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 tolerant to glyphosate herbicide and resistant to Lepidoptera (*cp4 epsps*, *cry1Ac*, *Gossypium hirsutum* L.) (1445 × 531, OECD UI : MON-Ø1445-2 × 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

Ⅰ. Information concerning preparation of living modified organisms

The cross progeny line (*cp4 epsps*, *cry1Ac*, *Gossypium hirsutum* L.) (1445×531, OECD UI : MON-Ø1445-2×MON-ØØ531-6) (hereinafter referred to as "this stack cotton") was from the crossing of the following two recombinant cotton with the use of traditional cross-breeding method. The two recombinant cottons are, i) Cotton tolerant to glyphosate herbicide (*cp4 epsps*、*Gossypium hirsutum*) (1445, OECD UI : MON-Ø1445-2) (hereinafter referred to as "1445"), and ii) Cotton resistant to Lepidoptera (*cry1Ac*、*Gossypium hirsutum* L.) (531, OECD UI: MON-ØØ531-6) (hereinafter referred to as "531"). Therefore, this stack cotton possesses the both characteristics of parent lines, 1445 and 531. The information concerning preparation of 1445 and 531 are explained individually in the followings.

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of 1445 are shown in Table 1.

The composition of donor nucleic acid and the origins of component elements used for the development of 531 are shown in Table 2. The 531 expresses modified 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 modified Cry1Ac protein expressed in 531 is hereinafter referred to as "modified Cry1Ac protein".

(2) Functions of component elements

【Target gene used for development of 1445】

a) The functions of component elements of donor nucleic acid used for the development of 1445 are shown in Table 1.

cp4 epsps gene

Glyphosate is the active ingredient in Roundup, a nonselective herbicide, and inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis by specifically binding to the enzyme. As a result, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. A *cp4 epsps* gene, which is the target gene, expresses the CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The activity of the CP4 EPSPS protein that is produced

by *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus, the recombinant plants that express this protein have normal functions of shikimate synthesis and can grow.

EPSPS is located in chloroplasts or plastids in plants. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway, but it has been clarified to be extremely unlikely that the stages from DAHP, through the production of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSP) which is catalyzed by EPSPS, to the synthesis of chorismic acid are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids. In addition, Monsanto Co. examined amino acid contents in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the Roundup herbicides, and confirmed that there is no difference in the content of aromatic amino acid between the original non-recombinant plants and the recombinant plants. These facts support that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS is the enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), and is known to specifically react with these substrates. The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

1445 possesses tolerance to glyphosate herbicide by expressing CP4 EPSPS protein. Therefore, 1445 can control weed by 1-2 times of spraying of glyphosate during weed growth, after one time of spraying glyphosate to non-tillage fields before sowing. It is considered that 3-5 times of herbicide spraying per one cropping season is required for cotton cultivation. However, thanks to 1445 cultivation, the reduction of using herbicide and “non-tillage cultivation” in which chemical fertilizer and agrichemicals lose less into river by wind and rain are expected.

nptII gene

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 CP4 EPSPS protein and NPTII protein share functionally important amino acid sequences with known contact allergens, the CP4 EPSPS protein and NPTII protein were compared with allergens in the database. As a result, the CP4 EPSPS protein and NPTII protein did not share structurally related homologous sequences with any of the known allergens examined.

【Target gene used for development of 531】

a) Functions of component elements of donor nucleic acid that was used for the development of 531 are shown in Table 2.

Modified *cry1Ac* gene

The modified *cry1Ac* gene has been created by modifying amino acid sequence of the wild-type Cry1Ac protein which is produced in *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain. Cry1Ac protein, including the modified Cry1Ac protein, exhibits insecticidal activity against Lepidoptera including Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm, otherwise Corn earworm (*Heliocoverpa zea*) which are the major insect pests 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 European corn borer (*Ostrinia nubilialis*) of the family *Pyralidae*. However, it is known that the protein exhibits no insecticidal activity against larvae of any insects 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 531 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. 531 confers the resistance by expressing modified Cry1Ac protein to Lepidoptera, including Cotton bollworm (*Heliocoverpa zea*) and Tobacco budworm (*Heliothis virescens*), the main insect pest

for cotton cultivation.

