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<br>(*cry1Ac*, *cry2Ab*, *Gossypium hirsutum* L.)<br>(15985, OECD UI : MON-15985-7) |
| 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

Cotton resistant to Lepidoptera (*cry1Ac*, *cry2Ab*, *Gossypium hirsutum* L.) (15985, OECD UI : MON-15985-7) (hereinafter referred to as "this recombinant cotton") has been developed by newly introducing *cry2Ab* gene derived from *Bacillus thuringiensis* subsp. *kurstaki (B.t.k)* to the recombinant cotton DP50B which has been created by repeated crossing of cotton resistant to Lepidoptera (*cry1Ac*, *Gossypium hirsutum* L.) (531, OECD UI : MON- ØØ 531-6) (hereinafter referred to as "531") ,expresses modified Cry1Ac protein by introducing *cry2Ab* gene derived from *B.t.k*, and the non-recombinant control cotton DP50. Therefore, the information concerning 531 and this recombinant cotton are shown in the following individually.

## 1. Information concerning donor nucleic acid

### (1) Composition and origins of component elements

Composition of the donor nucleic acid that was used for the development of 531 and this recombinant cotton and the origins of component elements are shown in Table 1 (p4) and Table 2 (p5).

### (2) Functions of component elements

Functions of component elements of donor nucleic acid that was used for the development of 531 are shown in Table1 (p4). Functions of component elements of donor nucleic acid that was used for the development of this recombinant cotton are shown in Table 2 (p5).

【Modified *cry1Ac* gene】

a) 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 in order to enhance its expression level in plants, and its homology of amino acid sequence is 99.4%. The Cry1Ac protein expressed in this recombinant cotton is hereinafter referred to as "modified Cry1Ac protein". 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 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 above mentioned important cotton insect pests: 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 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 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.

b) In order to investigate whether the Cry1Ac protein shares functionally important amino acid sequences with known contact allergens, the Cry1Ac protein was compared with allergens in the database (SwissProt, GenPept, PIR, GenBank/EMBL). As a result, the Cry1Ac protein did not share structurally related homologous sequences with any of the known allergens examined.

【*cry2Ab* gene】

a) Cry2Ab protein which is encoded by the *cry2Ab* gene is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium, universally exists in soil. Cry2Ab protein is also called Cry2Ab2、CryIIB, CryB2 or CryIIAb. The Cry2Ab protein, as well as Cry1Ac 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. Cry2Ab protein exhibits insecticidal activity also against insects of order Lepidoptera: for example, Fall Armyworm (*Spodoptera frugiperda*), Beet Armyworm (*Spodoptera exigua*) and Soybean Looper (*Pseudoplusia includens*), which do not show much sensitivity against Cry1Ac protein.

b) In order to investigate whether the Cry2Ab protein shares functionally important amino acid

sequences with known contact allergens, the Cry2Ab protein was compared with allergens in the database (SwissProt, GenPept, PIR, GenBank/EMBL). As a result, the Cry2Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

【Modified *cry1Ac* gene＋*cry2Ab* gene】

Since Cry2Ab protein is newly expressed in this recombinant cotton, as well as Cry1Ac protein derived from 531, it can control Fall Armyworm, Beet Armyworm and Soybean Looper, which have not been controlled by 531.

In addition, this recombinant cotton expresses Cry1Ac protein and Cry2Ab protein, both proteins have relatively overlapped insecticidal spectrum. Therefore, insects of order Lepidoptera, which shows sensitivity against both Bt proteins, could not become resistant insects without obtaining resistance for each of Bt proteins. Based on the above, it is expected that this recombinant cotton can lower the probability to obtain the resistance of insects of order Lepidoptera that shows sensitivity against both Bt proteins, as compared with 531 that expresses only Cry1Ac protein.

