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

Name: Bayer Crop Science K.K.
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (Modified *bar,* Modified *cry1Ac, cry2Ab, Gossypium hirsutum* L.) (LLCotton25×15985, OECD UI:ACS-GH001-3×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 Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

The LLCotton25 and the 15985, the parent strains of this stack line cotton, are genetically modified cotton cultivars produced respectively by Bayer Crop Science K.K. and Monsanto Company in the US.

In Japan, in accordance with the provisions in Clause 2, Article 4 of the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Law No. 97 of 2003), the approval of the competent minister for the Type 1 Use Regulations was obtained in February 10, 2006 for the LLCotton25 and in December 10, 2004 for the 15985.

The description about the 15985 provided below is based on the summary of the published Biological Diversity Risk Assessment Report and other related reference materials. The cotton 15985 was produced by the newly transferring *cry2Ab* gene derived from *Bacillus thuringiensis* subsp. *kurstaki* to the recombinant cotton DP50B which was produced by repeated crossing of the cotton 531 and the non-recombinant cotton cultivar DP50.

1) Composition and origins of component elements

Composition of the donor nucleic acid that was used for the production of the individual recombinant cotton cultivars and the origins of component elements are shown in Table 1 for the LLCotton25, Table 2 for the 531 and Table 3 for the 15985.

Table 1 Composition of the donor nucleic acid used for the production of the LLCotton25 and the origins of component elements

Component elements	Size (bp)	Position in vector (bp)	Origin and function
Modified *bar* gene expression cassette			
P35S3	1385	250-1634	Promoter regions derived from cauliflower mosaic virus 35S. Initiates transcription (Reference 19).
Modified *bar**	552	1635-2186	It is a bialaphos resistance (*bar*) gene derived from *Streptomyces hygroscopicus*, and encodes the modified PAT protein and gives tolerance to glufosinate herbicide (Reference 25). The two codons at the N-terminal of the wild-type *bar* gene are replaced by ATG and GAC respectively.
3'nos	260	2206-2465	3' untranslated region of the nopaline synthase (NOS) gene derived from T-DNA of pTiT37. Terminates transcription and induces 3'-polyadenylation (Reference 6).
Other component elements			
RB	25	198-222	Right border repetitive sequence of T-DNA derived from TiB6S3 (Reference 8).
LB	25	2519-2543	Left border repetitive sequence of T-DNA derived from pTiB6S3 (Reference 8).
aadA	2075	2544-4618	Sequence including a gene tolerant to streptomycin/spectinomycin, derived from Tn7 transposon (Reference 14).
pVS1ori	3780	4619-8389	Replication origin of the plasmid pVS1 derived from *Pseudomonas* (Reference 12).
ColE1	1165	8390-9555	Sequence including replication origin ColE1 ori derived from plasmid pBR322 (Reference 4).

(Note: All the rights pertinent to the information in the table above and the responsibility for the content rest upon the applicant.)

* The wild-type *bar* gene obtained from *Streptomyces hygroscopicus* has GTG modified to ATG to make the codon suitable for use in plants and AGC modified to GAC to enhance the efficiency of translation. In the modification from GTG to ATG, the amino acid to be actually translated remains as methionine, though in the modification from AGC to GAC, the amino acid changes from serine to aspartic acid. However, it has been confirmed that the functions of the PAT protein, the modified *bar* gene product by the modifications (hereinafter referred to as the "modified PAT protein"), remains unchanged (References 20, 25, and 27).

Table 2 Origins and functions of the component elements of the vector PV-GHBK04 used for the production of the 531

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.
npt II 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 transferring gene, it is used as a marker to select recombinant plant.
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 (RB)	A DNA fragment 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 *Ataphylococcus aureus*. It gives tolerance to spectinomycin and streptomycin.
oriV	The replication origin derived from the broad-host 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 of DNA from *E. coli* to *Agrobacterium tumefaciens.*

(Excerpts from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985ap.pdf)

Table 3 Origins and functions of the component elements of the vector PV-GHBK11L used for the production of the 15985

Component elements	Origin and 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.
NOS'3	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/CTP2	The sequence that encodes the N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis thaliana* EPSPS gene.
cry2Ab	It is a gene derived from *Bacillus thuringinensis* subsp. *kurstaki*, and 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. The 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.

(Excerpts from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985ap.pdf)

2) Functions of component elements

(a) Functions of component elements of donor nucleic acid, including target genes, expression-regulating regions, localization signals andselectable markers

Functions of the component elements of the nucleic acid that was used for the production of the LLCotton25, the 531 and the 15985 are respectively shown in Table 1, Table 2 and Table 3.

(b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity.

[Modified *bar* gene]

In the process of nitrogen metabolism, plants produce ammonia by nitrate reduction, amino acid decomposition, photorespiration and so on. Glutamine synthase plays an important role in detoxification of the ammonia produced, though if glufosinate herbicide is sprayed, glutamine synthase is inhibited and ammonia accumulates, causing the plant to die.

The phosphinothricin acetyl transferase (modified PAT protein) encoded by the modified *bar* gene acetylates glufosinate to make N-acetylglufosinate, and inactivates the inhibitory action of glufosinate to the glutamine synthase. By virtue of this mechanism, ammonia is not accumulated, and the plant does not die, even if it is sprayed with glufosinate herbicide.

The modified PAT protein exhibits a high affinity to glufosinate classified into *L*-amino acid, though it does not cause any acetyl group transfer reaction to the other various amino acids and it has little affinity for the glutamic acid which has specifically high structural similarity to glufosinate and it causes virtually no transfer reaction *in vivo* (Reference 25). In addition, even in the presence of excessive amounts of various amino acids, the acetyl group transfer reaction to glufosinate by the modified PAT protein was never inhibited (References 20 and 27).

Based on the amino acid sequence of the modified *bar* gene product, overall homology search (Swiss Prot, trEMBL, GeneSeq-Prot, PIR, PDB, DAD, and GenPept) and allergen epitope search were conducted. As a result, this protein did not show any homology with known toxins or allergens.

[Modified *cry1Ac* gene]

The modified *cry1Ac* gene has been produced by the 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 the 15985 is hereinafter referred to as the "modified Cry1Ac protein". The Cry1Ac protein, including the modified type, exhibits insecticidal activity against Tobacco budworm (*Heliothis virscens*),

Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm (*Helicoverpa zea*)], which are the major pest insects of the 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 and the insecticidal activity against Lepidoptera is as high as the wild-type Cry1Ac protein. The Cry1Ac protein, including the modified Cry1Ac protein, exhibits insecticidal activity also against insects other than the above mentioned major cotton insect pests: for example, the 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 the 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 the 15985 is identical to the core of the Cry1Ac protein in Bt product, which is a commercialized microbial agricultural insecticide. In the US, European countries and Japan, Bt preparation, which contains the Cry1Ac protein, has been safely used for crops and trees as an insecticide to control the order Lepidoptera.

