 3.2.1.1). The α-amylase is an enzyme that catalyzes the hydrolysis of starch by randomly cleaving the internal 1,4-α-glucosidic bonds of starch components amylose and amylopectin into dextrins, maltose and glucose (Reference 31). The α-amylase is inherently distributed widely in the nature, including the body fluids of animals (salivary, pancreatic juice, blood, urine, etc.), plants and microorganisms. It is also contained in the seeds of maize and it is known to rapidly increase the activity at the time of germination (Reference 32). At the time of germination, the α-amylase breaks down the starch in the endosperm for use for the subsequent growth of embryo.

The modified *amy797E* gene uses the *GZein* promoter. In addition, the modified *amy797E* gene contains the γ-zein signal sequence derived from *Zea mays* added to the N-terminus, and the endoplasmic reticulum retention signal sequence added to the C-terminus (Reference 33) and then, it is considered that the modified AMY797E α-amylase expressed by the modified *amy797E* gene would be retained in the endoplasmic reticulum of endosperm in maize (Reference 16; Reference 17). This modification causing the endoplasmic

reticulum retention was selected with the intent to prevent the modified AMY797E α-amylase expressed by the transferred genes from contact with the starch serving as substrate, since the starch contained inherently in the seeds of maize is retained in the plastid in the form of starch grains. Therefore, the modified AMY797E α-amylase expressed in this recombinant maize is present in different sites in the cells from those where the substrate starch is present and then, it is considered that starch hydrolysis does not take place due to the modified AMY797E α-amylase unless the cells are disrupted.

This recombinant maize is primarily used for efficient production of ethanol from maize, though it can also find use as food and feed similarly as ordinary maize. In addition, the Distiller's Dried Grains Solubles (DDGS), the by-product of ethanol production from maize, is used as feed similarly as the ordinary maize. Conventionally, in the ethanol production from dry ground maize kernels, water is added to maize powder and is heated to dissolve the starch, which is then liquefied by addition of the microbially produced thermostable α-amylase (Figure 1). This recombinant maize expresses the highly thermostable modified AMY797E α-amylase derived from the hyperthermophilic microorganisms of the archael order *Thermococcales* specifically in the seeds of maize and thus, addition of the seeds of this recombinant maize to the seeds of conventional maize during the starch liquefaction process of ethanol production would contribute to simplify the production processes and reduce costs. In fact, as a result of investigation of the temperature dependency of the modified AMY797E α-amylase activity, it was found that the modified AMY797E α-amylase exhibits higher activity at higher temperatures while the activity is substantially suppressed at ordinary temperatures. The activity of α-amylase derived from maize is reported to be higher at 40°C and 50°C, though it becomes suppressed at 60°C (Reference 34). In addition, as a result of toxicity tests (13-weeks subacute toxicity test, acute oral toxicity test, mutagenicity test, etc.), it is confirmed that the α-amylase derived from *Thermococcales* causes no problems in terms of safety (Reference 35).



(1) Liquefaction: α-amylase

(2) Saccharization: Glucoamylase

(3) Fermentation: Yeast

(4) Distillation

Figure 1 Ethanol production using dry ground maize kernels (starch)

Regarding the allergenicity of the modified AMY797E α-amylase, possible amino acid sequence homology with any known allergens was carried with reference to the database (SWISS-PROT, FARRP, BLASTP, etc.). As a result, in the modified AMY797E α-amylase and the American cockroach (*Periplaneta americana*), the homologous sequence composed of eight (8) amino acid residues with the specific known allergen (Per a 3 allergen) was observed. However, this sequence does not match the IgE binding epitope sequence of Per a 3 allergen (Reference 36) and then, it is estimated extremely low that the modified AMY797E α-amylase would become a similar allergen. It was confirmed that the modified AMY797E α-amylase does not share structurally related homologous sequences with any of the known allergens other than this.

PMI protein

The *pmi* gene, derived from *E. coli*, encodes the PMI protein (Phosphomannose isomerase), which has the capability of catalyzing the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate and then it was used as a selective marker for recombinant plants during the production of this recombinant maize (Reference 22). Generally, maize and other many plant cells cannot use mannose as a carbon source for their growth. However, the cells containing the transferred *pmi* gene and producing the PMI protein can convert mannose into fructose 6-phosphate for use for their growth. For this reason, by incubation on the tissue culture medium containing mannose as the

sole carbon source, transformed plants can be selected. The PMI protein also exists widely in nature and in fact, it is found present in soybean and other plants, though it has not been identified in maize.