Table 1 Component elements of the vector PV-GHBK04 to be used for the development of 531

| Component elements | Origin and Function |
|---|---|
| *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. |
| NOS3' | 3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens*. It terminates transcription and induces polyadenylation. |
| 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*. |

Table 2 Component elements of the vector PV-GHBK11L to be used for the development of this recombinant cotton

| Component elements | Function |
|---|---|
| *uidA* gene expression cassette | |
| E35S | Promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). |
| *uidA* | A *uidA* gene derived from *E.coli* plasmid pUC19. Encodes GUS (β-D-glucuronidase) protein. |
| NOS3' | 3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens*. It terminates transcription and induces polyadenylation. |
| *cry2Ab* gene expression cassette | |
| E35S | Promoter with duplicated enhancer, from cauliflower mosaic virus (CaMV). |
| PetHSP70 leader | 5' untranslated region of hsp70 (heat shock protein) of *Petunia hybrida.* |
| AEPSPS/CTP 2 | The sequence that encodes the N-terminal chloroplast transit peptide sequence derived form the *Arabidopsis thaliana* EPSPS gene. |
| *cry2Ab* | A gene that encodes the Cry2Ab protein that 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. Cry2Ab protein exhibits insecticidal activity also against insects of order Lepidoptera: for example, Fall Armyworm (*Spodoptera frugiperda*), Beet Armyworm (*Spodoptera exigua*) and Soybean Looper (*Pseudoplusia includens*), which are the pest insects that damage cotton cultivation. |
| NOS3' | 3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens*. It terminates transcription and induces polyadenylation. |

## 2. Information concerning vector

(1) Name and origin

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

(2) Properties

The total number of base pairs of the vector PV-GHBK04531 used to generate 531 is 11,407bp.
The total number of base pairs of the vector PV-GHBK11 used to generate this recombinant cotton is 8,718bp.

The 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 vector is not known.

## 3. Method of preparing living modified organisms

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

To introduce gene to plant cells, PV-GHBK11 was treated by restriction enzyme *Kpn*I, and linear DNA fragment PV-GHBK11L to be composed of *uidA* gene expression cassette ([P-e35S]-[*uidA*]-[NOS 3']) and *cry2Ab* gene expression cassette ([P-e35S] )-[PetHSP70 leader]-[AEPSPS/CTP2]-[*cry2Ab*]-[NOS3']) was used.

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

Vector PV-GHBK04 was introduced into a current cotton cultivar, Coker 312, the recipient organism of 531, by the Agrobacterium method.

Vector PV-GHBK11L was introduced into the recombinant cotton cultivar, DP50B, the recipient organism of this recombinant cotton, by the particle gun bombardment. DP50B is a commercialized cotton cultivar to be developed by repeated crossing of 531 and the

non-recombinant control cotton cultivar DP50.

(3) Processes of rearing of living modified organisms

【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 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, Labor 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, 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".

【Processes of rearing of this recombinant cotton】

a) The recombinant cotton cultivar DP50B was used as the mother plant of recombinant, and PV-GHBK11L was introduced into its shoot apex cells by the particle gun bombardment. Selection of regenerated individuals was carried out by histochemical staining method to use GUS protein.

b) Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes derived from PV-GHBK11L and the expression level of the Cry2Ab protein and 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.

March, 2001: The US Environmental Protection Agency (EPA) exempted the Cry2Ab protein from the specification of a residual standard value.

July, 2002: The US Food and Drug Administration (FDA) approved the safety of the cultivar as food and feed.

November, 2002: The United States Department of Agriculture (USDA) approved unlimited cultivation of the cultivar.

September, 2002: Food Standards Australia New Zealand (FSANZ) approved the safety of the cultivar as food.

October, 2002: 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.

July, 2001: 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.

October, 2002: 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".

## 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 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

R5 generation genome DNA, the selfed line 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 (Figure 1, p12).

However, 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 (both are BC2F3), it was confirmed that the 1st and 2nd inserted genes were stably inherited in progeny. Incidentally, the 2 commercialized cultivars do not contain the 3rd inserted gene. 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 remain in the selfed line of R5 and R6 generations, on the other hand, the 3rd inserted gene may have been separated from the others during back-crossing process in the commercialized cultivars. 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, the stability of Cry1A protein expression in this recombinant cotton was confirmed by ELISA analysis during the selection in the rearing process.

【State of existence of nucleic acid transferred in this recombinant cotton and stability of expression of traits】

As a result of the analysis of inserted gene by Southern blotting analysis, it was confirmed that one copy of the inserted gene was inserted at one site in the genome of the chromosome of this recombinant cotton. Then, as a result to confirm the completeness of *cry2Ab* gene expression cassette and *uidA* gene expression cassette by using the component elements of each gene expression cassettes as proofs, it was indicated that *cry2Ab* gene expression cassette was inserted in complete condition; on the other hand, *uidA* gene expression cassette was inserted in partially defective condition. It was confirmed that the defective part of this *uidA* gene expression cassette was about 279bp at 5'-terminal side of P-e35S and polylinker from multi-cloning site of about 24bp, as a result of genome walking analysis around neighboring sequence of the inserted gene. Figure 2 (p13) shows the map of inserted gene.