531 confers the resistance to Lepidoptera by expressing modified Cry1Ac protein, including Tobacco budworm, Pink bollworm and Cotton bollworm, the main insect pest for cotton cultivation. In cotton cultivation so far, abundant insecticide application was necessary to inhibit the development of order Lepidoptera; for instance, approximately 25% of insecticide used in the world was used for cotton cultivation. We have been apprehensive for the biological diversity risk of agrichemicals, which have remained in environment for a long period. However, it has been reported that the used amount of agrichemicals was vastly reduced due to the introduction of 531 in cotton cultivating countries, including the US, Australia and China. In addition, since 531 shows insecticidal activity only against the limited species of order Lepidoptera that eat cotton, 531 does not affect to the benefit insects to catch and eat secondary insect pest, including aphid. This characteristic of 531 above is different from agrichemicals which have wide range of insecticidal spectrum. Therefore, in China, it is reported that the number of benefit insects in fields to cultivate 531 has been increasing up to 24% compared with the other fields using conventional cultivation method.

b) In order to investigate whether the modified Cry1Ac protein and NPTII protein share functionally important amino acid sequences with known contact allergens, those proteins were compared with allergens in the database. As a result, the modified Cry1Ac protein and NPTII protein did not share structurally related homologous sequences with any of the known allergens examined.

Table 1 Component elements of the expression vector PV-GHGT07

Component elements	Origin and Function
Cp4 epsps gene expression cassette	
CMoVb	35S promoter of figwort mosaic virus. Involved in the constant expression of the target gene in all tissues.
Ctp2	A sequence in the *epsps* gene of *Arabidopsis thaliana* that encodes chloroplast transit peptide located at N-terminal region of EPSPS protein. Transports CP4 EPSPS protein to the chloroplast where aromatic amino acids are synthesized.
Cp4 epsps	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived form *Agrobacterium tumefaciens* CP4 strain.
E9 3’	3’ untranslated region of pea ribulose-1, 5-bisphosphate carboxylase E9 gene. Terminates transcription of mRNA and induces polyadenylation.
nptII gene expression cassette	
P-35S	35S promoter region of cauliflower mosaic virus (CaMV). Involved in the constant expression of the target gene in all tissues.
nptII(Kan)	A gene that was isolated from Tn5 transposon of *E.coli*. Encodes neomycin phosphotransferase type II (NPTII) enzyme protein.
NOS 3’	3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. Terminates transcription of mRNA and induces polyadenylation.
Gox gene expression cassette (As a result of inserted gene analysis, the followings were not inserted in 1445.)	
CMoVb	35S promoter of figwort mosaic virus. Involved in the constant expression of the target gene in all tissues.
Ctp1	A sequence that encodes chloroplast transit peptide located at N-terminal region of small subunit 1A of rubisco derived from *A. thaliana*. Transports GOX protein to chloroplast where aromatic amino acids are synthesized.
Gox	A sequence that encodes C-terminal of v247, a variant derived from glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein dissolves glyphosate.
NOS 3’	3’ untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. Terminates transcription of mRNA and induces polyadenylation.
Other component elements (As a result of inserted gene analysis, the followings were not inserted in 1445.)	
Ori-V	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens*.
Aad	The gene encoding the Tn7 adenyltransferase (AAD). Confers resistance to spectinomycin or streptomycin.

Component elements	Origin and Function
Right border sequence (Right Border)	A DNA sequence containing right border sequence (25bp) of nopaline type T-DNA derived form Ti plasmid pTiT37. Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome.
Ori322	Replication origin region isolated from pBR322, a plasmid derived from *E. coli*. Permits autonomous replication of vectors in *E.coli*.
Rop	Derived from *E. coli*. Regulates the number of plasmids to be replicated in *E. coli*.