Regarding the allergenicity of the PMI protein, it has been confirmed as a result of amino acid sequence homology with any known allergens using the database (SWISS-PROT, FARRP, BLASTP, etc.) that the PMI protein does not share structurally related homologous sequences with any of the known allergens investigated.

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

The modified AMY797E α-amylase expressed by the modified *amy797E* gene is an enzyme classified as α-amylase (EC 3.2.1.1). The α-amylase is an enzyme that catalyzes the hydrolysis of starch into dextrins, maltose and glucose, and it is inherently distributed widely in the nature, including the body fluids of animals (salivary, pancreatic juice, blood, urine, etc.), plants and microorganisms. As a result of investigation on whether the modified AMY797E α-amylase is dissolved in the artificial human gastric juice containing pepsin, one of the peptidases, it was found that the modified AMY797E α-amylase was rapidly dissolved.

It was considered very unlikely based on the findings listed below that the modified AMY797E α-amylase expressed in this recombinant maize affects the metabolism of the recipient organism of maize.

- It is considered that the modified AMY797E α-amylase would be retained locally in the endoplasmic reticulum of endosperm of maize kernels, though the substrate starch exists in the form of starch grains in the plastid in the maize kernels.
- The modified AMY797E α-amylase exhibits very low enzyme activity at ordinary temperatures.
- As a result of observation of this recombinant maize and the non-recombinant control maize at the temperatures 20 to 30°C considered optimum for the germination and the initial growth of maize in natural

environment, and lower (10°C) and higher (40°C) temperatures than the optimum, there was no significant difference in the germination and the initial growth between this recombinant maize and the non-recombinant control maize at all the temperatures examined.

- As a result of analysis of the components in kernels of this recombinant maize cultivated in the US fields in 2003 and 2004, the starch content was found similar to that in the non-recombinant control maize. In addition, also regarding the analytical results of the major components in kernels and stems and leaves other than starch content, there was no significant difference observed between this recombinant maize and the non-recombinant control maize and a significant difference, if observed, was found falling within the range of values referenced in the literature.

Based on the above understanding, it is considered very unlikely that the modified AMY797E α-amylase affects the metabolic pathway of maize of the recipient organism.

The PMI protein expressed by the *pmi* gene is a catalytic enzyme protein that catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. The PMI protein reacts specifically with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein (Reference 37). Consequently, it is considered very unlikely that the expression of the PMI protein affects the other metabolic pathways of maize of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The vector used for the production of this recombinant maize is the plasmid pNOV7013. This plasmid was constructed based on the plasmid derived from *E. coli*.

2) Properties

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

The total number of base pairs of the vector is 11,439 bp.

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

The vector contains the *spec* gene as a bacterial cell selective marker, which confers the resistances to streptomycin, erythromycin and spectinomycin. However, this gene is located outside the region of transferred gene and then not transferred into this recombinant maize.

(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

The vector contains no sequence showing infectivity.

(3) Method of preparing living modified organisms

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

Regarding the structure of the entire nucleic acid transferred in the recipient organism, the modified *amy797E* gene expression cassette and the *pmi* gene expression cassette which were located in the T-DNA region (region between RB and LB) of the vector.

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

The T-DNA region of the pNOV7013 (containing the modified *amy797E* gene which encodes the modified AMY797E α-amylase, and the *pmi* gene which encodes the PMI protein) was transferred to the immature embryo of maize based on the *Agrobacterium* method.

3) Processes of rearing of living modified organisms

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

In the immature embryo of maize, to which the T-DNA was transferred based on the *Agrobacterium* method, the cells to which the *pmi* gene was transferred can grow using mannose as a carbon source. Using the characteristics, the immature embryo was incubated on the medium containing mannose as the sole carbon source to select the PMI protein encoding cells.

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

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* used for the transformation.

(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

After transferring of the T-DNA based on the *Agrobacterium* method, the cells that express the PMI protein were selected and regenerated in plants. Then the plants to which the modified *amy797E* gene and the *pmi* gene were both transferred were conditioned and cultivated in a greenhouse. Among the cultivated plants, the individual which was confirmed to express the modified AMY797E α-amylase was selected as the first generation of recombinant (T0), and the progeny were crossed with elite strains of dent type maize, which were investigated for the respective traits under authorization from the United States Department of Agriculture (USDA).

This recombinant maize was approved in May 2005 by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment for Type I Use Regulation (Cultivation in isolated field, storage, transportation and 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 Organism". In addition, in 2007, an application for approval of the safety as food would be submitted to the Ministry of Health, Labour and Welfare and an application for approval of the safety as feed would be 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

1) Place where the replication product of transferred nucleic acid exists (on the chromosome, in the cell organelle, or in the protoplasm)

As a result of genetic segregation tests on the progeny, the transferred traits were inherited across multiple generations in accordance with the law of Mendelian inheritance. Consequently, the nucleic acid transferred to the cells is considered to exist on the chromosome and be inherited stably across multiple generations.

