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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Modified *cry1Ab, bar, Zea mays* subsp. *mays* (L.) Iltis) (Event176, OECD UI：SYN-EV176-9)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

(i) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements that were used for the production of maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Modified *cry1Ab*, *bar*, *Zea mays* subsp. *mays* (L.) Iltis) (Event176) (hereinafter referred to as "this recombinant maize") are shown in Table 1.

(ii) 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 donor nucleic acid are shown in Table 1.

Table 1 Component elements of pCIB4431 and pCIB3064 and their sizes, origins and functions

pCIB4431 : Component elements	**Size (kb)**	**Origin and function**
Pollen-specific CDPK promoter	1.48	Derived from maize calcium-dependent protein kinase (CDPK) gene, making the target gene (Modified *cry1Ab*) expressed specifically by pollens of maize.
Modified *cry1Ab* gene	1.95	A gene derived from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 strain gene, and encodes modified Cry1Ab protein. It has some nucleotide sequences modified (increasing GC content and optimizing codon) and some amino acid sequences deleted, though the amino acid sequence in the core protein showing the insecticidal activity of Cry1Ab protein remains unchanged.
PEPC intron #9	0.11	Derived from maize phosphoenolpyruvate carboxylase gene, enhancing the expression level of the target gene (Modified *cry1Ab*) in plant body.
CaMV 35S terminator	0.07	Derived from cauliflower mosaic virus (CaMV) CM1841 strain, providing a polyadenylation site. This sequence terminates the transcription of the target gene (Modified *cry1Ab*).
amp^R(*bla*) gene	0.86	Derived from *Escherichia coli* gene, and it has the function to encode β-lactamase and confer the tolerance to antibiotic ampicillin. This gene is used as a selective marker.
ColE1 ori	0.81	The replication origin of *Escherichia coli* plasmid.
PEPC promoter	2.32	Derived from maize phosphoenolpyruvate carboxylase gene, making the target gene (Modified *cry1Ab*) expressed in the chlorenchyma.
pCIB3064 : Component elements	**Size(kb)**	**Origin and function**
CaMV 35S promoter	0.52	Derived from cauliflower mosaic virus (CaMV), making the target gene (***bar***) expressed in the entire plant.
bar gene	0.55	Derived from genes of the soil bacterium *Streptomyces hygroscopicus*, encoding the enzyme phosphinothricin acetyltransferase (PAT protein).
CaMV 35S terminator	0.07	Derived from cauliflower mosaic virus (CaMV) CM1841 strain, providing a polyadenylation site. This sequence terminates the transcription of the target gene (*bar*).
amp^R(*bla*) gene	0.86	Derived from *Escherichia coli* gene, and it has the function to encode β-lactamase and confer the tolerance to antibiotic ampicillin. This gene is used as a selective marker.
ColE1 ori	0.81	The replication origin of *Escherichia coli* plasmid.

(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

Modified Cry1Ab protein:

The insecticidal protein (=Bt protein), isolated from the soil microorganism *Bacillus thuringiensis*, exhibits its insecticidal activity against limited species of insects. It is known that the Bt protein, when fed and digested by sensitive species of insects, becomes an active polypeptide (= core protein) through specific digestion of protein, which specifically binds to the specific receptors on the surface of midgut of insects, causing cytoclasis or cell-destruction and leading to destructed digestive tracts and death of the insects. This mechanism of action also holds for the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*. Regarding the insecticidal activity of Cry1Ab protein, detail investigational results are listed in the database operated by the Canadian Government (Reference 7), showing that it exhibits its insecticidal activity against European corn borer (*Ostrinia nubilalis*), Corn earworm (*Helicoverpa zea*), Fall armyworm (*Spodeptera frugiperda*) and other order Lepidopteran insects which are the major pest insects for cultivation of maize. On the other hand, Cry1Ab protein exhibits no or little insecticidal activity against any insects other than the order Lepidoptera. The Cry1Ab protein is known to be very sensitive to the digestion by protease that exists in the digestive systems of mammals. Thus, the protein, even if ingested by humans and other mammals, will be digested together with the core protein, and it is unlikely to affect humans and other mammals that do not have any receptors for the core protein (Reference 21).