In order to investigate whether the modified Cry1Ac protein shares functionally important amino acid sequences with known contact allergens, the modified Cry1Ac protein was compared with allergens in the database (SwissProt, GenPept, PIR and GenBank/EMBL). The results showed the modified Cyr1Ac protein did not share structurally related homologous sequences with any of the known allergens examined.

[*cry2Ab* gene]

The Cry2Ab protein which is encoded by the *cry2Ab* gene is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium universally existing in soil. The Cry2Ab protein is also called Cry2Ab2, CryIIB, CryB2 or CryIIAb. The Cry2Ab protein, as well as the Cry1Ac protein, exhibits insecticidal activity against the order Lepidoptera, including Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm (*Helicoverpa zea*)], which are the major pest insects of the order Lepidoptera that damage cotton cultivation in the US and Australia. The Cry2Ab protein exhibits insecticidal activity also against insects of the order Lepidoptera: for example, Fall Armyworm (*Spodoptera frugiperda*), Beet Armyworm (*Spodoptera exigua*) and Soybean Looper (*Pseudoplusia includens*), which do not show much sensitivity against the Cry1Ac protein.

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). The results showed the Cry2Ab protein did not share structurally related homologous sequences with any of the known allergens

examined.

[Modified *cry1Ac* gene＋*cry2Ab* gene]

The Cry2Ab protein is newly expressed in the 15985, as well as the modified Cry1Ac protein derived from the 531. The modified Cry1Ac protein exhibits insecticidal activity against Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gassypiella*) and Cotton bollworm [also called Corn earworm (*Helicoverpa zea*)], which are the major pest insects of the order Lepidoptera that damage cotton cultivation in the US and Australia. On the other hand, the Cry2Ab protein exhibits insecticidal activity also against a family of armyworms (Fall Armyworm, Beet Armyworm) and a family of budworms (Soybean Looper) in addition to the above-mentioned insects of the order Lepidoptera against which the modified Cry1Ac protein exhibits insecticidal activity. Therefore, the 15985, which expresses both proteins, modified Cry1Ac and Cry2Ab, exhibits insecticidal activity against Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gassypiella*), Cotton bollworm (*Helicoverpa zea*), a family of armyworms (Fall Armyworm, Beet Armyworm) and a family of budworms (Soybean Looper). In addition, the results of comparison of the pest control effects of the 531 and the 15985 on Tobacco budworm (*Heliothis virescens*), which shows sensitivity to both Bt proteins, confirmed that the 15985 has nearly 4 times the pest control effects of the 531 (Reference 9). {Pest control effects were determined as follows: Powdered samples of each of the 15985 and the 531 were diluted with 0.2% agar solution by a factor of 20,000 and then fed to the larvae of Tobacco budworm, which is 12 to 24 hours after hutching. The rate of the larvae surviving up to the 3rd instar stage was determined in units of percentage (%). The survival rate was converted into the concentration of the modified Cry1Ac protein (μg/g dry weight) based on the predefined standard curve.} It is considered that insects of the order Lepidoptera, which shows sensitivity against both Bt proteins, could not become resistant insects without obtaining resistance for each of the Bt proteins.

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

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(2) Information concerning vector

1) Name and origin

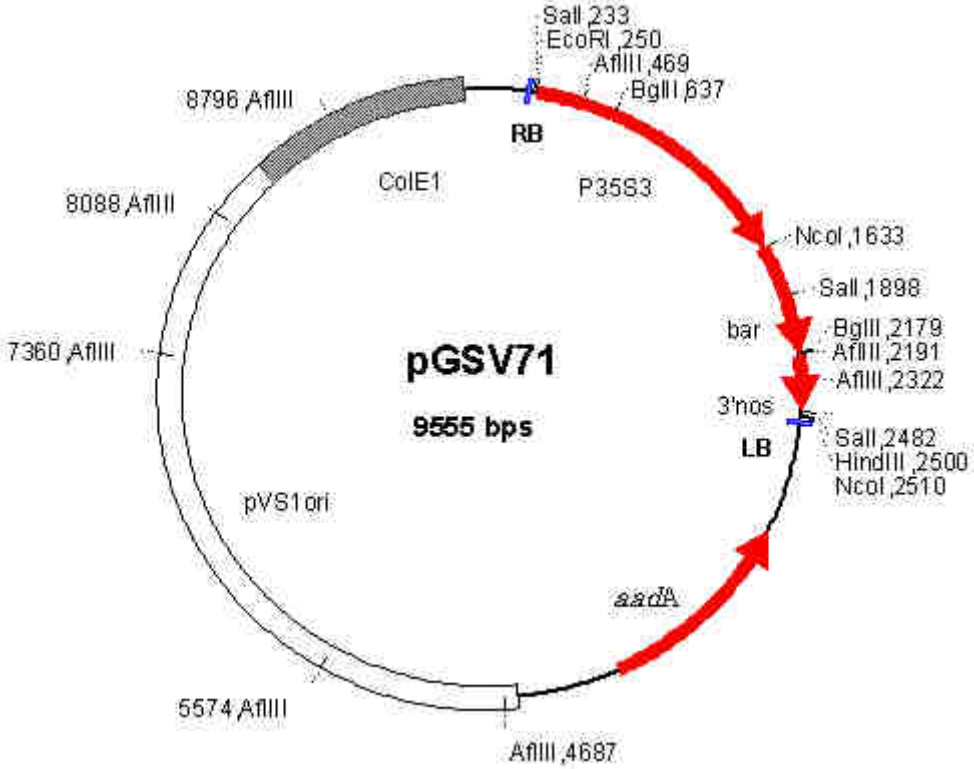
The plasmid vector pGSV71 used for the production of the LLCotton25 was constructed based on the plasmid pBR322 (Reference 4) derived from *Escherichia coli* and the plasmid vector pVS1 (Reference 12) derived from *Pseudomonas aeruginosa* (Reference 23). The vector used for the production of the 531 and the 15985 is derived from the plasmid pBR322.

2) Properties

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

The number of base pairs of the vector pGSV71 used for the production of the LLCotton25 is 9,555bp. The plasmid map and the sites cleavaged by restriction enzymes are shown in Figure 1.