Table 2 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 gene, it is used as a marker to select recombinant plant.
NOS3'	3' untranslated region of nopaline synthase (NOS) gene. Terminates transcription of mRNA and induces polyadenylation.
Other component elements	
Right border sequence (RB)	A DNA sequence containing right border sequence (24bp) of nopaline type T-DNA derived form Ti plasmid pTiT37. Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome.
Aad	A gene encoding 3''(9)-O-aminoglycoside adenylyltransferase (AAD) derived from *Staphylococcus aureus.* It terminates transcription and induces polyadenylation.

Component elements	Origin and Function
OriV	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens*.
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 form *E. coli* to *Agrobacterium tumefaciens.*

2. Information concerning vector

(1) Name and origin

The vector used to generate 1445 and 531 is derived from pBR322, which is a synthetic plasmid from *E.coli*.

(2) Properties

The total number of base pairs of the plasmid vector used to generate 1445 is 12,032bp.
The total number of base pairs of the plasmid vector used to generate 531 is 11,407bp.

The plasmid vector pBR322 is a double strand circular DNA which has tetracycline/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

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

Refer to Tables 1 and 2.

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

In developing 1445, T-DNA region of the plasmid vector PV-GHGT07 was introduced into a current cotton cultivar, Coker 312 by the Agrobacterium method.
In developing 531, T-DNA region of the plasmid vector PV-GHBK04 was introduced into a current cotton cultivar, Coker 312 by the Agrobacterium method.

(3) Processes of rearing of living modified organisms

【Processes of rearing of 1445】

a) T-DNA region of vector PV-GHG07 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.

b) In order to eliminate Agrobacterium from the regenerated plant, the regenerated plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics. It was confirmed that there was no Agrobacterium remained.

c) Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes and the expression level of the CP4 EPSPS protein. Tests in climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters (Morphological and growth characteristics, characteristics of harvested amount, sensitivity against insect pest, etc.) were examined in outdoor field tests. 1445 was selected upon the comprehensive evaluation of these results.

The following shows the approvals received from organizations abroad.

July 11, 1995: The United States Department of Agriculture (USDA) approved unlimited cultivation of the cultivar.

September 11, 1995: The US Food and Drug Administration (FDA) approved the safety of the cultivar as food and feed.

February 21, 1996: The US Environmental Protection Agency (EPA) approved glyphosate herbicide to use for growing cotton.

September 14, 2000: The Australian Interim Office of Gene Technology Regulator (IOGTR) approved the cultivar being safe as feed and for the environment.

November 24, 2000: Food Standards Australia New Zealand (FSANZ) approved the safety of the cultivar as food.

June 19, 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.

December 9, 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.

December 16, 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, Labor and Welfare.

January 12, 1998: 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 30, 2001: The Ministry of Health, Labor 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 27, 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".

【Processes of rearing of 531】

a) T-DNA region of 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.

b) In order to eliminate Agrobacterium from the regenerated plant, the regenerated plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics.

c) 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 (Morphological and growth characteristics, characteristics of harvested amount, sensitivity against insect pest, etc.) were examined in outdoor field tests. 531 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, Labor and Welfare.

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

March, 2001: The Ministry of Health, Labor 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".

【Process of rearing of 1445×531】

This stack cotton was developed by traditional breeding method with using two recombinant cottons, 1445 and 531.

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

【State of existence of nucleic acid transferred in cells of 1445 and stability of expression of traits caused by the nucleic acid】

As a result of the analysis of inserted gene by Southern blotting analysis, it was confirmed that one copy of gene is inserted into genome of 1445 chromosome at one site. Next, Southern blotting analysis was conducted with using CMoVb promoter, *gox* gene, *cp4 epsps* gene, *aad* gene, and *nptII* gene as probes. As a result, it was found that CMoVb promoter, *cp4 epsps* gene, *aad* gene, and *nptII* gene were inserted into 1445, but *gox* gene was not inserted. Therefore, it was confirmed that one copy of T-DNA region (*cp4 epsps* gene, *aad* gene, *nptII* gene; except *gox* gene) was inserted into the genome of 1445. In addition, simple selection has been conducted by PCR method for R_0 generation (R_0 shows the first generation of 1445, hereinafter, the number of selfed generations from R_0 is expressed by putting the digit after R). Regarding *gox* gene, it was confirmed that *gox* gene was not inserted in cotton genome from the first. Moreover, the neighborhood sequence of both sides of inserted gene was determined, and then the map of inserted gene was finalized.