The modified *cry1Ab* gene, used for the production of this recombinant maize, has some nucleotide sequences modified and some amino acid sequences deleted, though the amino acid sequence in the core protein showing the insecticidal activity of Cry1Ab protein remains unchanged (Annex 9).

Biological pesticides to which the protein produced by *Bacillus thuringiensis* has been applied as an active ingredient have been used since 1961 in the US and European countries for maize, cotton, apple, cabbage, tomato, avocado and other crops, stored grains and forests to control pest insects. Also in Japan, since the early 1980s, biological pesticides, which contain the Cry1Ab protein produced by the *Bacillus thuringiensis* subsp. *kurstaki* HD-1 strain as an active ingredient, have been used for vegetables and fruit trees as an insecticide to control the order Lepidoptera.

In order to investigate whether the modified Cry1Ab protein shares functionally important amino acid sequences with known allergens, the modified Cry1Ab protein was compared with allergens in the database (GenBank, EMBL, etc.). The results showed the modified Cry1Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

PAT protein:

The *bar* gene, used for the production of this recombinant maize, has some nucleotide sequences modified to enhance its expression in plants, though the amino acid sequence in the PAT protein expressed by this gene remains unchanged. The glufosinate herbicide inhibits glutamine synthase in plants and then it causes plants to die due to the accumulated ammonia in the cells. However, the expression of PAT protein acetylates and inactivates the glufosinate, which relieves the glutamine synthase from inhibition. Consequently, the plants, which express the PAT protein, exhibit the tolerance to glufosinate herbicide and thus they have been used as a selection marker for recombinant plants.

In order to investigate whether the PAT protein shares functionally important amino acid sequences with known allergens, the PAT protein was compared with allergens in the database (GenBank, EMBL, etc.). The results showed the PAT protein did not share structurally related homologous sequences with any of the known allergens examined.

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

Based on the fact that modified Cry1Ab protein does not possess any enzyme activity and it works independently from the metabolic system of recipient organism, modified Cry1Ab protein is unlikely to affect the metabolic pathway of recipient organism. In addition, PAT protein possesses very high substrate specificity, so it is unlikely to transfer the acetyl group to any compounds other than glufosinate; therefore, it is not considered that PAT protein can affect the metabolic system of recipient organism (Reference 9).

(2) Information concerning vectors

(i) Name and origin

The vectors used for the production of this recombinant maize are pCIB4431 and pCIB3064. These vectors were produced from pUC19 derived from *Escherichia coli*.

(ii) Properties

The total number of base pairs of pCIB4431 and pCIB3064 is 11,027bp and 3,980bp respectively.

This recombinant maize contains the ampicillin-resistant gene (amp^R), which encodes β-lactamase in *E. coli* and confers the ampicillin resistance. The β-lactamase is a hydrolytic enzyme that inactivates antibiotics by cleaving the β-lactam ring in the antibiotic ampicillin. However, the amp^R gene is derived from plasmid pUC19 and regulated by bacterial promoter; therefore, it is considered not to be recognized by plant RNA polymerase. The amp^R gene acts as a selective marker in the amplification of plasmid in *E. coli*, and it is not expressed in plants

since it does not contain any promoter that functions in plants.

In addition, this recombinant maize also contains the ColE1 ori, the replication origin region of *E. coli* plasmid, though the range of recipient organisms for the autonomous replication is limited to *E. coli* and some gram-negative bacteria.

(3) Method of preparing living modified organisms

(i) Structure of the entire nucleic acid transferred in the recipient organism

In the recipient organism, pCIB4431 and pCIB3064, which contain insect pest-resistant gene cassette (Pollen specific CDPK promoter/modified *cry1Ab*/PEPC intron/CaMV35S terminater and PEPC promoter/modified *cry1Ab*/PEPC intron/CaMV35S terminater) and herbicide-tolerant gene cassette (CaMV*35S* promoter/*bar*/CaMV35S terminater), are transferred.

(ii) Method of transferring nucleic acid transferred in the recipient organism

The pCIB4431 and pCIB3064 were transferred to immature embryo of dent type maize at the same time by the particle gun bombardment.