The total number of base pairs of the vector PV-GHBK04 used to produce the 531 is 11,407bp. The total number of base pairs of the vector PV-GHBK11 used to produce the 15985 is 8,718bp. The plasmid map and the sites cleavaged by restriction enzymes are shown in Figure 2 and Figure 3.



bar：Modified *bar* gene

Figure 1　Map of the plasmid pGSV71 used to produce the LLCotton25 and sites cleavaged by restriction enzymes

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

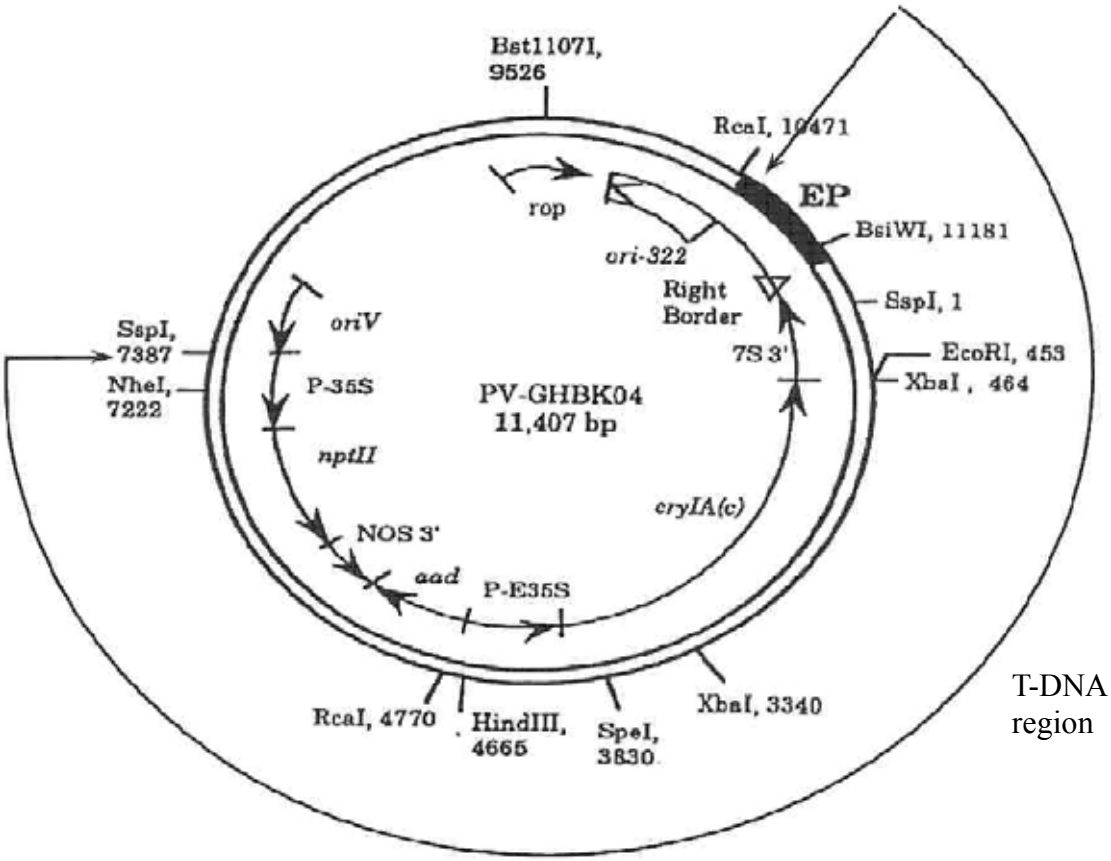


Figure 2 Map of the plasmid PV-GHBK04 used to produce the 531 and sites cleavaged by restriction enzymes

(Excerpt from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf)

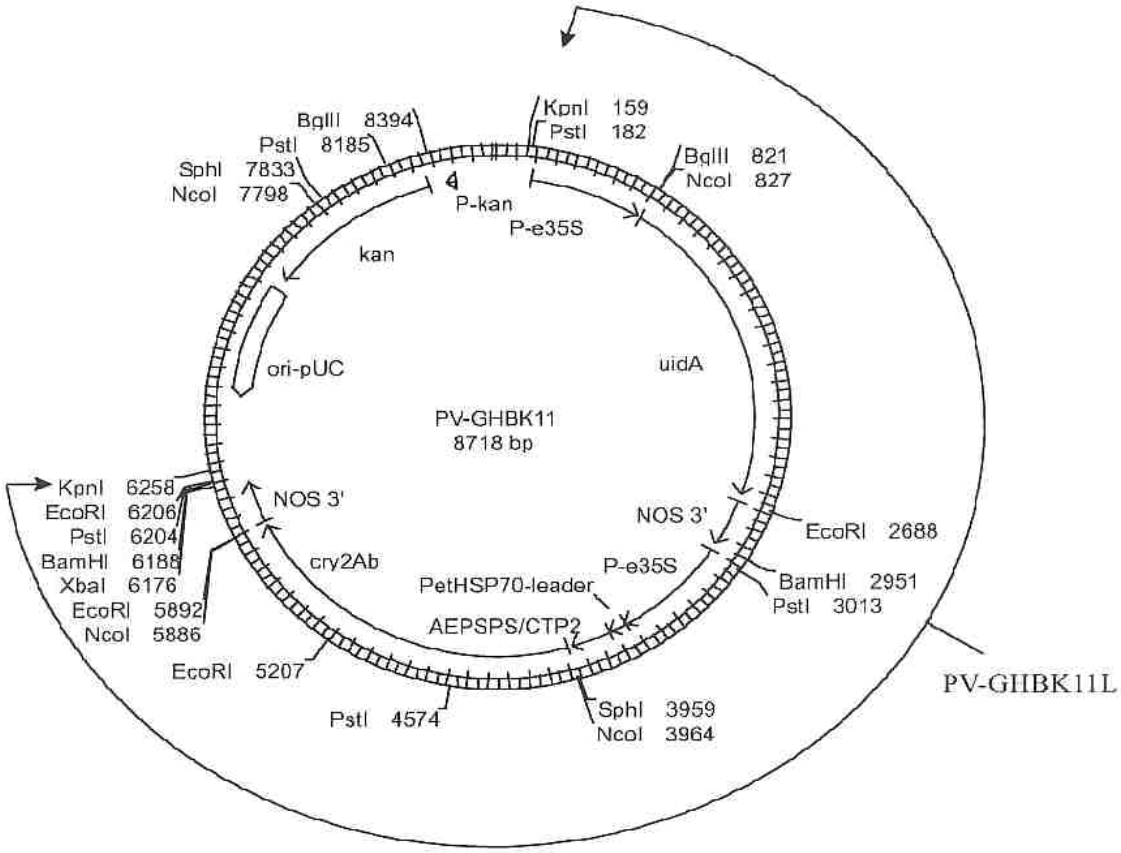


Figure 3 Map of the plasmid PV-GHBK11 used to produce the 15985 and sites cleavaged by restriction enzymes

(Excerpt from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf)

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

The plasmid pGSV71 used for the transformation of the LLCotton25 has a selective marker gene (*aadA* gene derived from *E. coli*) that confers tolerance to streptomycin and spectinomycin (Reference 14). The *aadA* gene is used as a selective marker when the plasmid pGSV71 for transformation of Cotton is constructed using *E. coli*, and since this gene does not have a promoter functioning in a plant, it cannot be considered that the gene is expressed in a plant. Furthermore, the plasmid pGSV71 has the replication origin ColE1ori derived from the plasmid pBR322 of *E. coli* and the replication origin pVS1ori of the plasmid vector pVS1 of *Pseudomonas aeruginosa*, and though they function to cause autonomous replication in *E. coli* and *Pseudomonas aeruginosa* respectively, they do not function in plants. These replication origins are positioned outside the T-DNA region, and it is confirmed based on the Southern blotting analysis that they are not transferred into the plant cells.

