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	Cotton resistant to Lepidoptera (*cry1Ac*, *Gossypium hirsutum* L.) (531, OECD UI : MON-ØØ531-6)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, 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 a recipient organism or the species to which the recipient organism belongs

(1) Taxonomical position and state of distribution in natural environment

a) The trivial common name: Cotton; The academic name: Gossypium hirsutum L.; Upland cotton

b) The recipient organism is a tetraploid cultivar of cotton (Gossypium hirsutum), Coker312, which belongs to the genus Gossypium of the family Malvaceae.

The wild types species of the genus Gossypium are distributed in arid regions, such as tropical and subtropical regions. Based on its geographical distribution, diploid wild types species are classified into 3 categories: an Australian group (11 species), an African/Arabian group (8 species), and an American group (12 species).

In addition to the diploid wild species, there are tetraploid wild species growing in the New Continent, which are classified into the following species: *G. tomentosum* (Hawaii), *G. mustelinium* (northwest Brazil), *G. darwinii* (Galapagos), *G. lanceolatum* (Mexico), *G. barbadense* (Antilles Islands, Central and South America), and *G. hirsutum* (Central America). Spontaneous *G.hirsutum* is rarely found in clumps, and in many cases they grow dispersed along the coastline or on islets. In Japan, natural distribution of *G. hirsutum* or any plant of the genus *Gossypium* that can hybridize with tetraploid cultivars of cotton has not been reported.

(2) History and present state of Use

a) The genus Gossypium consists of 41 species and subspecies. The wild type species of the genus is found in the Old and New Continent, Africa, and Australia, and its place of origin is believed to be India, Mexico and Peru. It is regarded that cotton was first introduced into Japan in 799 from India. However this cotton seems to have disappeared soon after. Cotton seeds were then introduced to Japan again through Kyushu between 1592 and 1595, and cultivation of cotton spread in the Kanto and southward area and southern parts of Japan. In 1882-1887, cotton cultivation expanded to 100,000 hectares and 24,000 tons were produced. However, the industry waned after that due to pressures from imported cotton. At present, commercial cultivation of cotton is scarcely carried out in Japan; it is mainly cultivated only as an ornamental plant. The cultivar that has long been cultivated in Japan is considered to be an Asian cotton, G. arboreum.

b) The genus Gossypium consists of 41 species. Among them, cultivars cultivated species are classified into 2 categories: diploid species (n=13) generically called "Asian cotton", G. herbaceum and G. arboret*um*; tetraploid species (n=26) generically called "Upland cotton", *G. hirsutum* and *G. barbadense*. At present, the

cultivation of "Asian cotton" is practiced only in certain limited areas in India, Africa and Asia. On the other hand, "Upland cotton" accounts for about 98% of cotton produced in the world, of which *G. hirsutum* represents 90%.

Since collected seed cotton contains seeds, it is ginned using a cotton ginning machine to separate lint, which is called cotton or raw cotton. Cotton is used for cotton products including such as cotton thread and cotton fabric, for guncotton, and for filling material. The remains after the separation of lint from seed cotton are seeds. On their surface, the seeds have short fiber of 3-5 mm in length on average (called short fuzz or original fuzz). Such fiber is scratched using a linter removing machine, and is called linter. Linter is produced as secondary product at oil mills, and used to make artificial fiber and guncotton. Relatively long linter can also be used for coarse yarn. Seeds without linter have 17-23% of oil content; these seeds are expressed, or extracted in solvent, to obtain cottonseed oil. About 130 kilograms of cottonseed oil is obtained from 1 ton of cottonseed, and is used as a raw material for margarine and soap, in addition to cooking oil. The oil-cake that remains after oil extraction is purified and used mainly for feed and manure.

According to statistical information of the US Department of Agriculture, the total area of cotton cultivation in the world in 2002 was 29.43 million hectares. Top cotton producing countries include India (7.60 million hectares), the US (5.03 million hectares), China (4.18 million hectares) and Pakistan (2.80 million hectares).

In 2002, Japan imported about 150,000 tons of cottonseed, 96% of which was from Australia. Among the imported cottonseed, about 40,000 tons were used for oil expression, and most of the rest was used for cattle feed. In Japan, only one oil company, in Osaka Prefecture, obtains oil by expressing cottonseeds imported from overseas. At present, cottonseeds for cultivation are mostly imported from the US, and are mainly cultivated as ornamental plants. These cottonseeds for cultivation are imported by a certain nursery company. According to this company, it is ensured by PCR method that the cottonseeds for cultivation imported through third parties from the US are those of non-recombinant cotton.