(iii) Processes of rearing of living modified organisms

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

Using the fact that the *bar* gene confers the glufosinate tolerant characteristics, regenerated individuals were obtained by selecting callus in media containing glufosinate.

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

This does not apply since *Agrobacterium* method has not been used for transferring nucleic acid.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line to which confirmed the state of existence of replication products of transferred nucleic acid, 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

The regenerated individuals that exhibit glufosinate herbicide tolerance were subjected to the immunoassay (ELISA) specific to the modified Cry1Ab protein and the bioassay for the resistance to European corn borer, and the individuals that express the traits stably were selected as the parent plant for this recombinant maize. In addition, the individuals that possess the traits of glufosinate herbicide tolerance and Lepidoptera resistance in the homozygous form were selected based on the observation of segregation ratio in the posterity by self-pollination to fixate the traits. For this recombinant maize, commercial varieties produced by crossing with dent type have been raised for

use as feed or food processing.

The permits and approvals regarding this recombinant maize obtained from the related regulatory agencies in Japan are listed below.

Environmental safety

The environmental safety in accordance with the "Guideline for the use of recombinant in agriculture, forestry and fisheries" was approved.

May, 1996: Isolated field test was conducted at the National Institute for Agro-Environmental Sciences (for the purpose of importing).

October, 1996: The environmental safety for use as food processing and feed (8th Agriculture, Forestry and Fisheries Research Council Notice No. 1897) was approved.

April, 2002: The environmental safety for the program for use of recombinant plants (environment use simulation) was approved.

May, 2002: Isolated field test was conducted at the National Institute for Agro-Environmental Sciences (for the purpose of cultivation).

Safety as feed

The safety for use as feed in accordance with the “6-(2) of the Guideline for the safety evaluation of feed derived from recombinant-DNA plants (8th Livestock Industry Department B Notice No. 585 dated April 19, 1996)” was approved.

September, 1996: Safety for use as feed (8th Livestock Industry Department B Notice No. 1365) was approved.

The safety for use as feed in accordance with the "Safety Evaluation Criteria for Feed and Additives Produced by Recombinant-DNA Techniques" was approved.

March, 2003: The safety for use as feed (disclosed March 27, 2003) was approved.

Safety as food

The safety for use as food in accordance with the "Guideline for the safety evaluation of food and additives derived from recombinant-DNA techniques" was approved.

September, 1996: The conformity with the "Guideline for the safety evaluation of food and additives derived from recombinant-DNA techniques (Enforcement of

Ordinance for Food Sanitation Law No. 229)" was approved.

The safety for use as food in accordance with the "Standards and regulations on food and additives derived from recombinant-DNA techniques" was approved.

March, 2001: The conformity with the "Guideline for the safety evaluation of food and additives derived from recombinant-DNA techniques (Ministry of Health, Labour and Welfare Notice No. 108)" was approvred.

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

(i) Location of the copy of transferred nucleic acid

The nucleic acid transferred to the cells is stably inherited in posterity in agreement with the Mendel's laws and thus, it is considered to exist on the chromosome.

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

As a result of the Southern blotting analysis for existence of the transferred genes, it was found that approx. four (4) copies of each of modified *cry1Ab* gene and *bar* gene exist. In addition, it was also confirmed that the modified *cry1Ab* gene and the *bar* gene are both inherited stably across multiple generations.

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

As a result of the ELISA analysis for determination of expression level of modified Cry1Ab protein in this recombinant maize, it was confirmed that the modified Cry1Ab protein is expressed in leaves and pollens of plant in the flowering period at stable levels across the multiple generations. It was also demonstrated by the results of field tests conducted in the US that the resistance to European corn borer is expressed stably across the individuals and generations.

Also for the PAT protein, the stable expression across the individuals and generations has been confirmed based on the ELISA analysis.

(5) Methods of detection and identification of living modified organisms

For the method of detection and identification of this recombinant maize, the analytical methods have been published by the Ministry of Health, Labour and Welfare and the Food and Agricultural Materials Inspection Center (FAMIC) respectively in accordance with the Food Sanitation Law and the JAS (Japanese Agricultural Standards) Law.