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

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(3) Method of preparing living modified organisms

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

The positions and directions of the component elements of the donor nucleic acid and the restriction enzyme cleavage sites in the vectors used for the production of the LLCotton25, the 531 and the 15985 are shown in Figure 1, Figure 2 and Figure 3 respectively.

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

For transferring the plasmid pGSV71 into a current cotton cultivar Coker312 for the production of the LLCotton25, the *Agrobacterium* method was used. A tissue fragment (region from hypocotyls to radicle) excised from an immature cultivar Coker312 was exposed to and infected with a culture solution of *A.tumefaciens* containing the Ti plasmid pGV3000 and the binary vector pGSV71, for integrating the T-DNA region placed between RB and LB on the plasmid pGSV71 into the cotton genome.

For transferring the vector PV-GHBK04 into a current cotton cultivar, Coker312, the recipient organism of the 531, the *Agrobacterium* method was used.

For transferring the vector PV-GHBK11L into the recombinant cotton cultivar, DP50B, the recipient organism of the 15985, the particle gun bombardment was used. The DP50B is a commercialized cotton cultivar to be produced by repeated crossing of the 531 and the non-recombinant cotton cultivar DP50.

3) Processes of rearing of living modified organisms

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

To create the LLCotton25, plant individuals were reproduced from the tissue fragment transplanted into a reproduction medium, and furthermore, a reproduction medium containing glufosinate was used to select strains tolerant to glufosinate.

To create the 531, the T-DNA region of the vector PV-GHBK04 was transferred into the hypocotyls of the Coker312 by the *Agrobacterium* method, and then regenerated individuals were obtained by culturing them in media containing kanamycin.

To create the 15985, the recombinant cotton cultivar DP50B obtained by repeated crossing of the 531 and the non-recombinant cotton cultivar DP50 was used as the mother plant of recombinant, and the PV-GHBK11L was transferred into its shoot apex cells by the particle gun bombardment. Selection of regenerated individuals was carried out by the histochemical staining method to use the GUS protein.

(b) Presence of any residual cell of *Agrobacterium*

For the LLCotton25, *Agrobacterium* is removed by a reproduction medium containing claforan.

For the 531, in order to eliminate *Agrobacterium* from the transgenic plant, the transgenic plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics.

(c) Process of rearing and pedigree tree

For the LLCotton25, regarding the obtained regenerated individuals, selective breeding was conducted, and then the LLCotton25 was selected based on the comprehensive evaluation of the expression level of the modified PAT protein, agronomic character and other factors.

For the 531, regarding the obtained regenerated individual, further selection was carried out based on the analysis of transferred genes and the expression level of the modified Cry1Ac protein. Tests in climate chambers and greenhouses were then carried out, and actual pest insect resistance and agronomic characters were examined in outdoor field tests. Then the 531 was selected based on the comprehensive evaluation of these results.

For the 15985, regarding the obtained regenerated individual, further selection was carried out based on the analysis of transferred genes derived from the PV-GHBK11L and the expression level of the Cry2Ab protein and the modified Cry1Ac protein. Tests in climate chambers and greenhouses were then carried out, and actual pest insect resistance and agronomic characters

were examined in outdoor field tests. Then the 15985 was selected based on the comprehensive evaluation of these results.

This stack line cotton was produced by crossing the two cotton products, the LLCotton25 and the 15985, with the use of the conventional breeding methods. A backcross of variety A with the LLCotton25 and the 15985 was repeated to transfer the variety A into the genetic background, and then the two lines were crossed with each other. Then, after repeating inbreeding, this stack line cotton was obtained.

Process of rearing of this stack line cotton is shown in Figure 4.

Regarding the LLCotton25, the 531 and the 15985, the approvals received from the related regulatory agencies in Japan are listed below.

LLCotton25

June, 2004: The approval was obtained in accordance with "Clause 2, Article 3 of the Procedure for the Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" from the Ministry of Health, Labour and Welfare.

February, 2006: The approval was obtained in accordance with "Article 4 of the Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques" from the Ministry of Agriculture, Forestry and Fisheries.

February, 2006: The approval on importing (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them) was obtained in accordance with the provisions in "Clause 2, Article 4 of the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms from the Ministry of Agriculture, Forestry and Fisheries.

531

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", the safety of use for food was approved by the Ministry of Health, Labour and Welfare.

June, 1997: Based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)" by the Ministry of Agriculture, Forestry and Fisheries,

safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries

March, 2001: The approval was obtained in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" was certified by the Ministry of Health, Labour and Welfare.

March, 2003: Based on the "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques", the safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

November, 2004: The approval on importing (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them) was obtained in the provisions in "Clause 2, Article 4 of the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organismsfrom the Ministry of Agriculture, Forestry and Fisheries.

15985

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 approval was obtained, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" from the Ministry of Health, Labour and Welfare.

March, 2003: Based on "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques", the safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries.

December, 2004: The approval on importing (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them) was obtained in accordance with the provisions in "Clause 2, Article 4 of the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms from the Ministry of Agriculture, Forestry and Fisheries.

Figure 4 Process of rearing this stack line cotton

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

1) Place where the replication product of transferred nucleic acid exists

The T1-generation transgenic plant of the LLCotton25 and the first generation of backcross (BC1F1) exhibited the segregation ratio corresponding to segregation patterns of single dominance (ratio of glufosinate herbicide tolerance to glufosinate herbicide sensitivity of 3:1 in the T1 generation and 1:1 in the BC1F1 generation). As a result, the transferred gene is considered to exist on one chromosome.

Regarding the 531, as a result of the analysis of the transferred gene by the Southern blotting analysis, the cosmid cloning technique and the genome walking method, the transferred gene was found to exist in the genome DNA of this recombinant cotton.

Regarding the 15985, as a result of the analysis of the transferred gene by the Southern blotting analysis, it was confirmed that one copy of the transferred gene was transferred at one site in the genome of the chromosome.

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

In the LLCotton25, one copy of the replication product of the transferred nucleic acid is transferred, and the stability of the transferred nucleic acid in multiple generations has been already confirmed in the assessment of Adverse Effect on Biological Diversity.