In addition, *E. coli* and *aad* gene that was used as a marker to select *Agrobacterium tumefaciens* were inserted in 1445. However, since *aad* gene does not possess the promoter to function in plant, *aad* gene was not expressed in plant. In the actual measurement, AAD protein produced by *aad* gene was under the limit value of detection in ELISA method (0.025ng per 1mg of raw tissue of seeds, 0.013ng per 1mg of raw tissue of leaves).

Also, as a result of Southern blotting analyses conducted in R3 and R5 generations, it was confirmed that the inserted gene has descended stably to progeny. In addition, as a result of ELISA method with using R4 and R5 generations' seeds, it was found that CP4 EPSPS protein and NPTII protein have also stably expressed.

【State of existence of nucleic acid transferred in 531 and stability of expression of traits 】

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 in the genome DNA of 531: 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 245bp.

Southern blotting analyses were 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 progeny. 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 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. Difference from the recipient organism or the species to which the recipient organism belongs

It is guessed that modified Cry1Ab protein and CP4 EPSPS protein are expressed in the plant body of this stack cotton by the function of genes which were inserted in parent lines, 1445 and 531. As mentioned in (2)-1-I, modified Cry1Ab protein does not possess enzyme activity, and functions independently of the metabolic system of recipient organism. Also, it is suggested that EPSPS protein, which possesses the same functions as CP4 EPSPS protein, is not a rate-determining enzyme. In addition, Monsanto Co. examined amino acid content in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the glyphosate herbicides, and confirmed that there is no difference in the aromatic amino acid content between the original non-recombinant plants and recombinant plants, thus it is considered not to affect to the metabolic pathway of

recipient organism. Furthermore, CP4 EPSPS protein has high substrate specificity. Based on the above understanding, it is not easy to consider that these two proteins would affect each other.

To confirm the above understanding in practice, regarding tolerance to glyphosate herbicide of this stack cotton, Roundup-spraying tests were carried out in fields of the US and the tolerance was observed. In general, since inhibition and retardation of growth are observed, as drug-induced suffering of glyphosate herbicide, number of nodes and total length in prime time of growth and number of days to reach prime time of flowering were set as objects of study. As a result, number of nodes in prime time of growth, total length and number of days to reach in prime time of flowering of this stack cotton was as same level as 1445 which expresses CP4 EPSPS alone (Table 3, p15). In addition, regarding resistance to Lepidoptera of this stack cotton, the number of insect pest was observed in the US fields. Two kinds of insect pest, Tobacco budworm (*Heliothis virescens*) and Cotton bollworm (*Helicoverpa zea*) emerged. As a result, the number of Tobacco budworm and Cotton bollworm of this stack cotton was as same level as 531 which express modified Cry1Ac protein alone (Table 4, p15). Based on the above results, it is suggested that the degree of expression of these proteins does not affect each other by crossing.

Based on the above understanding, regarding the difference between this stack cotton and cotton which is the taxonomic species to which the recipient organism belongs, it is guessed with the use of the results of individual examinations for the various characteristics of 1445 and 531.