(6) Difference from the recipient organism or the species to which the recipient organism belongs

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

With the modified Cry1Ab protein encoded by the modified *cry1Ab* gene and the PAT protein encoded by the *bar* gene, this recombinant maize shows the resistance to European corn borer (*Ostrinia nubilalis*) and other insects of the order Lepidoptera and the tolerance to glufosinate herbicide.

(ii) 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

For this recombinant maize, commercial varieties produced by crossing with dent type maize have been raised for use as feed or food processing. In 2002, isolated field tests were conducted at the National Institute for Agro-Environmental Sciences for the two (2) lines of this recombinant maize and the two (2) non-recombinant control lines which have the same genetic backgrounds as the two lines of this recombinant maize respectively. In addition, in 2006, in the special screened house at the National Institute of Livestock and Grassland Science, National Agriculture and Bio-oriented Research Organization, the test on the productivity of harmful substances (plow-in test) was carried out using the same lines.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was examined between this recombinant maize and the non-recombinant control maize regarding the uniformity of germination, time of tasseling, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, tiller number, total number of ears, number of productive ears, ear grain color, ear grain shape, culm length and height of ear, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, and fresh weight after harvesting. As a result, for one of the two lines of this recombinant maize examined, a significant difference from its control plant was observed in grain number per row, but in the other characteristics examined, no difference was observed between this recombinant maize and the non-recombinant control maize.

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

After germination, the seedlings were raised in a vinyl house and then, a cold snap visited temporarily and the inside of vinyl house dropped down to 0℃. In this condition, spots appeared in streaks, showing a possible cold-temperature injury. Then, the seedlings were put in a growth cabinet simulating the winter season (between 12 and 14℃ with a 12-hour lighting under sunshine lamp and 2℃ with a 12-hour darkness) to examine the

sensitivity to cold temperatures. In all the seedlings of this recombinant maize and the non-recombinant control maize examined, white spots in streaks, wilting and other signs of cold-temperature injury were observed. Regarding the progress of wilting, no difference was observed between this recombinant maize and the non-recombinant control maize.

Based on the above results, it was judged that, regarding the cold-tolerance at the early stage of growth, there is no difference between this recombinant maize and the non-recombinant control maize.

(c) Wintering ability and summer survival of the matured plant

Maize is a summer type annual plant, and after maturing it usually dies out, and does not regrow and propagate vegetatively, or produce seeds. It was observed in the isolated field that the maize plants died after maturation. Based on the above, an overwintering test for the matured plant of this recombinant maize was not carried out. In addition, there is no report in the use of this recombinant maize in foreign countries that the matured plant of this recombinant maize could successfully overwinter. It was observed in the isolated field tests that this recombinant maize and the non-recombinant control maize both died after ripening.

(d) Fertility and size of the pollen

For this recombinant maize and the non-recombinant control maize, the shape, fertility and size of pollen and other characteristics relating to reproduction and propagation were examined under a microscope to identify any difference between them.

Pollen was stained with iodine potassium iodine solution and examined under a microscope. As a result, no difference was observed between this recombinant maize and the non-recombinant control maize in the shape and size of pollen. In addition, the pollen was all found with the plasma stained, so they were considered fully matured. Based on the above results, also regarding the fertility of pollen, 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, in the measured results of ear length, ear diameter, row number per ear, and 100-kernel weight of the self-fertilized and harvested ears and the germination rate of harvested seeds, no significant difference was observed between this recombinant maize and the non-recombinant control maize. For the grain number per row, a significant difference was observed as mentioned in (a) between one of the two lines of this recombinant maize examined and the non-recombinant control maize.

Regarding shedding habit, shedding habit was not observed in the natural condition, since the ears of both of this recombinant maize and the non-recombinant control maize were covered with skins at the time of harvesting.

Regarding the dormancy, the seeds of both of this recombinant maize and the non-recombinant control maize harvested in the field tests were germinated at high rates, and no difference was observed between this recombinant maize and the non-recombinant control maize.

(f) Crossability

Crossability test was not performed for this recombinant maize, since no wild relatives that can be crossed with this recombinant maize exist in Japan.