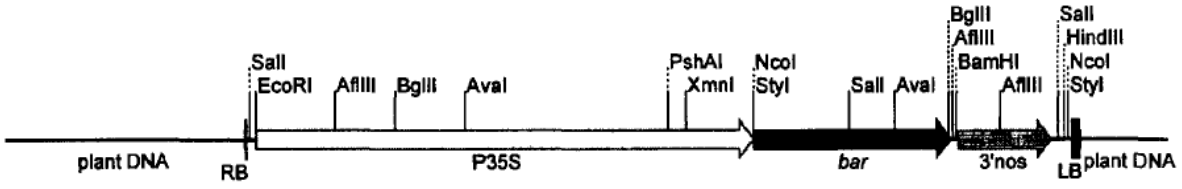
In the 531, the transferred genes were found in the following 3 regions: the 1st transferred gene consisting of the modified *cry1Ac* gene expression cassette, the *nptII* gene expression cassette, and the *aad* gene expression cassette; the 2nd transferred gene that consists of a 3' region fragment of the modified *cry1Ac* gene and the 7S3' terminator, which are transferred next to the 5' terminal of the 1st transferred gene, in the reverse direction; and the 3rd transferred gene consisting of a 7S3' terminator fragment of 245bp. In addition, as a result of the Southern blotting analysis, it was confirmed that the 1st and 2nd transferred genes were stably inherited in progeny. The commercialized cultivars do not contain the 3rd transferred gene, the fragment of 7S3' sequence, in the genome DNA. A possible reason is that the location of the 3rd transferred gene was, on the chromosome, distant from the 1st and 2nd transferred genes; therefore, the 3rd transferred gene may remain in the selfed line of R5 and R6 generations, on the other hand, the 3rd transferred gene may have been separated from the others during the back-crossing process in the commercialized cultivars. Besides, since the 3rd transferred gene was a fragment of the 7S3' sequence, which terminates transcription, it does not contribute to this recombinant cotton's resistance to Lepidoptera. Therefore, during backcrossing breeding, the 3rd transferred gene was not used for selection.

In the 15985, one copy of replication product of transferred nucleic acid is transferred, and the stability of its inheritance through multiple generations has

been already confirmed in the assessment of Adverse Effect on Biological Diversity.

The transferred T-DNA regions are shown in Figures 5, 6 and 7.

Regarding this stack line cotton (F6 generation), the levels of the modified PAT protein, the modified Cry1Ac protein and the Cry2Ab protein expressed respectively by the modified *bar* gene, the modified *cry1Ac* gene and the *cry2Ab* gene transferred into this stack line cotton were compared based on the ELISA method with those in the LLCotton25, the 531 and the 15985. The results showed no significant difference for the proteins compared (Table 6 and Annex 3), and it was confirmed that in this stack line cotton, the modified *bar* gene, the modified *cry1Ac* gene and the *cry2Ab* gene are expressed at the similar levels as in the parent lines.



bar：Modified *bar* gene

Figure 5　Map of the transferred gene to the LLCotton25

(Note: All the rights pertinent to the information in the table above and the responsibility for the content rest upon the applicant.)

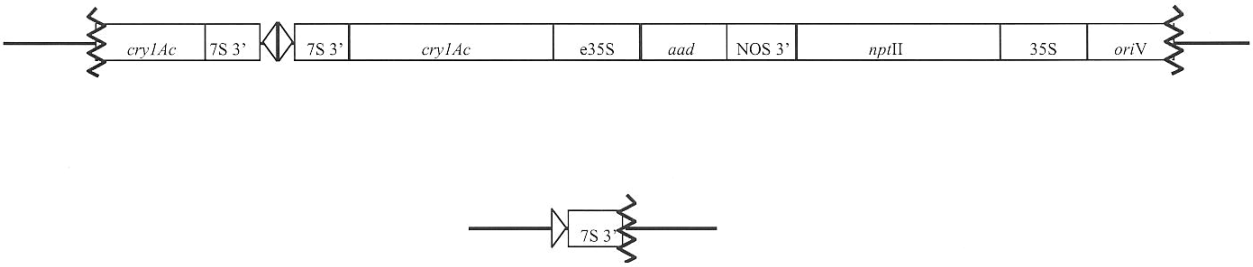


Figure 6　Map of the transferred gene to the 531

(Excerpt from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985ap.pdf)

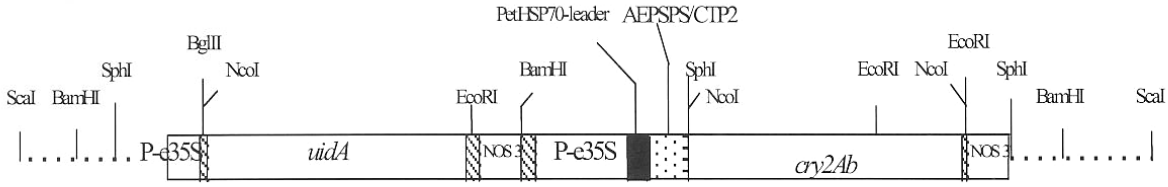


Figure 7　Map of the transferred gene to the15985

(Excerpt from http://www.bch.biodic.go.jp/download/lmo/public_comment/15985ap.pdf)

3) The position relationship in the case of multiple copies existing in chromosome

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4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

Regarding the LLCotton25, inter-individual or inter-generational expression stability due to spraying of glufosinate herbicide has been confirmed. In addition, the modified PAT protein in the seeds, lint coat and lint of the LLCotton25 was determined by the ELISA analysis. As a result, it is confirmed that the modified PAT protein is expressed in the respective tissues.

The stability of expression of the modified Cry1Ac protein in the 531 was confirmed by the ELISA analysis during the selection in the process of rearing.

Regarding the 15985, as a result of the Western blotting analysis, the Cry2Ab protein showed stable expression in R1, R3, R4 and BC2F3 generations of the 15985. In the 15985, the modified Cry1Ac protein and the Cry2Ab protein are expressed, though it was shown that the Cry2Ab protein is expressed in young leaves, leaves, seeds and plant body of the 15985. On the other hand, regarding the modified Cry1Ac protein, the amount of expression in the 15985 and the DP50B, the recipient organism, is examined with the use of young leaves, leaves, seeds, plant body and pollen. As a result, no difference was found between the 15985 and the DP50B, regarding the amount of expression of the modified Cry1Ac protein in each organ. Therefore, it was proved that the modified Cry1Ac protein and the Cry2Ab protein do not show interaction in the 15985. In addition, regarding the NPTII protein, the amount of expression is also examined in the 15985 and the DP50B with the use of leaves and seeds, no significant difference was found between the 15985 and the DP50B.

