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 and tolerant to glyphosate herbicide (*cry1Ac*, *cry2Ab*, *cp4 epsps*, *Gossypium hirsutum* L.) (15985×1445, OECD UI : MON-15985-7 ×MON-Ø1445-2)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them.
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment

I. Information concerning preparation of living modified organisms

The cross progeny line (*cry1Ac*, *cry2Ab, cp4 epsps*, *Gossypium hirsutum* L.) (15985×1445, OECD UI : MON-15985-7×MON-Ø1445-2) (herinafter referred to as "this stack cotton") was from the crossing of the following two recombinant cotton with the use of traditional cross-breeding method. The two recombinant cottons are, i) Cotton resistant to Lepidoptera (*cry1Ac*, *cry2Ab*, *Gossypium hirsutum* L.) (15985, OECD UI : MON-15989-7) (hereinafter referred to as "15985"), and ii) Cotton tolerant to glyphosate herbicide (*cp4 epsps*、*Gossypium hirsutum*) (1445, OECD UI : MON-Ø1445-2) (hereinafter referred to as "1445"). Therefore, this stack cotton possesses the both characteristics of parent lines,15985 and 1445. The information concerning preparation of 15985 and 1445 are explained individually in the followings.

15985 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- ØØ531-6) (hereinafter referred to as "531")**,** expresses modified Cry1Ac protein by introducing modified *cry1Ac* gene derived from *Bacillus thuringiensis* subsp. *kurstaki (B.t.k)*, and the non-recombinant cotton cultivar DP50. Therefore, the information concerning 531 is also shown in the following.

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 the origins of component elements are shown in Table 1.

Composition of the donor nucleic acid that was used for the development of 15985 and the origins of component elements are shown in Table 2.

Composition of the donor nucleic acid that was used for the development of 1445 and the origins of component elements are shown in Table 3.

(2) Functions of component elements

[Target gene used for development of 531 and 15985]

Functions of component elements of donor nucleic acid that was used for the development of 531 are shown in Table 1. Functions of component elements of donor nucleic acid that was used for the development of 15985 are shown in Table 2.

(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.

[Target gene used for development of 1445]

a) Functions of component elements of donor nucleic acid that was used for the development of 1445 are shown in Table 3.

(*cp4 epsps* gene)

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

EPSPS is located in chloroplasts or plastids in plants. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It has been clarified to be extremely unlikely that the stages from DAHP to the synthesis of chorismic acid, through the production of 5-enol-pyruvylshikimate-3-phosphate (EPSP) catalyzed by EPSPS, are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids. In addition, Monsanto Co. examined amino acid contents in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, canola, cotton and maize) that are tolerant to the glyphosate herbicides, and confirmed that there is no difference

in the content of aromatic amino acids, which are the final product of shikimate pathway, between the original non-recombinant plants and recombinant plants. These facts support that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS is the enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), and is known to specifically react with these substrates. The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living body.

1445 possesses tolerance to glyphosate herbicide by expressing CP4 EPSPS protein. Therefore, 1445 can control weed by 1-2 times of spraying of glyphosate during weed growth, after one time of spraying glyphosate to non-tillage fields before sowing. It is considered that 3-5 times of herbicide spraying per one cropping season is required for cotton cultivation. However, thanks to 1445 cultivation, the reduction of herbicide use and adoption of "non-tillage cultivation" are expected. In "non-tillage cultivation", chemical fertilizer and agrichemicals lose less into river by wind and rain. Furthermore, reduction of consuming fossil fuel can be expected thanks to the unnecessity of beating soil by agricultural machine to conduct tillage cultivation.

(*nptII* gene)

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

b) In order to investigate whether CP4 EPSPS protein and NTPII protein share functionally important amino acid sequences with known contact allergens, CP4 EPSPS protein and NTPII protein were compared with allergens in the database(GenBank, EMBL, PIR, NRL3D, Swiss Prot). As a result, both proteins did not share structurally related homologous sequences with any of the known allergens examined.

Table 1 Component elements of the vector PV-GHBK04 to be used for the development of 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.
nptII gene expression cassette	
35S	35S promoter region of cauliflower mosaic virus (CaMV).
nptII	A gene derived from a transposon of *E. coli*, Tn5 (Beck *et al*, 1982). 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 adenylyltransferase (AAD) derived from *Staphylococcus aureus.* Confers resistance to spectinomycin and streptomycin.
ori-V	The replication origin region derived from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* ABI strain.
ori322/rop	The replication origin region 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 15985

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/CTP2	The sequence that encodes the N-terminal chloroplast transit peptide sequence derived form the *Arabidopsis thaliana* EPSPS gene.
cry2Ab	A gene derived from *Bacillus thuringiensis* subsp. *kurstaki*, and it 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.