Table 3 Result of biological examination by spraying of glyphosate herbicide to cross progeny line, 1445×531

Cross progeny line	Number of nodes in prime of growth		Total length in prime time of growth		Number of days to reach in prime time of flowering	
	Number of nodes	SEM	Total length (cm)	SEM	Number of days	SEM
1445×531	20.9	5.61	101.4	29.0	10.4	0.74
1445	21.5	5.07	101.0	26.6	10.1	0.78
Non-recombinant plant	22.0	6.08	104.3	27.2	9.3	0.06

Fifty plant bodies for each cross progeny line in 4 fields were cultivated, and at the 3 to 4th stage, 350 ml of glyphosate herbicide (Product name: Roundup) per 10a were sprayed, then tolerance was observed. Normally, the non-recombinant cotton would easily die with the above amount of herbicide. As drug-induced suffering by glyphosate herbicide, inhibition and retardation of growth was observed in general. Therefore, number of nodes and total length in prime time of growth and number of days to reach prime time of flowering (the number of days to reach in prime time of flowering from the beginning of flowering) were set as objects of study. For the non-recombinant control cotton, plant bodies without spraying glyphosate herbicide were observed.

Table 4 Number of order Lepidoptera (Tobacco Budworm and Cotton bollworm) in cross progeny line, 1445×531

Cross progeny line	Number of insects (1st observation period)	Number of insects (2nd observation period)
1445×531	5	0
1445	16	24
531	6	2
Non-recombinant plant	17	24

Twenty plant bodies for each cross progeny line in 4 fields (80 plant bodies total) were cultivated, and at the 1st observation period (7/9-7/17) and the 2nd observation period (8/8-8/27), the number of insect pest was counted. The 1st observation period is the beginning of flowering, and the 2nd observation period is the beginning of boll dehiscence. The number of insect pest is the total number of the 80 plant bodies in 4 fields. The number of insect pest is the number of living insects at the

observation point of time, and all of them are larvae.

(1) Regarding 1445, it is confirmed by ELISA analysis that CP4 EPSPS protein, which is encoded by *cp4 epsps* gene to confer the tolerance to herbicide, has been expressed in leaves and seeds.

Regarding 531, it is confirmed by ELISA analysis that modified Cry1Ac protein, which is encoded by modified *cry1Ac* gene to confer the resistance to order Lepidoptera has been expressed in leaves, seeds, young leaves and plant bodies.

Therefore, for this stack cotton, it is considered that CP4 EPSPS protein and modified Cry1Ac protein have been expressed in leaves and seeds.

(2) To compare R5 generation of 1445 and recipient Coker312 as the control, isolated field tests were carried out at an isolated field in Kyusyu National Agricultural Experiment Station from May 1997 to October 1997. Also, to compare R5 generation of 531 and recipient Coker312 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. The characteristics of 1445 and 531 were investigated individually.

a) Morphological and growth characteristics

【Morphological and growth characteristics of 1445】

Differences in the following 19 items of morphological and growth characteristics were examined between 1445 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, 5 individual plants were selected from each row of each plot and 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, regarding germination rate, leaf length and short diameter of bolls, statistically significant difference ($P < 0.05$) was observed between 1445 and the non-recombinant control cotton. However no difference was found in other items.

Regarding germination rate , in which statistically significant difference was observed, the average germination rate of the non-recombinant control cotton was 95%, on the other hand , the average rate of three repetitions of 1445 was 55%. However, through the field test conducted in approximately 65 fields in the US and Puerto Rico for three years from 1992 to 1994, it was confirmed that there is no difference between 1445 and the non-recombinant control cotton in germination rate. In addition, there is a result of the test for the germination rate on 7th day in petri dishes under two conditions, those are 1) warm condition (31℃ / 24℃, Day/Night), and 2) cool condition (19℃), with using seeds of 1445 and Coker312 which were cultivated in Dominican Republic. However, there was no difference between 1445 and the non-recombinant control cotton in germination rate. Regarding seed sample used in this isolated field test, we asked for the information to the head office in the US. The head office reported that the both seeds were taken from the same test area in 1996, but it rained heavily at harvesting time of 1445 seeds, after harvesting the non-recombinant control cotton, and many 1445 seeds were torn of their seed coat. Therefore, it was reported that the quality of 1445 seeds was lower than that of the non-recombinant control cotton. Consequently, it was concluded that the difference of germination rate in this isolated field test was caused by the deterioration of 1445 seed sample torn at their seed coat; not caused by the inserted gene.