This stack line cotton was compared with the parent lines for the tolerance to glufosinate herbicide based on the glufosinate herbicide-spraying test (Table 4 and Annex 1), and for the resistance to Lepidoptera based on the target insect feeding test (Table 5 and Annex 2). In addition, the modified PAT protein, the modified Cry1Ac protein and the Cry2Ab protein in this stack line cotton was determined by the ELISA analysis, and the respective amounts of expression were compared with those in the parent lines (Table 6 and Annex 3).

For the tolerance to glufosinate herbicide, this stack line cotton (F6 generation), the LLCotton25 and the non-recombinant control cotton were sprayed with the glufosinate herbicide at four (4) different dilution ratios (1x, 8x, 16x and 32x) to determine the levels of damage induced by glufosinate herbicide at the individual concentrations. As a result, at all the concentrations examined, no significant difference was observed between this stack line cotton and the LLCotton25 (Table 4 and Annex 1).

In addition, regarding the resistance to Lepidoptera, squares of this stack line cotton (BC3F5 generation), the 15985 and the non-recombinant control cotton at early and mid squaring stages of squares were given to the larvae of Cotton bollworm

(*Helicoverpa zea*) to determine the mortality 3 and 6 days after feeding. As a result, 3 and 6 days after squares at the early squaring stage were fed, no significant difference was observed in the mortality between this stack line cotton and the 15985. On the other hand, the mortality 3 days after feeding of squares at the mid squaring stage was slightly higher in this stack line cotton and a significant difference was observed, though no significant difference was observed in the mortality 6 days after (Table 5 and Annex 2). Consequently, it is considered that the significant differences found in the examination are not always observed and that this stack line cotton and the 15985 are equivalent to each other in the insecticidal effects against target insects.

Moreover, the level of expression of the modified Cry1Ac protein, the Cry2Ab protein and the modified PAT protein in the leaves of this stack line cotton (F6 generation), the LLCotton25 and the 15985 were determined based on the ELISA analysis. The results showed no significant difference between this stack line cotton and the 15985 regarding the level of expression of the modified Cry1Ac protein and the Cry2Ab protein. Also regarding the level of expression of the modified PAT protein, no significant difference was observed between this stack line cotton and the LLCotton25 (Table 6 and Annex 3).

Based on the above understanding, it is considered that the modified PAT protein, the modified Cry1Ac protein and the Cry2Ab protein are expressed in this stack line cotton at similar levels as in the respective parent plants and that these proteins do not interact with each other to affect the expression of traits.

Table 4　Comparison of damage levels induced by spraying of glufosinate herbicide

Tested plant \ Dosage [2]	Levels of damage [1] (Mean±Standard deviation)			
	1x	8x	16x	32x
This stack line cotton	1.0±0.4	3.4±0.7	3.7±0.5	4.0±0.0
LLCotton25	0.5±1.4	3.7±0.5	3.7±0.5	3.9±0.3
Significant test	ns	ns	Ns	ns
Non-recombinant control cotton	4.9±0.3	5.0±0.0	5.0±0.0	5.0±0.0

Statistical method used: t-test, ns: No significant difference at a level of 5%

[1] Rating of damage level　(Subjective evaluation):
- 0: Damage <10%; Trace amount of bronzing to the cuticle of cotyledons
- 1: Damage between 10 and 20%; Moderate bronzing of cotyledons, and trace etching of the true leaves
- 2: Damage between 21 and 40%; Moderate bronzing of cotyledons, and minor etching and curling of the true leaves
- 3: Damage between 41 and 60%; Moderate to severe bronzing, moderate etching and curling of the true leaves
- 4: Damage between 61 and 80%; Moderate to severe etching , curling and necrosis to the cotyledons and true leaves
- 5: Damage between 81 and 100%; Severe chlorosis, necrosis and leaf drop to cotyledons and true leaves

[2] Dosage: 1x; Equivalent to approx. 884g/ha of glufosinate herbicide, 8x; Equivalent to approx. 7,072g/ha of glufosinate herbicide, 16x; Equivalent to approx. 14,144g/ha of glufosinate herbicide, 32x; Equivalent to approx. 28,288g/ha of glufosinate herbicide

Spraying method: Seeds were sown on November 15, 2005 in 4-inch pots in a greenhouse.　December 9, 2005, the plant body of each line at 2-3 leaf stages was sprayed with glufosinate herbicide at 4 different concentrations, and damage levels induced by spraying of the glufosinate were evaluated on December 12, 2005.　For each line, 12 plants were applied to the spraying at each concentration.

Table 5 Insecticidal effects against *Helicoverpa zea* in this stack line cotton

	Mortality (Mean±Standard deviation) (Unit: %)	
Early squaring stage [a]	3 days later	6 days later
This stack line cotton	58.3±19.2	99.1±3.9
15985	63.0±23.3	99.1±3.9
Significant test	ns	ns
Non-recombinant control cotton	0.0±0.0	3.7±7.1
Mid squaring stage [b]	3 days later	6 days later
This stack line cotton	61.1±18.1	99.1±3.9
15985	45.4±22.0	90.7±14.3
Significant test	*	ns
Non-recombinant control cotton	0.9±3.9	3.7±7.1

Statistical method used: Mann-Whitney U test, *: Significant at a level of 5%, ns: No significant difference

[a]: Early squaring stage (Approx. 8 weeks after planting) [b]: Mid squaring stage (Approx. 11 weeks after planting)

Nine (9) plants of each line were grown in a greenhouse. Squares obtained from each plant at the early and mid squaring stages were used to determine the pest control effects against Cotton bollworm (*Helicoverpa zea*). One square and one 3rd instar larva of *H.zea* were put into each well on the 6-wells plate in which a wet filter paper was placed. Three (3) days later, mortality was determined and an additional new square was supplied. An additional three (3) days later, mortality was again determined. For the number of samples, a total of 9 plants for each line were measured repeatedly twice, and a total of 18 measurements per line were obtained.

Table 6 Determination of the amount of the modified Cry1Ac protein, the Cry2Ab protein and the modified PAT protein contained in 1 g of leaf based on the ELISA analysis

	Protein content (Mean±Standard deviation) (μg/1 g of leaf)		
	Modified Cry1Ac	Cry2Ab	Modified PAT
This stack line cotton	1.19±0.2	80.3±12.8	60.9±8.7
15985	1.26±0.2	75.9±12.0	ND
LLCotton25	ND	ND	65.9±10.6
Significant test [a]	ns	ns	ns
Non-recombinant control cotton	ND	ND	ND

[a]: Significant test for the modified Cry1Ac protein and the Cry2Ab protein was carried out based on the t-test between this stack line cotton and the 15985. For the modified PAT protein, significant test was conducted between this stack line cotton and the LLCotton25. ns: No significant difference at a level of 5%

For the number of samples, twice-replicated extraction was conducted from 5 plants for each line, and the extracts obtained were subjected to the ELISA analysis repeatedly twice (a total of 20 measurements/line).