Table 3 Component elements of the vector PV-GHGT07 to be used for the development of 1445

Component elements	Origin and Function
Cp4 epsps gene expression cassette	
CMoVb	35S promoter of figwort mosaic virus. Involved in the constant expression of the target gene in all tissues.
ctp2	A sequence in the *epsps* gene of *Arabidopsis thaliana* that encodes chloroplast transit peptide located at N-terminal region of EPSPS protein. Transports CP4 EPSPS protein to the chloroplast where aromatic amino acids are synthesized.
Cp4 epsps	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium tumefaciens* CP4.
E9 3'	3' untranslated region of ribulose-1, 5-bisphosphate carboxylase E9 gene. Terminates transcription of mRNA and induces polyadenylation.
NptII gene expression cassete	
35S	35S promoter region of cauliflower mosaic virus (CaMV). Involved in the constant expression of the target gene in all tissues.
nptII (Kan)	A gene that was isolated from Tn5 transposon of *E.coli*. It encodes neomycin phosphotransferase type II (NPTII) enzyme protein, and confers resistance to kanamycin on plant. In introducing gene, it is used as a marker to select recombinant plant.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens*. It terminates transcription and induces polyadenylation.
Gox gene expression cassette (The followings were not inserted in 1445.)	
CMoVb	35S promoter of figwort mosaic virus. Involved in the constant expression of the target gene in all tissues.
Ctp1	A sequence that encodes chloroplast transit peptide located at N-terminal region of small subunit 1A of rubisco derived form *A. thaliana*. Transports GOX protein to chloroplast where aromatic amino acids are synthesized.
Gox	A sequence that encodes C-terminal of v247, a variant derived from glyphosate oxidoreductase (*gox*) of *Achromobacter* sp. strain LBAA. GOX protein dissolves glyphosate.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
Other component elements	
Ori-V	The replication origin region derived from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* ABI strain.
Aad	A gene encoding 3''(9)-0-aminoglycoside adenylyltransferase (AAD) derived from *Staphylococcus aureus.* Confers resistance to spectinomycin and streptomycin.
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.
Ori322	Replication origin region isolated from pBR322, a plasmid derived from *E. coli*. Permits autonomous replication of vectors in *E.coli*.
Rop	Derived from *E. coli*. Regulates the copy number of plasmids to be replicated in *E. coli*.

2. Information concerning vector

(1) Name and origin

The vectors used to generate 531, 15985 and 1445 are derived from pBR322, which is a synthetic plasmid from *E.coli*.

(2) Properties

The total number of base pairs of PV-GHBK04 used to generate 531 is 11,407bp, and the details of the component elements of the vector are shown in Table 1.

The total number of base pairs of PV-GHBK11 used to generate 15985 is 8,718bp, and the details of the component elements of the vector are shown in Table 2.

The total number of base pairs of PV-GHGT07 used to generate 1445 is 12,032bp, and the details of the component elements of the vector are shown in Table 3.

3. Method of preparing living modified organisms

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

Figure 1 shows the location, orientation and the section broken by restriction enzyme of the component elements of the nucleic acid in vector used for developing 531.

Figure 2 shows the location, orientation and the section broken by restriction enzyme of the component elements of the nucleic acid in vector used for developing 15985.
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.

Figure 3 shows the location, orientation and the section broken by restriction enzyme of the component elements of the nucleic acid in vector used for developing 1445.