(g) Productivity of harmful substances

As the succeeding crop test, the seeds of radish were sown in pots packed with the residual soil taken from individual experimental plots used for cultivation of this recombinant maize and the non-recombinant control maize, and the number of seeds germinated and the growth were examined. In addition, as the plow-in test, the seeds of radish were sown in pots packed with the mixture of residual soil, taken from individual plots used for cultivation of this recombinant maize and the non-recombinant control maize, and seedlings, thinned out in the individual plots and then dried and crushed, and the number of seeds germinated and growth were examined. As a result of the both tests, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the number of seeds of radish germinated and the fresh weight.

In 2006, as the plow-in test, the seeds of radish were sown in pots packed with the mixture of soil and dried and crushed leaves and stems of cultivated recombinant maize and non-recombinant control maize, and the germination rate was examined. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize. In addition, the radish was left to grow, and plant height, fresh weight and dry weight were examined. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

To identify whether cultivation of this recombinant maize could affect the soil microflora, soil samples were collected from individual experimental plots at two different periods of time (approx. 3 weeks after sowing and after harvesting of ears), the number of filamentous fungi, bacteria and actinomyces was examined. As a result, at the both periods of time, no significant difference was observed between this recombinant maize and the non-recombinant control maize. Consequently, it was judged that there is no difference in possible effects on the soil microflora between this recombinant maize and the non-recombinant control maize.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them

(2) Emergency measures which should be taken to prevent Adverse Effect on Biological Diversity in case Adverse Effect on Biological Diversity could arise

Refer to the attached “Plans of Emergency Measures.”

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.

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 self-seeding in Japan.

In the isolated field tests in Japan, the morphological and growth characteristics have been examined for two lines of this recombinant maize. As a result, one of the lines examined exhibited a significant difference from the control plant in grain number per row, though no significant difference was observed in the other characteristics examined. Consequently, it is considered unlikely that this difference alone can cause this recombinant maize to become competitive.

This recombinant maize is given the traits to be resistant to the order of insects Lepidoptera by the transferred modified *cry1Ab* gene and to be tolerant to glufosinate herbicide by the transferred *bar* gene. However, it is not generally considered that the insect damage by Lepidoptera is the major cause making the maize difficult to grow and glufosinate exerts pressure for selection in the natural environment; therefore it is considered unlikely that these characteristics cause this recombinant maize to become competitive.

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

Productivity of harmful substances of this recombinant maize (including secretion from roots to affect the other plants, secretion from roots to affect microorganisms in soil, and the possession in the plant body to affect the other plants after dying) has been investigated in the isolated field tests in Japan, but there is no significant difference between this recombinant maize and the non-recombinant control maize.

This recombinant maize produces the modified Cry1Ab protein, which possesses an insecticidal activity against Lepidopteran insects, and the PAT protein, which confers the tolerance to glufosinate.

The modified Cry1Ab protein possesses an insecticidal activity against Lepidopteral insects. As a result, there is a possibility that the modified Cry1Ab protein expressed by the pollens could affect the order Lepidoptera inhabiting around the fields where this recombinant maize is cultivated. However, based on the results including bioassay in which pollens were actually ingested together with feed plants, the effects, if any, will be limited to a very confined range. Consequently, it is considered extremely low that the insects of the order Lepidoptera could be affected at the level of individual population by the pollens dispersed from this recombinant maize.

On the other hand, there is no report that the PAT protein is harmful to wild animals and wild plants. In addition, the PAT protein possesses high substrate specificity and thus it is considered not to affect the metabolic system of the recipient organism.

As a result of homology search with amino acid sequences, it was confirmed that the modified Cry1Ab protein and the PAT protein 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 Japan, the growth of wild plants that can be crossed with maize in natural environment has not been reported. Based on the above understanding, no wild plants 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 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 is valid.

Bibliography

Confidential: Not made available or disclosed to unauthorized person

Maize resistant to Lepidoptera and tolerant to glufosinate herbicide

(Modified *cry1Ab, bar*, *Zea mays* subsp. *mays* (L.) Iltis)
(Event176, OECD UI：SYN-EV176-9)

Biological Diversity Risk Assessment Report

Annex List

Syngenta Seeds K.K.