In addition, as a result of Western blotting analysis, Cry2Ab protein showed stable expression in

R1, R3, R4 and BC2F3 generations of this recombinant cotton. It was considered that the R1 band is thin, because dominant homo individuals and recessive homo individuals exist together in R1, which is a segregating population.

As a result of base sequence analysis of the inserted gene, the 1,490th base from 5' terminal of *uidA* gene changed from guanine (G) to adenine (A) in comparison with *uidA* gene sequence in plant expression plasmid to be introduced into *E.coli*. As a result, it emerged that the 377th amino acid residue from N terminal of amino acid sequence changed from glutamine (E) to lysine (K) (hereinafter referred this protein to as "GUSE377K").

Regarding this GUSE377K, it was considered that GUSE377K is equal to normal GUS protein in its component and function, based on the following understanding: i) the 377th amino acid from N terminal of amino acid sequence in which the change of amino acid was confirmed is not the amino acid to be included in active region that is preserved commonly in all GUS protein family expressed in plants, microorganisms and mammals; ii) this amino acid variation does not effect the active region and its three-dimensional structure of GUS protein; iii) as a result of examining whether GUSE377K shares amino acid sequence with known allergens or not with use of the protein data base (SwissProt ver.30, PIR ver.41), GUSE377K shows no homology of sequence between known allergens.

In addition, the generation analyzed of the inserted gene is the R3 generation and the multiple BC2F3 generations derived from the R1 generation, that were evaluated by environmental safety tests in the US. In all generations analyzed, the 1,490th base from 5' terminal of *uidA* gene appeared to be adenine (A). Therefore, it was concluded that the change from guanine (G) to adenine (A) of the 1,490th base from 5' terminal of *uidA* gene was brought by replication of plasmid in *E. coli* for expression in plant or by gene introduction by particle gun method, not brought during transmission to progeny. Based on the above, it was suggested that GUSE377K is expressed in R1 and R4 generations evaluated by environmental safety test in Japan.

In addition, the hereditary stability of this recombinant cotton was confirmed by Southern blotting analysis in multiple generations (R1, R2, R3 and R4, which are selfed lines from 15985; and BC2F3 generation developed by crossing with two current cotton cultivars).