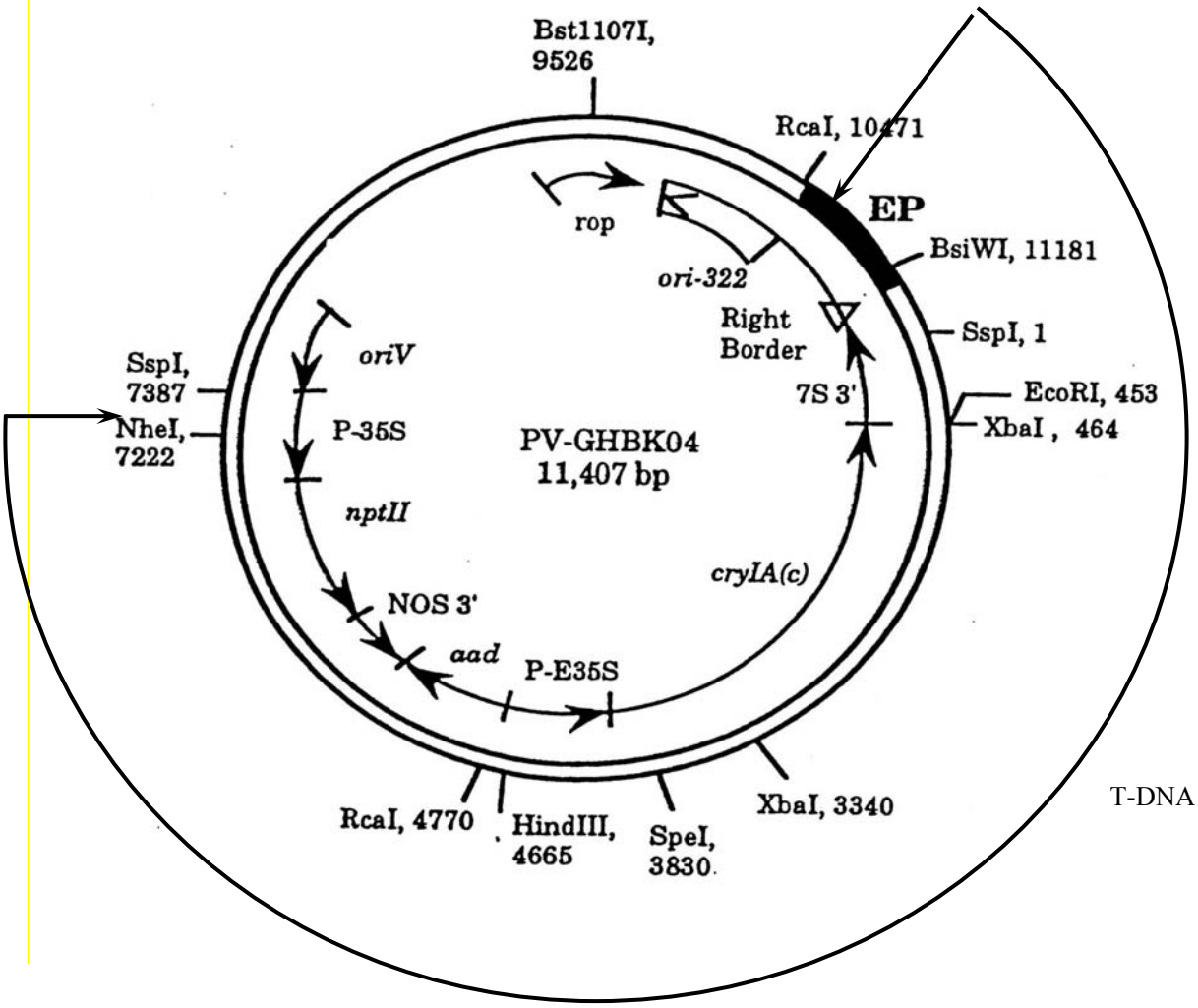

Figure 1 Map of the PV-GHBK04 plasmid to be used for developing Cotton resistant to Lepidoptera 531

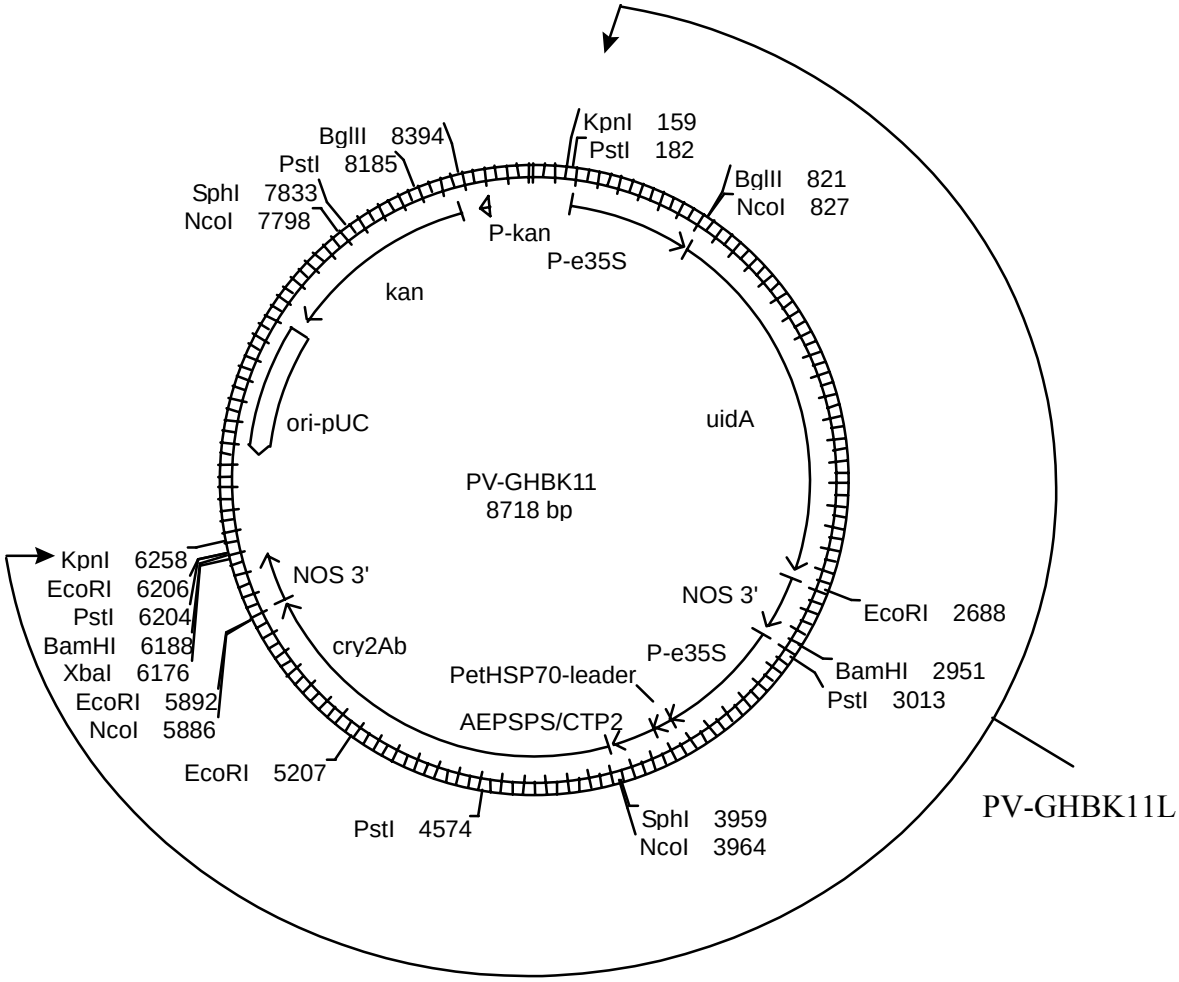

Figure 2 Map of the PV-GHBK11 plasmid to be used for developing Cotton resistant to Lepidoptera 15985