(3) Physiological and ecological properties

i) Basic properties

Cotton is a perennial plant of the family *Malvaceae*, that reproduce by seed propagation. It grows to a plant height of 90-120 cm, having 15-20 nodes with a leaf and 2 buds each, and forms vegetative and bearing shoots. In principle, cotton is perennial only in tropical regions, since it has no wintering ability. Japan is situated in the template regions, where cotton is annual because it dies during winter seasons.

ii) Environmental conditions allowing inhabiting or growth

The average temperature of 25ºC is optimum and 20-28ºC is suitable for inhabitation and growth of cotton. A considerable amount of rainfall is required during the growing season, and a precipitation of 1,000-1,500 mm/year is suitable. A lot of rain after the flowering season enhances abscission of flowers and bolls,

while insufficient rain reduces the lint percentage. In North America, cotton is grown in the range latitude 37-39ºN. Cotton is distributed up to latitude 42ºN in Europe, latitude 44.3ºN in Central Asia, and latitude 43ºN is the northern limit of the hemisphere for cotton growing in general. In Japan, the southern end of Ouu District (37.5ºN) is regarded as the northern most limit. Well-drained sandy loam being the most suitable, cotton prefers alkaline soil to acidic soil. The crop also grows on reclaimed land containing considerably high salt concentrations.

iii) Mode of propagation or reproduction

a) Ripe seeds appear when a boll opens, and usually do not easily shed because they are covered with lint. The level of seed dormancy is extremely low.

b) The propagation of cotton is based on seed propagation, not on vegetative propagation with tubers and subterranean stems. So far, there were no reports that tissues or organs have budding property that can regenerate the plant body under natural conditions.

c) The mode of pollination of cotton is basically self-pollination, though it is known that hybridization is also possible. In Japan, wild relatives that can hybridize with cotton are not known.

d) Since the pollen of cotton is relatively heavy and sticky, hybridization by wind pollination is unlikely to occur. Instead, the pollen is sometimes transferred by bumble bees (*Bombus* sp.) or honey bees (*Apis mellifera*). However, it is only in a limited range that pollens are dispersed by insect pollination. According to a study where fluorescent particles were applied to pollens so as to trace pollens dispersed to neighbor flowers, dispersed pollens were observed in only about 1.6% of flowers in flower fields 45-60 m away from a cotton field around which beehives were arranged. Besides, it was reported that another test showed that the percentage of hybridization was under 0.4% in flowers planted 1 m away from a cotton field, while it was down to under 0.3% in flowers 16 m away. Moreover, according to a result of hybridization tests using a marker gene of genetically recombinant cotton, the percentage of hybridization was 5% in flowers planted 1 m away from a cotton field of 30×136 m, while it was under 1% in flowers 7 m away. However, the percentage of hybridization under 1% was also found in some flowers planted farthest away from—or 25 m away from—the cotton field.

After placed under laboratory conditions for about 8 hours, 98% of cotton pollen maintained its germinability; on the other hand, 30% did after 24 hours, and as low as 1% did after 32 hours.

iv) Productivity of harmful substances

Production of substances that may have an effect on the inhabitation or growth of wild animals or plants, such as allelo chemicals, is not known.

v) Other information

Cotton contains gossypol, a kind of terpenoid. This physiologically active substance exists in secretory organs in all plant tissues including the seeds. Gossypol causes inflammation in the visceral organs and lungs of mammals, and is known as one of the toxic substances that causes dyspnea and paralysis in experimental animals. However, wild mammals that eat cottonseeds are not reported.

By the way, there has been no report stating that seeds of cotton spilled on the ground during transportation germinated and became self-seeding in Japan.

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

Composition of the donor nucleic acid that was used for the development of cotton tolerant to glyphosate herbicide (*cry1Ac, Gossypium hirsutum* L.) (531, OECD UI: MON-ØØ531-6) (hereinafter referred to as "this recombinant cotton") and the origin of components are shown in Table 1. This recombinant cotton expresses Cry1Ac protein which has been created by modifying the amino acid sequence of the wild-type Cry1Ac protein, in order to enhance its expression levels in plants. The two proteins show 99.4% of amino acid sequence homology. The Cry1Ac protein expressed in this recombinant cotton is hereinafter referred to as "modified Cry1Ac protein".

ii) Functions of component elements

a) Functions of component elements of donor nucleic acid that was used for the development of this recombinant cotton are as shown in Table 1 (P7-8).