In addition, regarding leaf length and short diameter of bolls, statistically significant difference was observed between 1445 and the non-recombinant control cotton. The average value of cotton leaf length for 1445 and the non-recombinant control cotton were 17.8cm and 17.1cm, and the average value of short diameter for 1445 and the non-recombinant control cotton were 3.5cm and 3.2cm.

【Morphological and growth characteristics of 531】

Differences in the following 19 items of morphological and growth characteristics were examined between 531 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 531 and the non-recombinant control cotton, in the number of bolls per plant: in this recombinant cotton, the number of bolls per plant 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 531, the dry weight of a boll was 7.7g on average, while, in the non-recombinant control cotton, it was 8.1g 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 borer (*Ostrinia furnacalis*). As a result, the number of bolls per plant decreased in the non-recombinant control cotton, becoming less than in 531. Thus, each individual boll of the non-recombinant control cotton grew heavier. Therefore, it was judged that there is no difference between 531 and the non-recombinant control cotton in the number of bolls per plant and the dry weight of a boll.

【Stack cotton 1445×531】

Thus, there is a possibility that statistically significant difference is observed in leaf length and short diameter of bolls between this stack cotton and the cotton which is the taxonomic species to which the recipient organism belongs. However, it is considered that there is no difference in other morphological and growth characteristics between this stack cotton and cotton which is the taxonomic species to which the recipient organism belongs.

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

【Chilling-tolerance and heat-tolerance at the early stage of 1445 growth】

A chilling-tolerance test was not conducted during the isolated field tests. Instead, the seeds collected in 1994 from R5 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 of examination, the seeds sown in October 18 at the field in Loxley, AL, had germinated slightly (0.3%) in December 15, but they had died in next January 17. Thereafter, they had not germinated until April 27, the last day of observation. No germination of the seeds

in the other two fields was observed.

【Chilling-tolerance and heat-tolerance at the early stage of 531 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 531 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 spilled 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 531 is as low as that of the non-recombinant control cotton at the early stage of its growth.

【Stack cotton 1445×531】

Consequently, germination of the sown seeds of this stack cotton was not found, and it was considered that even if they germinate, they would die in next spring.

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 1445 and 531 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 the seeds of 1445 and 531 to be commercialized, and commercial cultivation has not been conducted. In addition, natural distribution of genus *Gossypium* that has crossability with 1445 and 531 has not been reported in Japan. Therefore, if 1445 and 531 causes 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) Production, dormancy, and germination rate

【Production, dormancy, and germination rate of 1445】

Regarding seed production, the differences between 1445 and non-recombinant control cotton were examined in "a) Morphological and growth characteristics", as the number of bolls, the number of segments of a boll, the number of seeds per boll. As a result, no difference was found between 1445 and the non-recombinant control cotton.

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, 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, 1445 seeds decayed in soil because of low soil temperature, like the non-recombinant control cotton seeds. Therefore, it was considered that 1445 seeds have extremely poor survival ability in low temperature and extremely low wintering ability, and they cannot keep their germinating ability under the winter condition in Japan, so the test concerning dormancy was not conducted.

Germination rate was examined in "a) Morphological and growth characteristics" of 1445. As a result, statistically significant difference was found between 1445 and the non-recombinant control cotton, and the average germination rate of 1445 was 55% and the average germination rate of the non-recombinant control cotton was 95%. However, as described in "a) Morphological and growth characteristics", the difference above was not caused by the inserted gene, and it was concluded that the background was the deterioration of 1445 seed sample which was torn at the seed coat.

【Production, dormancy, and germination rate of 531】

Regarding seed production, the differences between 531 and non-recombinant control cotton were examined in "a) Morphological and growth characteristics", as the number of bolls, the number of segments of a boll, the number of seeds per boll. As a result, a statistically significant difference was found between 531 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 531, and was 6.5 in the non-recombinant control cotton.

As described in "a) Morphological and growth characteristics" of 531, 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 borer (*Ostrinia furnacalis*). As a result, the number of bolls per plant decreased in the non-recombinant control cotton, becoming less than in 531. Therefore, it was judged that there is no difference between 531 and the non-recombinant control cotton in the number of bolls per plant.