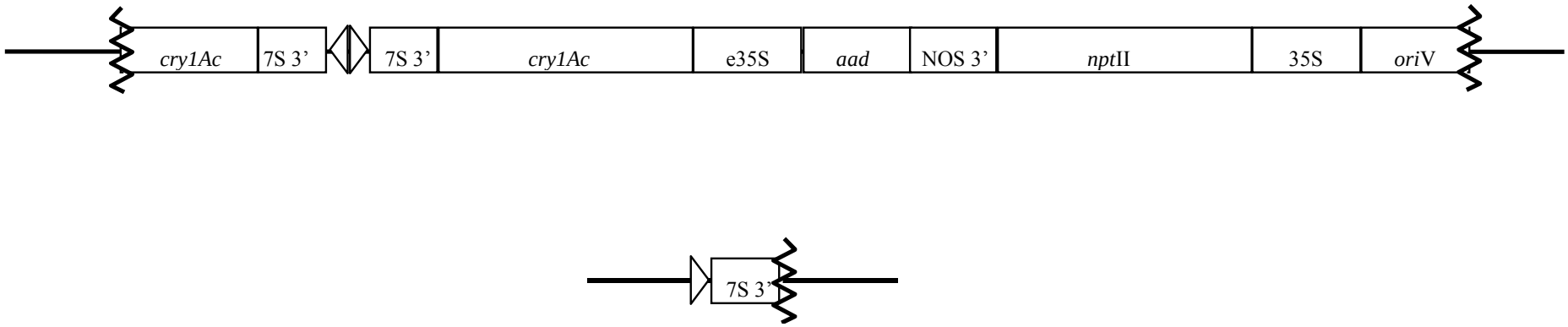


Figure 1 Map of inserted gene of Cotton resistant to Lepidoptera 531

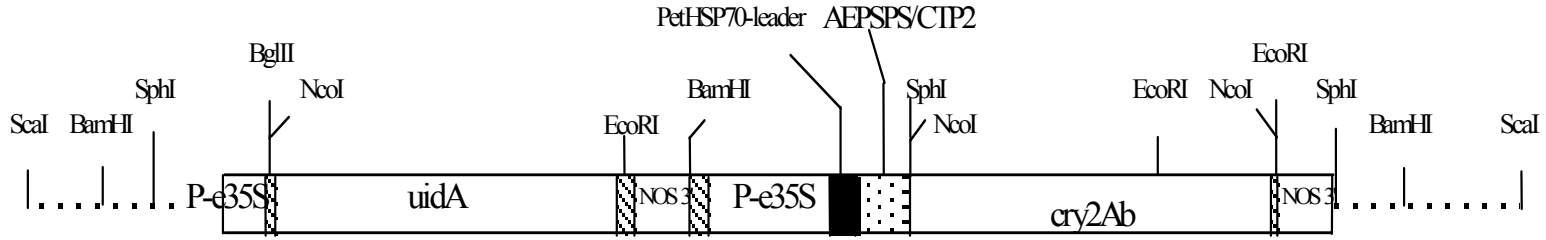


Figure 2 Map of inserted gene of Cotton resistant to Lepidoptera 15985

## 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

（1）In this recombinant cotton, modified Cry 1Ac protein encoded by modified *cry 1Ac* gene and Cry2Ab protein encoded by *cry2Ab* gene are expressed. It was shown that Cry2Ab protein is expressed in young leaves, leaves, seeds and plant body of this recombinant cotton. On the other hand, regarding modified Cry1Ac protein, the amount of expression in this recombinant cotton and DP50B, the recombinant mother plant is examined with the use of young leaves, leaves, seeds, plant body and pollens. As a result, no difference is found between this recombinant cotton and DP50B, regarding the amount of expression of Cry1Ac protein in each organs. Therefore, it was proved that modified Cry1Ac protein and Cry2Ab protein do not show interaction in this recombinant cotton. In addition, regarding NPTII protein, the amount of expression is also examined in this recombinant cotton and DP50B with the use of leaves and seeds, no significant difference was found between this recombinant cotton and DP50B.

（2） Isolated fields tests of this recombinant cotton were carried out in isolated fields of Kyusyu National Agricultural Experiment Station and Kawachi Research Farm (KRF), Monsanto Japan Limited, with the use of R4 and R1 generations' seeds for both locations from May 2000 to March 2001. The original plan was that isolated field tests would be carried out with the use of R4 generation in Kyusyu National Agricultural Experiment Station only. However, the back crossing program of this recombinant cotton with the other cultivars started in R2 generation. In April, 2000 it was pointed out that in case of using seeds of R4, safety evaluation test could be conducted only for R4 generation downward. Therefore, isolated field tests were hurriedly carried out in Kawachi Research Farm (KRF), Monsanto Japan Limited, other than Kyusyu National Agricultural Experiment Station, with the use of seeds of R1 generation.

In addition, this isolated field test was conducted with using this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50. DP50B is the recombinant commercialized cotton cultivar derived from repeated crossing of 531 and DP50 that is the non-recombinant control cotton cultivar.

a) Morphological and growth characteristics

Differences in the following 20 items of morphological and growth characteristics were

examined between this recombinant cotton and the 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 non-harvested bolls; 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. For characteristics concerning balls, two balls per plant were measured. 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, no difference was observed among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 in isolated field tests conducted in Kawachi Research Farm (KRF) with using R1 generation.

On the other hand, in isolated field tests in Kyusyu National Agricultural Experiment Station with using R4 generation, there was a significant difference in leaf shape (leaf length) the weight of under-ground part. However, no difference was observed in other items. Regarding leaf length, the significant difference from this recombinant cotton was observed both in the recombinant mother plant DP50B and the non-recombinant control cotton DP50, and the average leaf length of this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 were 16.5cm, 17.8cm and 17.9cm. Regarding the weight of under-ground part, the significant difference from this recombinant cotton was observed only in the non-recombinant control cotton DP50, and there was no difference between this recombinant cotton and recombinant mother plant DP50B. The average weight of Under-ground part of this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 were 163.3g, 156.7g and 133.3g.