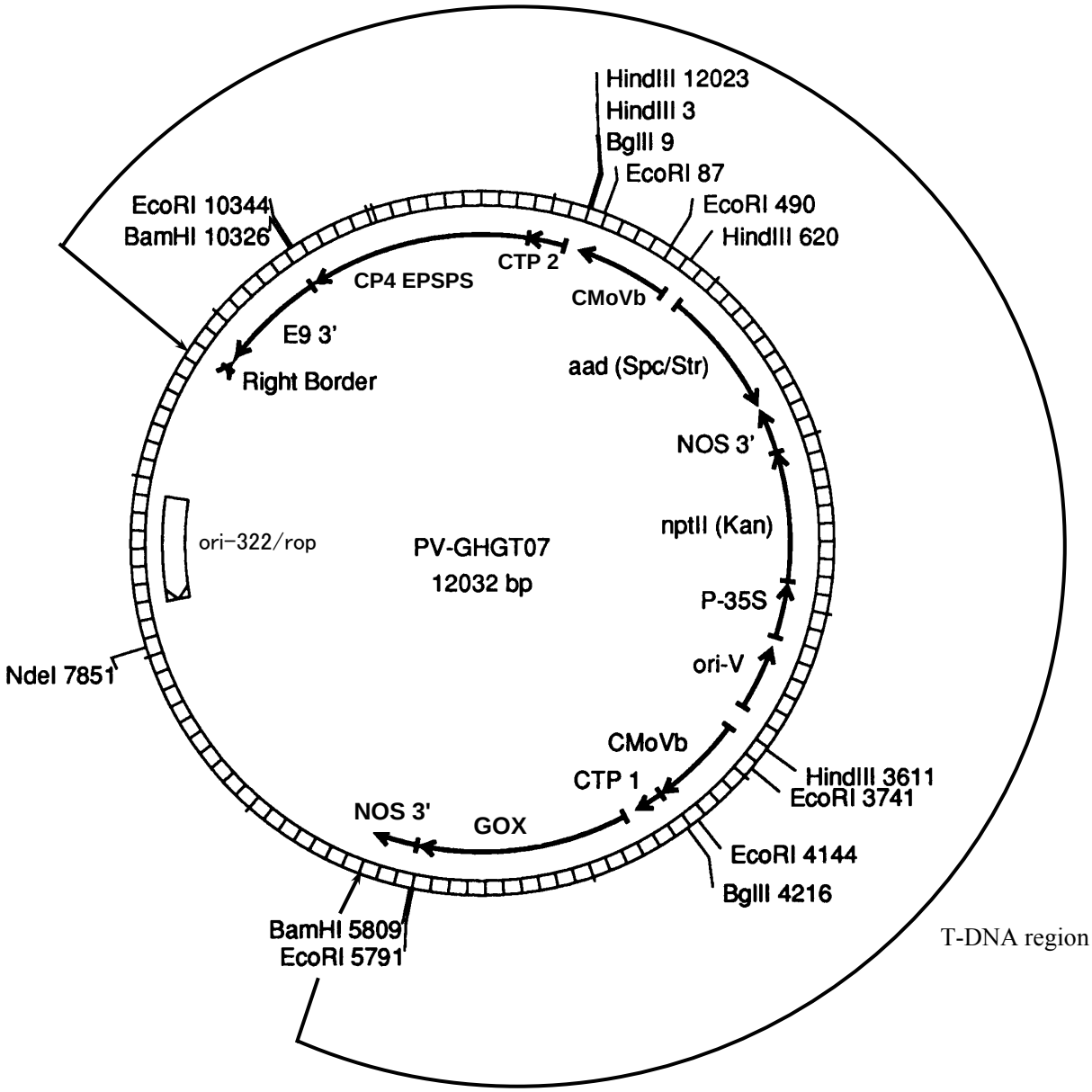

Figure 3 Map of the PV-GHGT07 plasmid to be used for developing Cotton tolerant to glyphosate herbicide

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

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

In developing 15985, the linear plasmid vector PV-GHBK11L was introduced into the recombinant cotton cultivar, DP50B by the particle gun bombardment method. DP50B is a commercialized cotton cultivar developed by repeated crossing of 531 and the non-recombinant control cotton cultivar DP50.

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

(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 confirmed that there was no Agrobacterium remained by culture in regenerating media containing no antibiotics.

c) Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes and the expression level of the modified Cry1Ac protein. Tests in climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters (morphological and growth characteristics, yield characteristics, sensitivity against insect pest, etc.) were examined in outdoor field tests. 531 were 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.

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

May, 1997: The Ministry of Health, Labor and Welfare ensured the safety of use for food, based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4".

June, 1997: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of use of the cultivar for feed, 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 15985]

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 method. 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 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.

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: The Ministry of Agriculture, Forestry and Fisheries ensured the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed), based on the "Guideline for the use of recombinant in 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".

[Processes of rearing of 1445]

a) The plasmid vector PV-GHGT07 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) The regenerated plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics. It was confirmed that there was no Agrobacterium remained.

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

The following shows the approvals received from organizations abroad.

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

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

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

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

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

June 19, 2003: The Australian Office of Gene Technology Regulator (OGTR) approved the cultivar being safe as feed and for the environment.

The following shows the approvals received from organizations in Japan.

December 9, 1997: The Ministry of Agriculture, Forestry and Fisheries ensured the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed), based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries".

December 16, 1997: The Ministry of Health, Labor and Welfare ensured the safety of use for food, based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4".