The modified *cry1Ac* gene is the target gene contributing to the resistance to Lepidoptera; it has been created by modifying the amino acid sequence of the wild-type Cry1Ac protein which is produced in *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain. Cry1Ac protein, as well as the modified protein, exhibits insecticidal activity against order Lepidoptera, including Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm] (*Heliocoverpa zea*), which are the major pest insects of order Lepidoptera that damage cotton cultivation in the US and Australia. The modified Cry1Ac protein was produced by modifying amino acid sequence only for the N-terminal sequence of the wild-type Cry1Ac protein in order to enhance its expression level in plants. Therefore, the modified Cry1Ac protein has insecticidal activity against Lepidoptera as high as the wild-type Cry1Ac protein. Cry1Ac protein, including the modified Cry1Ac protein, exhibits insecticidal activity also against insects other than order Lepidoptera: for example, European corn borer (*Ostrinia nubilialis*) of the family *Pyralidae*. However, it is known that the

protein exhibits no insecticidal activity against larvae of any insect other than order Lepidoptera.

B.t. proteins which are produced by the bacterium *B.t.*, including the modified Cry1Ac protein, bind to the specific receptors on the midgut epithelium of the target insects, and form cation selective pores, which lead to the inhibition of the digestive process and result in the insecticidal activity. Also, the core protein—i.e. the active site of the modified Cry1Ac protein produced in this recombinant cotton—is identical to the core of the Cry1Ac protein in Bt preparation, which is a commercialized microbial agricultural insecticide. In the US, European countries and Japan, Bt preparation, which contains Cry1Ac protein, has been safely used for crops and trees as an insecticide to control order Lepidoptera.

The expression of the modified Cry1Ac protein confers resistance to Lepidoptera to this recombinant cotton, including Tobacco budworm, Pink bollworm and Cotton bollworm that damage cotton cultivation. In the traditional cotton cultivation methods, it was required to use a great deal of insecticide to control such order Lepidoptera. Because of that, the amount of insecticide used for cotton cultivation accounted for no less than 25% of all the insecticide used in the world. But it is reported in cotton-cultivating countries, such as the US, Australia and China, that the introduction of this recombinant cotton has dramatically reduced the amount of used chemical insecticide.

In addition, this recombinant cotton exhibits insecticidal activity against the only limited order Lepidoptera that damage cotton. Unlike chemical insecticides with wide insecticidal spectrum, this recombinant cotton does not affect on the existence of beneficial insects that prey on secondary pest insects such as aphids. As a result, it has been reported that, in China, there has been a 24% increase in the number of beneficial insects in the fields where this recombinant cotton is cultivated, compared to the fields where cotton is cultivated in a traditional method.

An *nptII* gene encodes an enzyme protein, neomycin phosphotransferase type II (NPTII), which transfers the terminal phosphate group of adenosine 5'-triphosphate (ATP), to a hydroxyl group at the aminoglycoside region of antibiotics. As a result, aminoglycoside antibiotics such as paromomycin and kanamycin become inactivated. In general, these aminoglycoside antibiotics bind specifically with a protein on ribosome in a cell; then protein synthesis is inhibited, and the cell is killed. However, when these antibiotics are phosphorylated by NPTII protein, they cannot bind with the target protein on ribosome any longer. Consequently, they cannot kill cells due to the absence of the ability to inhibit protein synthesis.

b) In order to investigate whether the modified Cry1Ac protein and NPT II protein share functionally important amino acid sequences with known allergens, the modified Cry1Ac protein and NPT II protein were compared with contact allergens in the database (SwissProt, GenPept, PIR, GenBank/EMBL). As a result, the modified Cry1Ac protein and NPT II

protein did not share structurally related homologous sequences with any of the known allergens examined.