ND: Not detected

5) Presence or absence, and if present, degree of transmission of nucleic acid

transferred through virus infection and/or other routes to wild animals and wild plants

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

A PCR method using respective pairs, each consisting of a 20-mer primer and a 21-mer primer, obtained by using the DNA transferred in the LLCotton25 and its surrounding genome sequences can be used to specifically identify this event. Furthermore, if a template DNA of usually 50ng is used, efficient detection can be made. If a very slight amount of a seed or plant body of the LLCotton25 is available, detection and identification can be made. In replicated tests, highly reproducible results could be obtained. This PCR method has been effectively used also for actual management of cultivation of the LLCotton25.

For the detection and identification of the 15985, a qualitative PCR method has been produced where the DNA sequences of the transferred genes and the adjacent regions of the plant genome are used as primers. This method makes it possible to specifically detect the 15985.

For the detection and identification of this stack line cotton, a single seed or a plant body is analyzed by the above-mentioned two methods. When the seed or plant body is found positive by both methods, it refers to the detection and identification of this stack line cotton.

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

1) Specific contents of physiological or ecological properties that were accompanied as a result of the expression of replication products of the transferred nucleic acid

With the modified PAT protein encoded by the modified *bar* gene, the modified Cry1Ac protein encoded by the modified *cry1Ac* gene and the Cry2Ab protein encoded by the *cry2Ab* gene, this stack line cotton shows tolerance to glufosinate herbicide and resistance to Lepidoptera.

As mentioned in I-1-(1)-2), the modified PAT protein has high substrate specificity and thus is considered extremely unlikely to affect the metabolic pathway of a recipient organism. In addition, the modified Cry1Ac protein and the Cry2Ab protein do not possess any enzyme activity and function independently from the metabolic system of the recipient organism. Moreover, as mentioned in I-1-(4)-4), it was confirmed that as a result of the glufosinate spraying and target insects feeding tests for traits expressed in this stack line cotton that the traits are expressed at similar levels as in the parent lines. Furthermore, the results of determination of the levels of expression of proteins based on the ELISA analysis showed no significant difference between the stack line and the parent lines. Based on the above understanding, there is no reason to suspect that these three proteins would affect each other.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between recombinant plant and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

As mentioned in I-1-(6)-1), it is hard to consider that the modified Cry1Ac protein, the Cry2Ab protein and the modified PAT protein derived from the genes transferred into the respective parent lines might interact with each other in this stack line cotton. Therefore, for the difference between this stack line cotton and the taxonomic cotton species to which the recipient organisms belong, evaluation is made based on the results of individual examinations for the various traits of the parent lines, the LLCotton25 and the 15985. Regarding the LLCotton25, isolated field tests were carried out using the T5 generation in FY 2003 at the National Agricultural Research Center for Kyushu Okinawa Region, National Agricultural Research Organization. In addition, for reference, evaluation was made based on the results of the comparison tests conducted in 2000 and 2001 in the US for comparing the LLCotton25 and the non-recombinant control cotton and the reproductive test conducted in 2001 in France. Isolated field tests of the 15985 were carried out in isolated fields of the Kyushu 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 2004 to March 2005. In addition, this isolated field test was conducted with the use of the 15985, the recombinant mother plant DP50B and the non-recombinant control cotton DP50. The DP50B is the recombinant commercialized cotton cultivar derived from repeated crossing of the 531 and the DP50, which is the non-recombinant cotton cultivar.

(a) Morphological and growth characteristics

Differences in the following items of morphological and growth characteristics were examined between the recombinant cotton and the non-recombinant control cotton: Regarding the LLCotton25, uniformity of germination; germination rate; plant type; plant height; total number of branches; number of nodes; leaf shape; leaf size; flower color; flower shape; petal color; flowering date; number of appearing flower buds; boll (fruit) shape; boll size; number of segments per boll and number of seeds per boll; boll opening time; fiber color (lint color); seed color and shape; harvesting time; number of harvested bolls per plant; number of non-harvested bolls and total number of bolls; fresh weight per boll; and weights of aerial and under-ground parts at harvesting time, and for the 15985, uniformity of germination; germination rate; plant type; culm length; flowering time; flower color; leaf shape; number of effective flower buds; number of bearing shoots; boll opening time; fiber color (lint color); shape of bolls (fruits of cotton); number of bolls per plant; number of non-harvested bolls; number of segments of a boll; number of seeds per boll; color of seeds; harvesting time; dry weight of a boll; weights of aerial- and under-ground parts at the harvest time. As a result, there was no difference observed that could cause Adverse Effect on Biological Diversity. Consequently, it is considered that there is no difference in this stack line cotton that could cause Adverse Effect on Biological Diversity.

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

Twenty (20) seedlings of the LLCotton25 (two-leaf stage) were placed in a climate chamber of 4°C with a 12-hour lighting interval and the reaction to the low temperature was observed with the lapse of time. As a result, on the 6th day after they were placed, all the individuals died, showing no cold tolerance.

Regarding the 15985, cold-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 was judged that these regions provide better climatic conditions for cotton growth than Japan. The results of observations showed that some seeds spilt on the field had germinated in fall after harvesting. However, it was reported that all of them had died by the following spring. Based on the above understanding, it was judged that cold tolerance of the 15985 is as low as that of the non-recombinant control cotton at the early stage of its growth.

Based on the above understanding, it is considered that cold tolerance of this stack line cotton is as low as that of the parent lines at the early stage of its growth.

(c) Wintering ability of the matured plant

Basically cotton cultivated in Japan naturally dies in winter seasons after fruit-bearing.

The matured plant of the LLCotton25 grown in the isolated field (open field) completely died by the last 10 days of December due to low temperatures and frost, showing no wintering ability. Regarding the 15985, it was observed that the plants were partly dead when the isolated field tests were completed (November 27, 2000).

Based on the above, it is also considered that the matured plant of this stack line cotton is not conferred with any overwintering ability.

(d) Fertility and size of the pollen

In Japan, cotton is not cultivated for commercial use, and there are no plans for this stack line cotton to be commercialized. Therefore, if this stack line 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 Japan's 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, shedding habit, dormancy, and germination rate of the seed

Regarding seed production, the differences between the LLCotton25 and the non-recombinant control cotton have been examined in terms of boll size, number of segments per boll, number of seeds per boll, seed shape, number of non-harvested bolls per plant, number of harvested bolls per plant and total number of bolls per plant. The differences between the 15985 and the non-recombinant control cotton have been examined in terms of number of bolls per plant, number of segments of a boll, and number of seeds per boll. The results showed no significant difference between the recombinant cotton and the non-recombinant control cotton.