January 12, 1998: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of use of the cultivar for feed in accordance with "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)".

March 30, 2001: The Ministry of Health, Labor and Welfare ensured the safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques".

March 27, 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of the use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques".

[Process of rearing of 15985×1445]

This stack cotton was developed by traditional breeding method with using two recombinant cottons, 15985 and 1445.

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 genome DNA of 531: the 1st inserted gene consisting of the modified *cry1Ac* gene expression cassette, *nptII* gene expression cassette, and *aad* gene expression cassette; the 2nd inserted gene that consists of a 3' region fragment of the modified *cry1Ac* gene and 7S3' terminator, which are inserted next to the 5' terminal of the 1st inserted gene, in the reverse

direction; and the 3rd inserted gene consisting of a 7S3' terminator fragment of 245bp (Figure 4, p20).

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

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

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

A possible reason is that the location of 3rd inserted gene was, on the chromosome, distant from the 1st and 2nd inserted genes; therefore, the 3rd inserted gene may have been segregated from the others during back-cross process. Besides, since the 3rd inserted gene was a fragment of 7S3' sequence, which terminates transcription, it does not contribute to 531's resistance to Lepidoptera. Therefore, during back-cross breeding, the 3rd inserted gene was not functional for selection.

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

[State of existence of nucleic acid transferred in 15985 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 15985. 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 5 (p21) 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 15985. 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 nucleotide 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 15985 was confirmed by Southern blotting analysis in multiple generations (R1, R2, R3 and R4, which are selfed progenies from 15985; and BC2F3 generation developed by crossing with two current cotton cultivars).

[State of existence of nucleic acid transferred in 1445 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 gene is inserted into genome of 1445 chromosome at one site. Next, Southern blotting analysis was conducted with using CMoVb promoter, *gox* gene, *cp4 epsps* gene, *aad* gene, and *nptII* gene as probes. As a result, it was found that CMoVb promoter, *cp4 epsps* gene, *aad* gene, and *nptII* gene were inserted into 1445, but *gox* gene was not inserted. Therefore, it was confirmed that one copy of T-DNA region (*cp4 epsps* gene, *aad* gene, *nptII* gene; except *gox* gene) was inserted into the genome of 1445. In addition, simple selection has been conducted by PCR method for R_0 generation (R_0 shows the first generation of 1445, hereinafter, the number of successive selfed generations is shown as the suffix to R). Regarding *gox* gene, it was confirmed that *gox* gene was not inserted in cotton genome from the first. Moreover, the neighborhood sequence of both sides of inserted gene was determined, and then the map of inserted gene was finalized (Figure 6, p21).

In addition, *E. coli* and *aad* gene that was used as a marker to select *Agrobacterium tumefaciens* were inserted in 1445. However, since *aad* gene does not possess the promoter to work in plant, *aad* gene was not expressed in plant. In the actual measurement, AAD protein

produced by *aad* gene was under the limit value for detection in ELISA method (0.025ng per 1mg fresh weight of seed tissue, 0.013ng per 1mg fresh weight of leaf tissue).