Table 1 Component elements of the expression vector PV-GHBK04

Component elements	Origin and Function
Modified *cry1Ac* gene expression cassette	
e35S	Promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV).
Modified *cry1Ac*	A gene that encodes the modified Cry1Ac protein that exhibits insecticidal activity against order Lepidoptera that damage cotton cultivation, such as Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm] (*Heliocoverpa zea*). It encodes the protein which shows 99.4% of amino acid sequence homology with the wild-type Cry1Ac protein produced by *Bacillus thuringiensis* subsp. *kurstaki*.
7S 3'	3' untranslated region of soybean *β-conglycinin* gene. Contains a signal for the polyadenylation of mRNA, and functions to terminate transcription of the target gene.
nptII gene expression cassette	
35S	35S promoter region of cauliflower mosaic virus (CaMV).
nptII	A gene derived from a transposon of *E. coli*, Tn5. Encodes neomycin phosphotransferase type II. It confers resistance to kanamycin. In introducing genes, it is used as a marker to select recombinant plants,
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene. Functions to terminate transcription of the target gene.
Other component elements	
Right border sequence (Right Border)	A DNA sequence containing right border sequence (24bp) of nopaline type T-DNA derived from Ti plasmid pTiT37. Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome.
aad	A gene encoding 3''(9)-0-aminoglycoside adenyltransferase (AAD) derived from *Staphylococcus aureus*. Confers resistance to spectinomycin and streptomycin.
oriV	The replication origin derived from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* ABI strain.
ori322/rop	The replication origin derived from *E. coli* plasmid pBR322. Permits autonomous replication of vectors in *E. coli*. This region contains not only replication origin, but also rop region that is involved in the regulation of the replication initiation, and *oriT* sequence that is necessary for conjugal transfer from *E. coli* to *Agrobacterium tumefaciens*.

(2) Information concerning vector

i) Name and origin

The plasmid vector used to generate this recombinant cotton is assembled from plasmids including pBR322, which is a synthetic plasmid from *E.coli.*

ii) Properties

The total number of base pairs of this plasmid vector is 11,407 bp.

The plasmid vector pBR322 is a double strand circular DNA which has tetracyclin/ampicillin resistance as a selectable marker for construction vector in *E.coli*, and ori sequence, the origin of DNA replication.

The infectivity of this plasmid vector is not known.

(3) Method of preparing living modified organisms

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

The component elements of this plasmid vector which was transferred into the recipient organism are shown in Table 1

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

T-DNA region of the plasmid vector PV-GHBK04 was introduced into a current cotton cultivar, Coker 312, by the Agrobacterium method.

iii) Processes of rearing of living modified organisms

a) Plasmid vector PV-GHBK04 was introduced into the hypocotyls of Coker 312 by the Agrobacterium method, and then regenerated individuals were obtained by culturing them in media containing kanamycin.

In order to eliminate Agrobacterium from the regenerated plant, the regenerated plant was cultivated in media containing carbenicillin andcefotaxime , and then it was cultivated in embryo germination media containing no antibiotics.

Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes and the expression level of the modified Cry1Ac protein. Tests in climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters were examined in outdoor field tests. This recombinant cotton was selected upon the comprehensive evaluation of these results.

The following shows the approvals received from organizations abroad.

June, 1995: The US Food and Drug Administration (FDA) approved the safety of the cultivar as food and feed.

July, 1995: The United States Department of Agriculture (USDA) approved unlimited cultivation of the cultivar.

August, 1995: The US Environmental Protection Agency (EPA) exempted the Cry1Ac protein from the specification of a residual standard value.

August, 1996: The Australian Interim Office of Gene Technology Regulator (IOGTR) approved the cultivar being safe as feed and for the environment.
July, 2000: Food Standards Australia New Zealand (FSANZ) approved the safety of the cultivar as food.

June, 2003: The Australian Office of Gene Technology Regulator (OGTR) approved the cultivar being safe as feed and for the environment.

The following shows the approvals received from organizations in Japan.

April, 1997: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was certified by the Ministry of Agriculture, Forestry and Fisheries.

May, 1997: Based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4", safety of use for food was approved by the Ministry of Health, Labour and Welfare.

June, 1997: The safety of use of the cultivar for feed was approved in accordance with "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)".

March, 2001: The Ministry of Health, Labour and Welfare ensured the safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA techniques".

March, 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of the use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques".