In both 531 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) of 531, 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, recombinant cotton 531 showed no germination. Based on the result, 531 seeds decayed in soil because of low soil temperature, like the non-recombinant control cotton seeds. Therefore, it was considered that 531 seeds have extremely poor survival ability in low temperature and extremely low wintering ability, and they cannot keep their germinating ability under the winter condition in Japan, so the test concerning dormancy was not conducted.

Germination rate was examined in "a) Morphological and growth characteristics" of 531. As a result, no statistical differences were observed between 531 and the non-recombinant control cotton.

【Stack cotton 1445×531】

Thus, it is considered that there is no difference in characteristics of production (the number of bolls, the number of segments of a boll, and the number of seeds per boll) and germination rate between this stack cotton and cotton which is the taxonomic species to which the recipient organism belongs.

f) Crossability

In Japan, no wild relatives exist that belong to *Gossypium* which can cross with tetraploid cotton cultivar (*Gossypium hirsutum)* to which 1445 and 531 belong. Thus, crossability was not assessed.

g) Productivity of harmful substances

As described in d), if 1445 and 531 produce 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. Therefore, in isolated fields of 1445 and 531, it was mainly examined that the possibility of cotton seeds spilled during transportation to germinate at places and grow or become self-seeding. 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.

II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." Results of the review are listed below.

This stack cotton was produced by cross of cotton tolerant to glyphosate herbicide (MON-01445-2) and cotton resistant to Lepidoptera (MON-00531-6) by traditional cross-breeding method. Regarding the parent lines above, it was already judged there is no risk that the use of each of these recombinant cotton in accordance with Type I Use Regulation in the same way as this stack cotton, causes Adverse Effect on Biological Diversity.

It is suggested that CP4 EPSPS protein which is encoded by modified *cp4 epsps* gene (gene tolerant to glyphosate herbicide) derived from MON-01445-2 has high substrate specificity, and Cry1Ac protein, which is encoded by modified *cry1c* gene (gene resistant to Lepidoptera) derived from MON-00531-6 does not have enzyme activity. Thus, it is considered that these two proteins have no interaction.

In addition, resistance to Lepidoptera of this stack cotton was examined by biological examination in number of observed insects like Tobacco Budworm [*Heliothis virescens* (Fabricius)] and Cotton Bollworm (*Helicoverpa zea*), and tolerance to glyphosate herbicide was examined by glyphosate-spraying test. As a result, significant difference was not confirmed between this stack cotton and the parent lines.

Based on the above understanding, regarding this stack cotton, it is considered that there is no change of significant characteristics except having the characteristics of parent lines.

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

(1) Competitiveness

This stack cotton has tolerance to glyphosate herbicide derived from MON-01445-2, and also resistance to Lepidoptera derived from MON-00531-6. However, it is not considered that the glyphosate exerts pressure for selection under a natural environment, and also the insect damage by Lepidoptera is not the main factor to inhibit the growth of cotton under a natural environment in Japan. Thus, it is considered that these characteristics are not the characteristics to raise competitiveness, and this stack cotton is not considered to become dominant in competition as compared with parent lines. Based on the above understanding, the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to dominance in competition is valid.

(2) Productivity of harmful substances

This stack cotton has the production of CP4 EPSPS protein from MON-01445-2, and also the production of Cry1AC protein from MON-00531-6. Cry1Ac protein has the insecticidal activity to Lepidoptera, but CP4 EPSPS protein has the same functions as EPSPS inhering in plant body, except the function that CP4 EPSPS is not inhibited the activity by glyphosate, and it is considered not to possess the characteristic to raise productivity of harmful substances. Thus, it is considered that the productivity of harmful substances of this stack cotton would not become higher than that of parent lines. Based on the above understanding, it was concluded that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances.

（3）Crossability

In the Japanese natural environment, there are no wild species which cross 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.

2. Conclusion

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant cotton in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above is valid.