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

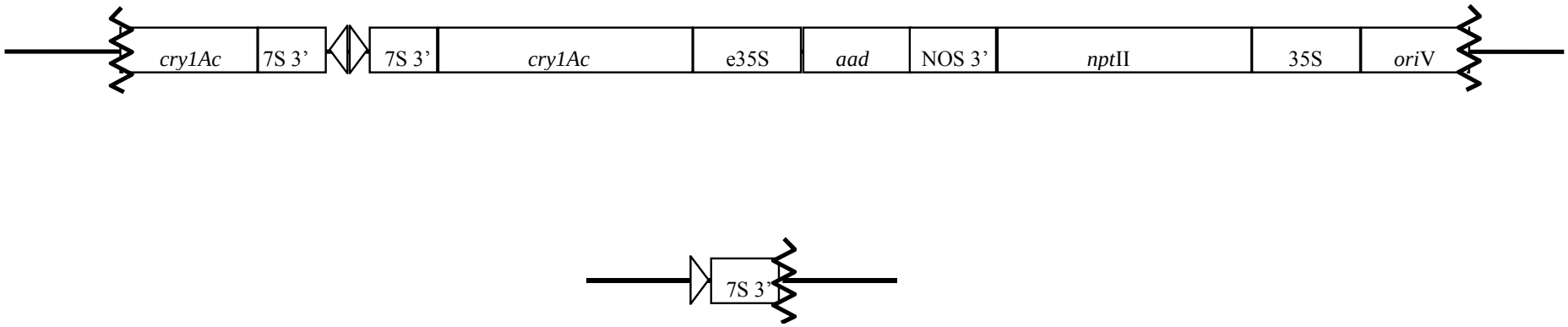

Figure 4 Map of inserted gene of Cotton resistant to Lepidoptera 531

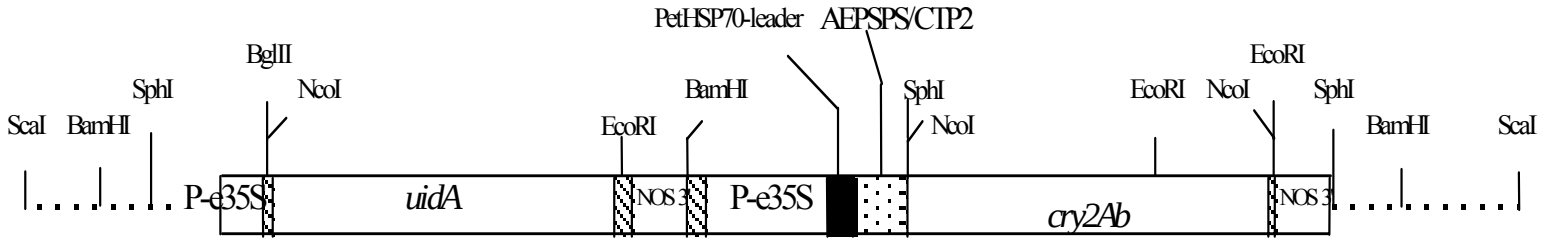

Figure 5 Map of inserted gene of Cotton resistant to Lepidoptera 15985

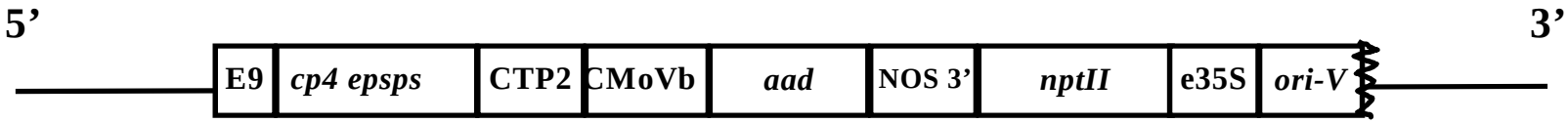

Figure 6 Map of inserted gene of Cotton tolerant to glyphosate herbicide 1445

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

For the detection and identification of 15985, 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 15985.

For the detection and identification of 1445, 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 1445.

To detect and identfy this stack cotton, the two methods on the above should be applied to each seed of the cotton.

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

It is guessed that modified Cry1Ab protein, Cry2Ab protein and CP4 EPSPS protein are expressed in the plant body of this stack cotton by the function of genes which were inserted in parent lines, 15985 and 1445. As mentioned in (2)-1-I, modified Cry1Ab protein and Cry2Ab protein do not possess enzyme activity, and function independently of the metabolic system of recipient organism. Also, it is suggested that EPSPS protein, which possesses the same functions as CP4 EPSPS protein, is not a rate-determining enzyme in the shikimate cycle. In addition, Monsanto Co. examined amino acid content in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton and maize) that are tolerant to the glyphosate herbicides, and confirmed that there is no difference in the aromatic amino acid content between the original non-recombinant plants and recombinant plants, thus it is considered not to affect to the metabolic pathway of recipient organism. Furthermore, CP4 EPSPS protein has high substrate specificity. Based on the above understanding, it is impossible to consider that characteristics directed by these three proteins would affect each other.

To confirm in practice, regarding resistance to Lepidoptera, the examination was carried out as follows. Young leaves and buds were taken from 15985 and this stack cotton, and powdered each of them to mix with artificial feeds. The artificial feeds were given to larvae of tobacco budworm, the target insect of the examination. Then the survival rate by the 3rd instar period of the larvae was examined. The expression level of Bt protein was derived by applying the survival rate of each to the standard curve which had been already prepared, and then comparison was made between the values of 15985 and this stack cotton. As a result, it was confirmed that the amount of Bt protein expression of 15985 and this stack cotton are almost same, and there is no statistically significant difference between 15985 and this stack cotton (Table 4).

In addition, regarding tolerance to glyphosate herbicide, glyphosate herbicide was sprayed onto 1445 and this stack cotton, then the level of glyphosate-specific chemical antagonism (growth inhibition, etiolation and malformation) were examined. As a result, it was confirmed that 1445 and this stack cotton show tolerant to glyphostae herbicide in normal amount of spraying (Table 5).

Based on the above understanding, regarding the difference between this stack cotton and cotton which is the taxonomic species to which the recipient organism belongs, it is guessed with the use of the results of individual examinations for the various characteristics of 15985 and 1445.

Table 4 Comparison of amount of Bt protein expression between 15985 and this stack cotton, 15985×1445 by quantitative biological examination

	Young leaves		Buds	
Variety	Amount of Bt protein expression (μg/g dry tissue weight)[1]	Standard error	Amount of Bt protein expression (μg/g dry tissue weight) [1]	Standard error
15985	145.4[2]	6.7	160.8[2]	7.3
15985×1445	156.2[2]	4.2	181.6[2]	5.6

[1] To show the amount of Bt protein expression, the following process was carried out; young leaves and buds were taken from 15985 and this stack cotton, 15985×1445, and powdered each of them to mix with artificial feeds. The artificial feeds were given to larvae of tobacco budworm, and the survival rate by the 3rd instar period of the larvae was examined. The amount of Bt protein expression was derived by applying the survival rate of each to the standard curve to show the correlation with modified Cry1Ac protein consistency and growth inhibition rate, which had been already prepared based on the biological examination to do the same process for modified Cry1Ac purified protein. The amount of Bt protein expression was shown by modified Cry1Ac protein equivalent.

15985, which expresses two Bt proteins (modified Cry1Ac protein and Cry2Ab protein) shows approximately 4 times high value of expression amount on the average in this quantitative biological examination, compared to 531 which expresses modified Cry1Ac protein only. So that, this quantitative biological examination is used for confirming the segregation of modified *cry1Ac* gene or *cry2Ab* gene, as well as for evaluation of resistance toward target insects, in the process of 15985 selections.