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

The inserted genes were analyzed in Southern blotting analysis, cosmid cloning technique and genome walking method. As a result, gene insertion was found in the following 3 regions: the 1st inserted gene consisting of the modified *cry1Ac* gene expression cassette, *nptII* gene expression cassette, and *aad* gene expression cassette; the 2nd inserted gene that consists of a 3' region fragment of the modified *cry1Ac* gene and 7S3' terminator, which are inserted next to the 5'-terminal of the 1st inserted gene, in the reverse direction; and the 3rd inserted gene consisting of a 7S3' terminator fragment of 245 bp.

Southern blotting analysis was conducted in combination of 7 varieties of probes (from Probe 1 to Probe 6, and 5'-terminal flanking sequence of the inserted genes) and 7 varieties of restriction enzymes treatment (*Ase*I + *Bst*Z17I, *Ssp*I, *Xmn*I, *Bam*HI, *Bam*HI + *Nde*I, *Bam*HI + *Pme*I).

DNA fragments obtained in cosmid cloning technique and genome walking method were analyzed so as to determine the 5'-terminal flanking sequence of the 2nd inserted gene; the 3'-terminal flanking sequence of the 1st inserted gene; and the 3- and 5'-terminal flanking sequences of the 3rd inserted gene. In order to conclusively analyze the structure of the 1st and 2nd inserted genes, a PCR analysis was performed with a primer designed based on the base sequence of PV-GHBK04. As a result, PCR products with an expected size were detected. In addition, by analyzing the DNA sequence of these PCR products, the complete base sequence of the 1st and 2nd inserted genes was determined.

In addition, as a result of Southern blotting analysis of the genome DNAs extracted from the recombinant in R5 and R6 generations and from 2 commercialized cultivars, it was confirmed that the 1st and 2nd inserted genes were stably inherited in posterity. Incidentally, the 2 commercialized cultivars does not contain the 3rd inserted gene, i.e. the fragment of 7S3' sequence.

A possible reason is that the location of 3rd inserted gene was, on the chromosome, distant from the 1st and 2nd inserted genes; therefore, the 3rd inserted gene may have been separated from the others during back-cross process. Besides, since the 3rd inserted gene was a fragment of 7S3' sequence, which terminates transcription, it does not contribute to this recombinant cotton's resistance to Lepidoptera. Therefore, during back-cross breeding, the 3rd inserted gene was not used for the target of selection.

In addition, it was confirmed that the resistance to Lepidoptera was also stably expressed in multiple generations, according to simple ELISA analysis which only detects the expression of the modified Cry1Ac protein.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

For the detection and identification of this recombinant cotton, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and neighboring areas of plant genome are used as primers. This method makes it possible to specifically detect this recombinant cotton.

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

i) ELISA analysis confirmed that the Cry1Ac protein, which is encoded by the modified *cry1Ac* gene, is expressed at leaves, seeds, young leaves, and the plant body of this recombinant cotton.

ii) To compare R5 generation of this recombinant cotton and recipient Coker 312 as the control, isolated field tests were carried out at an isolated field in Kyusyu National Agricultural Experiment Station from June 1996 to December 1996.

(a) Morphological and growth characteristics

Differences in the following 19 items of morphological and growth characteristics were examined between this recombinant cotton and non-recombinant control cotton: the uniformity of germination; germination rate; plant type; stem height; flowering time; flower color; leaf shape; the number of effective flower buds; the number of bearing shoots; boll opening time; the color of fiber (lint); the shape of bolls (fruits of cotton); the number of bolls per plant; the number of segments of a boll; the number of seeds per boll; the color of seeds; harvest time; the dry weight of a boll; and the weights of above and under-ground parts at the harvest time .

For the following items among those above, 3 or more individual plants were selected from the central row of each plot, and totally 10 or more individual plants were analyzed: plant type; stem height; the number of effective flower buds; the number of bearing shoots; the color of fiber (lint); the shape of bolls (fruits of cotton); the number of bolls per plant; the number of segments of a boll; the number of seeds per boll; the color of seeds; the dry weight of a boll; and the weights of above- and under-ground parts at the harvest time. As for the analysis of bolls among these items, 2 bolls were selected from each individual plant. With regard to the analysis of the following items, including uniformity of germination, germination rate, flowering time, boll opening time, and harvest time, all individuals were analyzed.