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

As a result of Southern blotting analysis for the number of copies of the transferred gene, it was confirmed that one copy of each of the modified *amy797E* gene and the *pmi* gene exists and that the transferred genes are all inherited stably through 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)

For determination of expression level of the modified AMY797E α-amylase in this recombinant maize, ELISA analysis was conducted on the samples collected from

this recombinant maize cultivated in the US fields at individual stages of growth. As a result, it was found that the modified AMY797E α-amylase was expressed in the maize kernels at stable levels. In addition, it was also confirmed by the results of isolated field tests conducted in Japan that the modified AMY797E α-amylase is expressed in the seeds of this recombinant maize. Based on the findings, it was confirmed that the modified AMY797E α-amylase is expressed stably across the individuals and generations.

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

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 does not contain any sequence allowing transmission. Therefore, it is considered unlikely that the nucleic acid transferred to this recombinant maize 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

Existence of target genes in this recombinant maize can be confirmed based on the results of Southern blotting analysis using the modified *amy797E* gene as a probe after cleaving the genome DNA by the restriction enzyme. In addition, for specific detection of this recombinant maize, a method has been developed based on the nucleotide sequences of the transferred genes and the both neighboring nucleotide sequences of genome.

For the modified AMY797E α-amylase produced by the expression of target genes, the specific quantitative analysis is available based on the ELISA method.

(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

With the expression of replication products of the transferred nucleic acid, the recipient organism is given the following new properties: productivity of the modified AMY797E α-amylase, the thermostable α-amylase, conferred by expression of the modified *amy797E* gene; and productivity of the PMI protein conferred by expression of the *pmi* gene.

Modified AMY797E α-amylase

The expressed modified AMY797E α-amylase is an enzyme classified as α-amylase (EC 3.2.1.1). The α-amylase is an enzyme that catalyzes the hydrolysis of starch into dextrins, maltose and glucose and it is inherently distributed widely in nature, including the body fluids of animals (salivary, pancreatic juice, blood, urine, etc.), plants and microorganisms. This recombinant maize is primarily used for efficient production of ethanol from maize, though it can also be used as food and feed similarly as ordinary maize. This recombinant maize expresses the highly thermostable modified AMY797E α-amylase in the seeds of maize and thus, it would contribute to simplify the starch liquefaction process in the ethanol production and reduce the production costs. In fact, as a result of investigation on the temperature dependency of the activity of the modified AMY797E α-amylase, it was found that the modified AMY797E α-amylase exhibits higher activity at higher temperatures while the activity is substantially suppressed at ordinary temperatures.

As a result of measurement of starch content in the kernels of this recombinant maize, the starch content was found equivalent as in the non-recombinant maize. In addition, also regarding the analytical results of major components in kernels and stems and leaves other than starch content, no significant difference was observed between this recombinant maize and the non-recombinant control maize, and a significant difference, if observed, was found falling within the range of values referenced in the literature. Furthermore, under the conditions at 10 to 40°C, the

seeds of this recombinant maize and the non-recombinant control maize were germinated and the initial growth was observed. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the germination and the initial growth at all the temperatures examined.

PMI protein

The PMI protein reversibly interconverts mannose 6-phosphate and fructose 6-phosphate. Generally, maize and other many plant cells cannot use mannose as a carbon source for their growth, though the cells containing the *pmi* gene transferred and producing the PMI protein can convert mannose into fructose 6-phosphate for use for their growth. For this reason, by incubation on the tissue culture medium containing mannose as a sole carbon source, transformed cells can be selected. The PMI protein also exists widely in nature and in fact, it is found present in soybean and other plants, though it has not been identified in maize.

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

At the National Institute of Livestock and Grassland Science (NILGS) of National Agriculture and Food Research Organization (NARO), an isolated field test in 2005 and a harmful substances productivity test using kernels (seeds) in 2006 were 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 tasseling, 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, fresh weight of above ground part at harvesting time between this

recombinant maize and the non-recombinant control maize. As a result, in all characteristics evaluated, no significant difference nor difference was observed between this recombinant maize and the non-recombinant control maize.

(b) Cold-tolerance and 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. As a result of evaluation on the growth under the low temperature conditions representing the winter season, this recombinant maize and the non-recombinant control maize both exhibited interrupted growth. In addition, as a result of transfer of seedlings to open-air conditions, all the seedlings of this recombinant maize and the non-recombinant control maize died. 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.