[2] The values of the amount of Bt protein expression are the mean value of the 14 varieties of 15985 and 10 varieties of this stack cotton, 15985×1445. This statistical analysis was conducted by two-way analysis of variance of SAS (Statistical Analysis System) (version 8.2).

Table 5 Data of biological examination of 15985 and this stack cotton, 15985×1445 by spraying glyphosate herbicide (Roundup)

	Degree of tolerance after spraying Roundup on 4th leaf stage (%)		Degree of tolerance after spraying Roundup on 10th leaf stage (%)	
	Sprayed amount of Roundup			
Variety	0.84 kg/ha	1.68 kg/ha	0.84kg/ha	1.68kg/ha
1445	100	99	100	100
15985×1445	100	99	100	100

The 10 individuals were prepared for each of the 4th leaf stage and the 10th leaf stage of 1445 and 15985×1445. Then the degree of tolerance (the degree of tolerance in all 10 individuals) was observed after 10 days of spraying glyphosate herbicide as effective amount of 0.84kg/ha or 1.68kg/ha. The degree of tolerance was examined by comparing the level of glyphosate-specific chemical antagonism (growth inhibition, etiolation and malformation) with non-sprayed individuals.

(1) In 15985, 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 15985. On the other hand, regarding modified Cry1Ac protein, the amount of expression in 15985 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 15985 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 15985. In addition, regarding NPTII protein, the amount of expression is also examined in 15985 and DP50B with the use of leaves and seeds, no significant difference was found between this recombinant cotton and DP50B.

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

Therefore, for this stack cotton, it is considered that modified Cry1Ac protein, Cry2Ab protein and CP4 EPSPS protein have been expressed in leaves and seeds.

(2) Isolated fields tests of 15985 were carried out in isolated fields of Kyusyu National Agricultural Experiment Station and Kawachi Research Farm (KRF), Monsanto Japan Limited, with the use of 15985, the recombinant control mother plant DP50B, and the non-recombinant cotton DP50 from May 2000 to March 2001. DP50B is the recombinant commercialized cotton cultivar derived from repeated crossing of 531 and the non-recombinant cotton DP50.

To compare R5 generation of 1445 and the recombinant mother plant, Coker312 as the control cotton, isolated field tests were carried out at an isolated field in Kyusyu National Agricultural Experiment Station from May 1997 to October 1997.

a) Morphological and growth characteristics

[Morphological and growth characteristics of 15985]

Differences in the following 20 items of morphological and growth characteristics were examined among 15985, the recombinant mother plant DP50B and the non-recombinant control cotton DP50: 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. 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 15985, 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) and 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 15985, 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 15985 was observed only in the non-recombinant control cotton DP50, and there was no difference between 15985 and the recombinant mother plant DP50B. The average weight of under-ground part of 15985, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 were 163.3g, 156.7g and 133.3g.

[Morphological and growth characteristics of 1445]

Differences in the following 19 items of morphological and growth characteristics were examined between 1445 and non-recombinant control cotton: the uniformity

of germination; germination rate; plant type; stem height; flowering time; flower color; leaf shape; the number of effective flower buds; the number of bearing shoots; boll opening time; the color of fiber (lint); the shape of bolls (fruits of cotton); the number of bolls per plant; the number of segments of a boll; the number of seeds per boll; the color of seeds; harvest time; the dry weight of a boll; and the weights of above and under-ground parts at the harvest time . For the following items among those above, 5 individual plants were selected from the central row of each plot and analyzed: plant type; stem height; the number of effective flower buds; the number of bearing shoots; the color of fiber (lint); the shape of bolls (fruits of cotton); the number of bolls per plant; the number of segments of a boll; the number of seeds per boll; the color of seeds; the dry weight of a boll; and the weights of above- and under-ground parts at the harvest time. As for the analysis of bolls among these items, 2 bolls were selected from each individual plant. With regard to the analysis of the following items, including uniformity of germination, germination rate, flowering time, boll opening time, and harvest time, all individuals were analyzed.

As a result, regarding germination rate, leaf length and short diameter of boll, statistically significant difference ($P < 0.05$) was observed between 1445 and the non-recombinant control cotton. However no difference was found in other items.

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

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

[Stack cotton 15985×1445]

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

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

[Chilling-tolerance and heat-tolerance at the early stage of 15985 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 main 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 spilled on the field had germinated in fall after harvesting. However, it was reported that all of them had died by the following spring. Based on the above understanding, it was judged that chilling-tolerance of this recombinant cotton is as low as that of the non-recombinant cotton.

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

Chilling-tolerance tests are not conducted at the early stage of growth in isolated fields.
Instead, the seeds collected in 1994 from R5 generation cultivated in 3 fields in the US (Tifton, Georgia [GA]; Starkville, Mississippi [MS]; and Loxley, Alabama [AL]) were sown directly to the ground in all three locations; and the germination rate and the wintering ability of the seeds, up to the following spring were examined. All of these 3 regions are representative for cotton cultivation in the South of the US. Besides, compared to average climatic conditions in Japan, the winter coldness in these regions is relatively mild. Therefore, it is considered that these regions provide better climatic conditions for cotton growth than Japan.

As a result of observation, it was found that few seeds (0.3%) sown on the field in Loxley, AL on October 18 had germinated on December 15 after harvesting. However, it was reported that all of them had died by January 17, and had not germinated until April 27, the last day of the observation. No germination of the seeds was found in the other two fields.