As a result, a statistically significant difference was found between this recombinant cotton and the non-recombinant control cotton, in the number of bolls per plant: in this recombinant cotton, the number of bolls per plant of was 9.6 on average, while in the non-recombinant control cotton, it was 6.5 on average. Likewise, a statistically significant difference was found in the dry weight of a boll: in this recombinant cotton, the dry weight of a boll was 7.7 g on average, while, in the non-recombinant control cotton, it was 8.1 g on average. No statistically significant differences were found in the other items.

A possible reason can be as follows: Though insecticide was sprayed, the non-recombinant control cotton was damaged by order Lepidoptera: cotton leafroller (*Notarcha derogata*) and Asian corn boer (*Ostrinia furnacalis*). As a result, the number of bolls per plant decreased in the non-recombinant control cotton, becoming less than in this recombinant cotton. Thus, each individual boll of the non-recombinant control cotton grew heavier.

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

A chilling-tolerance test was not conducted during the isolated field tests. Instead, the seeds collected in 1994 from R4 generation cultivated in 3 fields in the US (Tifton, Georgia [GA]; Starkville, Mississippi [MS]; and Loxley, Alabama [AL]) were sown directly to the ground in all three locations; and the germination rate and the wintering ability of the seeds, up to the following spring were examined. All of these 3 regions are famous for cotton cultivation in the South of the US. Besides, compared to average climatic conditions in

Japan, the winter coldness in these regions is relatively mild. Therefore, it is considered that these regions provide better climatic conditions for cotton growth than Japan.

As a result, the sown seeds of this recombinant cotton showed no germination in all the 3 fields up to the following spring.

In addition, field tests were carried out in 21 fields in the US, for 3 years from 1991 to 1993. Then, in some fields where harvest time was relatively early, it was found that some seeds spilt on the field had germinated in fall after harvesting. However, it was reported that all of them had died by the following spring. Based on the above understanding, it was judged that chilling-tolerance of this recombinant cotton is as low as that of the non-recombinant control maize at the early stage of its growth.

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

Basically cotton is a perennial plant, but only in tropical regions. In other cotton cultivation regions in the world, including Japan, cotton naturally dies in winter seasons after fruit-bearing. In practice, it was observed that the plants were partly dead when the isolated field tests of this recombinant cotton were completed. Based on the above, the wintering ability test was not conducted for adult plants.

(d) Fertility and size of the pollen

In Japan, there are no plans for this recombinant cotton to be commercialized, and it is not cultivated for commercial use. Therefore, if this recombinant cotton caused Adverse Effect on Biological Diversity in Japan, it would be in the following way: cotton seeds imported for oil extraction and feed are spilled during transportation, into Japanese natural environment; then, the spilled seeds grow or become self-seeding, and expel other plants from the area. However, the fertility and the size of pollens were not examined, because pollens are not formed until spilled cotton seeds germinate, grow or become self-seeding, and become adult; and because there have been no reports that seeds spilled during transportation grow or become self-seeding under natural conditions in Japan.

(e) Productivity, dormancy, and germination rate of the seeds

Regarding seed production, the differences between this recombinant cotton and non-recombinant control cotton were examined in “a) Morphological and growth characteristics”, as the number of bolls per plant, the number of segments of a boll, the number of seeds per boll. As a result, a statistically significant difference was found between this recombinant cotton and the non-recombinant control cotton, in the number of bolls per plant ($P < 0.05$). However, no significant differences were found in other items. The number of bolls per plant—in which a statistically significant difference was found—was 9.6 in this recombinant cotton, and was 6.5 in the non-recombinant control cotton.

As described in “a) Morphological and growth characteristics”, the following is a possible reason for the statistically significant difference found in the number of bolls per plant: Though insecticide was sprayed, the non-recombinant control cotton was damaged by order Lepidoptera: cotton leafroller (*Notarcha derogata*) and Asian corn boer (*Ostrinia furnacalis*). As a result, the number of bolls per plant decreased in the non-recombinant control cotton, becoming less than in this recombinant cotton.

In both this recombinant cotton and the non-recombinant control cotton, seeds are covered with lint at harvest time. Therefore, we did not observe shedding habits of the seed under natural conditions.