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

Chilling-tolerance tests are not conducted at the early stage of growth in isolated fields. Instead, the observation of volunteer individuals up to the following spring is carried out in 22 isolated fields in the US. All of these fields are located in famous regions for cotton cultivation in the South of the US. Besides, compared to average climate 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 observation, it was found that some seeds split 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 cotton 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. So, in this isolated field test, mainly the possibility that seeds spilled during transportation would germinate, grow and become self-seeding was investigated. 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, shedding habit, dormancy and germination rate of the seed

Regarding seed production, the differences among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 have been 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, no statistically significant difference was observed in all items for both R1 and R4 generations.

In both of 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.

To evaluate dormancy, in 3 isolated fields in Texas (TX) , South Caroline (SC) and Louisiana (LA) in the US in 1999, germination rate of the seed was examined with using the harvested

seeds of this recombinant cotton, the recombinant mother plant DP50B, the non-recombinant control cotton DP50 and 11 current cultivars, under the different temperature condition from 5 to 40℃.

As a result, under some temperature conditions, statistically significant difference ($p \leq 0.05$) was observed between this recombinant cotton and the recombinant mother plant DP50B. However, the difference was within the value range of 11 current cultivars which were added for reference. On the other hand, in various temperature conditions, the seeds of this recombinant cotton, the recombinant mother plant DP50B and 11 current cultivars were germinated, Viable Firm Swollen or degenerated, and no seed of Viable Hard was observed.

Germination rate was examined in "a) Morphological and growth characteristics". As a result, no difference was observed between the recombinant mother plant DP50B and the non-recombinant control cotton DP50 in both R1 and R4 generations.

f) Crossability

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

g) Productivity of harmful substances

Plow-in test, succeeding crop test and soil microflora test were carried out among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50.

【Plow-in test】

As a result of plow-in test by using plant body of R1 generation, regarding germination rate and early stage growth of lettuce as the indicator plant, no statistically significant difference was observed among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50.

In addition, as a result of plow-in test by using plant body of R4 generation, no statistically significant difference was observed among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 in all items.

Based on the above understanding, it was judged that the effect of plowing this recombinant cotton in soil on the growth of indicator plants is equal to those of the recombinant mother plant DP50B and the non-recombinant control cotton DP50.

【Succeeding crop test】

As a result of succeeding crop test of lettuce using soil cultivated with R1generation, regarding germination rate and early stage growth of lettuce, no statistically significant difference was observed among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50.

In addition, as a result of succeeding crop test by using plant body of R4 generation, no statistically significant difference was observed among this recombinant cotton, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 in all items.

Based on the above understanding, it was judged that the effect on succeeding crops of the soil in which this recombinant cotton was cultivated is equal to the recombinant mother plant DP50B and the non-recombinant control cotton DP50.

【Soil microflora test】

The number of each microorganism is almost equal in the soil in which R1generation was cultivated and before transplantation. In addition, regarding the number of microorganisms in soil at harvesting, no statistically significant difference was observed among the cultivation areas of the non-recombinant control cotton DP50, the recombinant mother plant DP50B and this recombinant cotton.

The number of each microorganism is almost equal in the soil in which R4generation was cultivated and before sowing. In addition, regarding the number of microorganisms in soil and soil microflora in lateral root at harvesting, no statistically significant difference was observed among the cultivation areas of the non-recombinant control cotton DP50, the recombinant mother plant DP50B and this recombinant cotton.

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

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

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

(1) Competitiveness

The plant body of cotton species（*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 available 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 and *cry2Ab* gene confer resistance to Lepidoptera to this recombinant cotton. However, the damage by Lepidoptera is not the main factor to inhibit the cotton growth under natural environment in Japan. In addition, from the result of the examination of characteristics in competition (examination such as morphological and growth characteristics, and productivity of the seeds in isolated fields), 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 cotton 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 it was concluded that the use of such cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness. It was judged that the conclusion made by the applicant above is valid.

(2) Productivity of harmful substances

Into this recombinant cotton, the modified *cry1Ac* gene and *cry2Ab* gene have been introduced, conferring to the ability to produce Cry1Ac protein and Cry2Ab protein that have 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 and Cry2Ab 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 conclusion 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, which was made by the applicant, is valid.

(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. It was judged that the conclusion above made by applicant is valid.

## 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.