[Stack cotton 15985×1445]

Based on the above understanding, it was also judged that chilling-tolerance of this stack cotton is extremely low 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 15985 and 1445 were completed. Based on the above, the wintering ability test was not conducted for adult plants of 15985 and 1445.

d) Fertility and size of the pollen

In Japan, there are no plans to sell seeds of 15985 and 1445, and they are not cultivated for commercial use. Therefore, if 15985 and 1445 caused Adverse Effect on Biological Diversity in Japan, it would be in the following way: cotton seeds imported for oil extraction and feed are spilled during transportation, into Japanese natural environment; then, the spilled seeds grow or become self-seeding, and expel other plants from the area. However, the fertility and the size of pollens of 15985 and 1445 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

[Production, dormancy and germination rate of the 15985 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.

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°C.

As a result, under some temperature conditions, statistically significant difference ($p < 0.05$) 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 dormant 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

[Production, dormancy and germination rate of the 1445 seed]

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

It is known that the level of seed dormancy of cotton is extremely low. Also, it is known that the longevity of cotton seeds is short under natural conditions, and most of them decay in soil if sown before a period when soil temperature reaches 15-16°C. As described in b), seeds collected in autumn were soon sown in 3 fields in the US (Tifton, GA, Starkville, MS, Loxley, AL), and observed until the next spring; as a result, like the non-recombinant control cotton, 1445 showed no germination. Based on the result, 1445 seeds decayed in soil because of low soil temperature, like the non-recombinant control cotton seeds. Therefore, it was considered that 1445 seeds have extremely poor survival ability in low temperature and extremely low wintering ability, and they cannot keep their germinating ability under the winter condition in Japan, so the test concerning dormancy was not conducted.

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

[Stack cotton 1445×531]

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

f) Crossability

In Japan, no wild relatives exist that belong to *Gossypium* which crosses with tetraploid cotton cultivar (*Gossypium hirsutum*) to which 15985 and 1445 belongs. Thus, crossability of 15985 and 1445 was not assessed.

g) Productivity of harmful substances

As described in d), if 15985 and 1445 produce harmful substances and cause an Adverse Effect on Biological Diversity in Japan, it would be in the following way: cotton seeds imported for oil extraction and feed are spilled during transportation;

after that, such spilled seeds grow or become self-seeding at places which are not under human control, and expel other plants from the area. The productivity of harmful substances of 15985 and 1445 was not examined, because it is considered that, until spilled cotton seeds germinate and become matured to a certain extent, harmful substances would not be produced in the root or aerial parts of the plant, up to a level where the environment may be affected; and because there are no reports that seeds spilled during transportation grow or become self-seeding under natural conditions in Japan.

However, regarding 15985, plow-in test, succeeding crop test and soil microflora test were carried out as reference to use R1 and R4 generation plants.
As a result, no statistically significant difference was observed among 15985, the recombinant mother plant DP50B and the non-recombinant control cotton DP50 in all items.

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

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

This stack cotton was produced by crossing cotton resistant to Lepidoptera (MON-15985-7) with cotton tolerant to glyphosate herbicide (MON-01445-2) by taditional cross-breeding method. Regarding the parent line above, it was already judged by the investigative commission of Adverse Effect on Biological Diversity there is no risk that the use of each of these recombinant cotton in accordance with Type I Use Regulation.

It is suggested that CP4 EPSPS protein which is encoded by modified *cp4 epsps* gene (gene tolerant to glyphosate herbicide) derived from MON-01445-2 has high substrate specificity. Modified Cry1Ac protein which is encoded by modified *cry1Ac* gene (gene resistant to Lepidoptera) derived from MON-15985-7, and Cry2Ab protein which is encoded by *cry2Ab* gene (gene resistant to Lepidoptera) do not have enzyme activity. Thus, it is considered that the characteristics which were conferred by CP4EPSPS protein and two Cry proteins have no possibility to interact each other.

In addition, it was confirmed by biological examination with the use of Tobacco Budworm [*Heliothis virescens* (Fabricius)], that this stack cotton possesses resistance to Lepidoptera at the same level as MON-15985-7. Furthermore, it was confirmed by glyphosate-spraying test that this stack cotton possesses tolerance to glyphosate herbicide as same as MON-01445-2.

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

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

(1) Competitiveness

This stack cotton has tolerance to glyphosate herbicide derived from MON-01445-2, and also resistance to Lepidoptera derived from MON-15985-7. However, it is not considered that the glyphosate exerts pressure for selection under a natural environment, and also the insect damage by Lepidoptera is not the main factor to inhibit the growth of cotton under a natural environment in Japan. Thus, it is considered that these characteristics are not the characteristics to raise competitiveness, and this stack cotton is not considered to become more competitive as compared with parent lines. Based on the above understanding, the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

This stack cotton has the productivity of CP4 EPSPS protein from MON-01445-2, and also the productivity of Cry1Ac protein and Cry2Ab protein from MON-15985-7. Cry1Ac protein and Cry2Ab protein have the insecticidal activity to Lepidoptera. CP4 EPSPS protein has the same functions as EPSPS which is inhered in plant body, except that CP4 ESPSP is not inhibited its activity by glyphosate. It is considered not to possess the characteristic to raise productivity of harmful substances. Thus, it is considered that the productivity of harmful substances of this stack cotton would not become higher than that of parent lines. Based on the above understanding, the conclution made by applicant that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances 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.