It is known that the level of seed dormancy of cotton is extremely low. Also, it is known that the longevity of cotton seeds is short under natural conditions, and most of them decay in soil if sown before a period when soil temperature reaches 15-16°C. As described in b), seeds collected in autumn were soon sown in 3 fields in the US (Tifton, GA; Starkville, MS; and Loxley, AL), and observed until the next spring; as a result, like the non-recombinant control cotton, this recombinant cotton showed no germination. Based on the result, this recombinant cotton seeds decayed in soil because of low soil temperature, like the non-recombinant control cotton seeds. Therefore, it was considered that this recombinant cotton seeds have extremely poor survival ability in low temperature and extremely low wintering ability, so the test concerning dormancy was not conducted.

Germination rate was examined in “a) Morphological and growth characteristics”. As a result, no statistical differences were observed between this recombinant cotton and the non-recombinant control cotton.

(f) Crossability

In Japan, no wild relatives exist that belong to *Gossypium* which is hybridized with tetraploid cotton cultivar (*Gossypium hirsutum)* to which this recombinant cotton belongs. Thus, Crossability was not assessed.

(g) Productivity of harmful substances

As described in d), if this recombinant cotton produces harmful substances and cause an Adverse Effect on Biological Diversity in Japan, it would be in the following way: cotton seeds imported for oil extraction and feed are spilled during transportation; after that, such spilled seeds grow or become self-seeding at places which are not under human control, and expel other plants from the area. The productivity of harmful substances was not examined, because it is considered that, until spilled cotton seeds germinate and become matured to a certain extent, harmful substances would not be produced in the root or aerial parts of the plant, up to a level where the environment may be affected; and because there are no reports that seeds spilled during transportation grow or become self-seeding under natural conditions in Japan.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

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

(2) Information obtained from Use abroad

Between 1991 and 1993, field tests were conducted for this recombinant cotton at 21 fields in the US. In the tests, the cotton was evaluated by observing the following items: characteristics regarding weediness; characteristics regarding morphology and growth; characteristics regarding yields; pest insect sensitivity; and ability to become self-seeding (volunteers). However, no difference was observed between this recombinant cotton and the non-recombinant control cotton.

It is estimated that the cultivation area of this recombinant cotton in 2002 was about 1 million hectares in the world. So far, there is no report that this recombinant cotton caused Adverse Effect on Biological Diversity.

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

1. Competitiveness

The plant body of cotton (*Gossypium hirsutum* L), to which the recipient organism belongs, dies during winter seasons in Japan. Moreover, the level of seed dormancy is extremely low. Therefore, it is considered unlikely that cotton becomes self-seeding in Japan. Cotton has long been distributing as seed cotton in Japan, however, there has been no report that cotton becomes self-seeding in Japan.

The introduction of the modified *cry1Ac* gene confers resistance to Lepidoptera to this recombinant cotton. However, from the result of the examination of characteristics in competition (examination such as morphological and growth characteristics, and productivity of the seeds), it is considered unlikely that a significant difference will arise in such characteristics between this recombinant cotton and the non-recombinant control cotton, under natural conditions in Japan. Therefore, it is considered unlikely that this recombinant will grow or become self-seeding in Japan. Consequently, it is judged that, even though this recombinant cotton is resistant to Lepidoptera, it will unlikely become dominant in competition with the non-recombinant control cotton.

Based on the above understanding, it was judged that there are no specific wild plants and wild animals that are possibly affected by this recombinant cotton, and that the use of such cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

2. Productivity of harmful substances

Into this recombinant cotton, the modified *cry1Ac* gene has been introduced, conferring to the ability to produce Cry1Ac protein that has insecticidal activity against larvae of order Lepidoptera. However, the content of Type 1 Use of this recombinant cotton does not include "cultivation". Therefore, it is only when this recombinant cotton grew after spilled during transportation that order Lepidoptera could be exposed to Cry1Ac protein. Also, it is considered that, even if cotton (seeds) grew after spilled during transportation, such seeds would not grow and become self-seeding, as was discussed in the section of "Competitiveness". Therefore, it was judged that the use of this recombinant cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to the productivity of harmful substances.

3. Crossability

In the Japanese natural environment, there are no wild species which can hybridize with cotton. Therefore, it was judged that there are no specific wild plants or wild animals that are possibly affected by this recombinant cotton, and that the use of such cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to Crossability.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

Consequently, it was judged that there is no risk of Adverse Effect on Biological Diversity in Japan attributable to the use of this recombinant cotton for provision as food, for provision as feed, processing, storage, transportation, disposal and acts incidental to them.