(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. In addition, it was observed in the isolated field tests that the maize plants died after maturation, and no plant body re-grew. It was also observed in the isolated field tests in the US that, after maturating, this recombinant maize and the non-recombinant control maize both died out.

(d) Fertility and size of the pollen

For this recombinant maize and the non-recombinant control maize, the shape, size and fertility of pollen were examined under a microscope to identify any difference between them. As a result of the observation with pollen stained with Acetocarmine solution, no difference was observed between this recombinant maize and the non-recombinant control maize. In addition, the pollens were all found with the plasma stained about 100% with Acetocarmine; therefore, also regarding the fertility, 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 the number of grains in ear and as a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize. In addition, also for the number of ears, number of productive ears, row number per ear, grain number per row and other items relating to the production of the seed, no significant difference was observed between the recombinant plant and the non-recombinant control plant.

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

Regarding the germination rate, no significant difference was observed between this recombinant maize and the non-recombinant control maize, since the germination rate was found equivalent for both the sowing seeds and harvested seeds from this recombinant maize and the non-recombinant control maize. Dormancy has not been examined, though the possibility is considered low that the dormancy of this recombinant maize 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 for this recombinant maize and the non-recombinant control maize.

(f) Crossability

Crossability test was not performed for this recombinant maize 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

Regarding the productivity of harmful substances under the natural environment in Japan, a plow-in test, succeeding crop test and soil microflora test were carried out.

In the plow-in test, the dried powder of leaves and stems was mixed with soil, to which the seeds of radish were sown 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 fresh weight and dry weight of radishes after germination, no significant difference was observed between this recombinant maize and the non-recombinant control maize. Furthermore, the powder of kernels (seeds) of this recombinant maize and the non-recombinant control maize was mixed with soil, to which the seeds of radish were sown to measure the germination rate, fresh weight and dry weight of radishes. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

In the succeeding crop test, soil was collected from the root zones of maize in individual experimental plots, to which the seeds of radish were sown 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 fresh weight and dry weight of radishes after germination, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

In the soil microflora test, soil was sampled from isolated fields to measure the number of colonies of the microorganisms in soil (filamentous fungi, bacteria and actinomyces) based on the dilution plate technique. As a result, no significant difference was observed in the number of colonies of filamentous fungi, bacteria and actionmyces 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 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

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

(1) Competitiveness

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

As a result of examination of the morphological and growth characteristics of this recombinant maize in the isolated fields in Japan, no significant difference nor difference observed from the non-recombinant control maize.

This recombinant maize is given the capability of producing the modified AMY797E α-amylase protein, which exhibits high activity at higher temperatures while lower activity at ordinary temperatures, due to the transferred modified *amy797E* gene. The α-amylase protein is an enzyme related to germination and then, examination was conducted regarding the germination and the initial growth in the temperature conditions from 10 to 40°C. As a result, at all the temperatures examined, no significant difference was observed between this recombinant plant and the non-recombinant control plant regarding the germination and the initial growth.

The α-amylase protein is an enzyme that catalyzes the hydrolysis of starch to dextrins, maltose and glucose, though the content of starch in the grains of this recombinant maize is similar as in the non-recombinant control maize, and also regarding the results of analysis on the major components in the grains and stems and leaves other

than starch content, no significant difference was observed between this recombinant maize and the non-recombinant control maize, otherwise a significant difference, if observed, was found falling within the range of values referenced in the literature.

Based on the above understanding, it is considered unlikely that the given trait of productivity of the modified AMY797E α-amylase protein causes this recombinant maize to become competitive in the natural environment in Japan.

In addition, due to the transferred *pmi* gene, the PMI protein is expressed and then, mannose can be a carbon source, though it is considered unlikely that this trait enhances the competitiveness of this recombinant maize.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant maize poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable

(2) Productivity of harmful substances

Regarding 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 is given traits to produce the modified AMY797E α-amylase protein and the PMI protein. The α-amylase protein is found present extensively in the natural world, though there is no report that it is harmful for living organisms. In addition, the PMI protein possesses substrate specificity and there is no other natural substrate known. Therefore, it is considered unlikely that the PMI protein would affect the other metabolic pathway of recipient organism and produce any harmful substances.

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), no significant difference

from the non-recombinant control maize was observed.

As a result of the investigation on whether the modified AMY797E α-amylase protein and the PMI protein share functionally important amino acid sequences with known allergens, it was confirmed that they do not share structurally related homologous sequences with any of the known allergens examined.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant maize poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(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