The seeds of cotton are unlikely to be separated from each other since the lints are entangled with each other (Reference 11), and the shedding habit of seeds is considered to be low (Reference 13). In isolated fields, it was observed that the LLCotton25 and the non-recombinant control cotton were similar to each other in the shape of opened bolls and the seeds are covered with lint. In addition, in both of the 15985 and the non-recombinant control cotton, the seeds at harvested time were found covered with fiber and lint, and no shedding habit was observed in the natural conditions. Consequently, it is considered that there is no difference between this stack line cotton and the non-recombinant control cotton.

With regard to the dormancy, it is reported that the presently cultivated cotton cultivars do not have deep dormancy. For the dormancy and germination rate of the LLCotton25, when natural crossing rate was investigated, in the second 10-day period of September 2003, seeds were taken from the LLCotton25 and the non-recombinant control cotton cultivated in isolated fields and kept at room temperature after harvesting, and on October 20, 2003, the seeds were sown in small vinyl greenhouses installed in isolated fields. As a result, all the seeds of both easily germinated. From these facts, it is considered that the LLCotton25 is shallow in the dormancy of seeds like the non-recombinant control cotton and that even in the germination rate the LLCotton25 is equivalent to the non-recombinant control.

Regarding the 15985, to evaluate dormancy the germination rate of the seeds was examined using of the seeds of the 15985 harvested in 3 isolated fields in the US, the recombinant mother plant DP50B, the non-recombinant control cotton DP50 and 11 current cultivars, which were added for reference under the different temperature conditions from 5 to 40°C. As a result, under some temperature conditions, a statistically significant difference ($p \leq 0.05$) was observed between the 15985 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 the 15985, the recombinant mother plant DP50B and 11 current cultivars for reference were germinated, Viable Firm Swollen or degenerated, and no seed of Viable Hard was observed. Moreover, with regard to germination rate, as mentioned in I-1-(6)-2) "(a) Morphological and growth characteristics," no difference was observed between the recombinant

cotton and the non-recombinant control cotton.

Based on the above, it is considered that also regarding dormancy and germination rate, there is no difference between this stack line cotton and the non-recombinant control cotton.

(f) Crossability

In Japan, no wild relatives exist which can cross with tetraploid cotton cultivar (*Gossypium hirsutum*) to which this stack line cotton belongs. Thus, crossability of this stack line cotton was not assessed.

(g) Productivity of harmful substances

To examine the productivity of harmful substances of the LLCotton25 and the non-recombinant control cotton, succeeding crop tests, plow-in tests and soil microflora tests were carried out and no significant differences were observed in any of the tests conducted.

In addition, plow-in tests, succeeding crop tests and soil microflora tests were carried out among the 15985, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 and no significant differences were observed in any of the tests conducted.

Moreover, as mentioned in I-1-(6)-1), the bioassay on the tolerance to glufosinate herbicide (Table 4 and Annex 1) and the bioassay on the resistance to pest insects (Table 5 and Annex 2) for traits expressed in this stack line cotton confirmed that the traits are expressed at similar levels as in the parent lines. Furthermore, determination of the levels of expression of the modified PAT protein, the modified Cry1Ac protein and the Cry2Ab protein in this stack line cotton based on the ELISA analysis showed no significant difference from the parent lines for all the proteins examined (Table 6 and Annex 3).

Based on the above understanding, this stack line cotton is found to possess those proteins, though it is considered extremely unlikely that these proteins could affect each other in the plant body and newly produce any harmful substances.

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 line cotton was produced by the crossbreeding method of cotton tolerant to glufosinate herbicide (ACS-GH001-3) and cotton resistance to Lepidoptera (MON-15985-7), and the parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied in the Type 1 Use the same as for this stack line cotton.

It is reported that the modified PAT protein, which is encoded by the gene tolerant to glufosinate herbicide (modified *bar* gene) derived from the ACS-GHØØ1-3, is an enzyme that specifically acetylates the phosphinothricin, an active ingredient of glufosinate herbicide, though it has high substrate specificity.

In addition, it is suggested that the modified Cry1Ac protein and the Cry2Ab protein, which are encoded by the genes resistant to Lepidoptera (modified *cry1Ac* gene and *cry2Ab* gene) derived from the MON-15985-7, do not possess any enzyme activity.

For the traits conferred to this stack line cotton, it was confirmed that expressions of traits are the same levels as in the parent lines based on (1) the herbicide spraying tests for the tolerance to glufosinate herbicide, (2) the bioassay for the resistance to Lepidoptera, and (3) the ELISA analysis for the productivity of the modified Cry1Ac protein, the Cry2Ab protein and the modified PAT protein.

Based on the above understanding, it is considered unlikely that the modified Cry1Ac protein, the Cry2Ab protein and the modified PAT protein could affect each other, and it is considered that there is no specific change in the traits in this stack line cotton except it possesses the same characteristics as the parent lines do.

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

(1) Competitiveness

The plant of cotton cannot propagate and become competitive under the natural environment in Japan without human intervention. This stack line cotton possesses the glufosinate herbicide tolerance derived from the ACS-GH001-3 and the Lepidoptera resistance derived from the MON-15985-7. However, it is considered that the glufosinate does not exert selective pressure under a natural environment in Japan, and that the insect damage by Lepidoptera is not the major cause making the cotton difficult to grow in the natural environment in Japan. Consequently, it is considered that these traits do not increase the competitiveness.

Therefore, it is considered that this stack line cotton is not predominant over the parent lines. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack line cotton possesses the productivity of the modified PAT protein derived from the ACS-GH001-3 and the productivity of the modified Cry1Ac protein and the Cry2Ab protein derived from the MON-15985-7.

The modified Cry1Ac and the Cry2Ab proteins possess the insecticidal activity against insects of the order Lepidoptera, though they do not have enzyme activity and thus are considered not to affect the metabolic system of recipient organism. On the other hand, there is no report that the modified PAT protein is harmful to animals and plants, but it has high substrate specificity. This stack line cotton possesses the productivity of these proteins, though it is considered that the traits expressed are at the same levels as in the respective parent lines and that the traits would not affect each other. Consequently, the productivity of harmful substances of this stack line cotton would not become higher than that of the parent lines.

Therefore, it was judged that the conclusion made by the applicant that there are no specific wild plants or wild animals that are possibly affected by the productivity of harmful substances and thus there is no risk of Adverse Effect on Biological Diversity is valid.

(3) Crossability

In the Japanese natural environment, there are no wild species which can 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 Biological Diversity Risk Assessment Reports

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

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex List

Annex 1： Bioassay on the glufosinate herbicide tolerance of the LLCotton25 and this stack line cotton

Confidential: Not made available or disclosed to unauthorized person

Annex 2： Bioassay on the pest control of the 15985 and this stack line cotton

Confidential: Not made available or disclosed to unauthorized person

Annex 3： Determination of the modified PAT protein, the modified Cry1Ac protein and the Cry2Ab protein based on the ELISA analysis

Confidential: Not made available or disclosed to unauthorized person