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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton tolerant to glyphosate herbicide and resistant to Lepidoptera (*cp4 epsps*, *cry1Ac*, *cry2Ab, Gossypium hirsutum* L.) (MON 88913 × 15985, OECD UI : MON-88913-8 × 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 concerning preparation of living modified organisms

The cotton tolerant to glyphosate herbicide and resistant to Lepidoptera (*cp4 epsps*, *cry1Ac*, *cry2Ab, Gossypium hirsutum* L.) (OECD UI : MON-88913-8×MON-15985-7) (hereinafter referred to as "this stack line cotton") was from the crossing of the following two recombinant cotton products with the use of traditional crossbreeding methods. The two recombinant cottons are: i) Cotton tolerant to glyphosate herbicide (*cp4 epsps*, *Gossypium hirsutum*) (MON 88913, OECD UI : MON-88913-8) (hereinafter referred to as "MON 88913") and ii) Cotton resistant to Lepidoptera (*cry1Ac*, *cry2Ab*, *Gossypium hirsutum* L.) (15985, OECD UI : MON-15985-7) (hereinafter referred to as "15985"). Therefore, this stack line cotton possesses the both characteristics of these two parent recombinant cotton products, MON 88913 and 15985. Then the information concerning preparation of MON 88913 and 15985 are explained individually in the following sections.

15985 was developed by newly introducing modified *cry2Ab* gene derived from *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*) into 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") to which modified *cry1Ac* gene derived from *B.t.k* was introduced, and the non-recombinant control cotton DP50. Therefore, the information concerning 531 is also described in the following sections.

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 MON 88913 and the origins of component elements are shown in Table 1 on Pages 8 and 10. The nucleotide sequences of all the component elements of the donor nucleic acid of MON 88913 are shown in Annex 2 of Biological Diversity Risk Assessment Report for MON 88913.

Composition of the donor nucleic acid that was used for the development of 531 and the origins of component elements are shown in Table 2. The nucleotide sequences of all the component elements of the donor nucleic acid of 531 are shown in Annex 1 of Biological Diversity Risk Assessment Report for 531.

Composition of the donor nucleic acid that was used for the development of 15985 and

the origins of component elements are shown in Table 3. The nucleotide sequences of all the component elements of the donor nucleic acid of 15985 are shown in Annex 1 of Biological Diversity Risk Assessment Report for 15985.

(2) Functions of component elements

[Target gene used for the development of MON 88913]

1) Functions of component elements of donor nucleic acid that was used for the development of MON 88913 are shown in Table 1.

(Modified *cp4 epsps* gene)

Glyphosate, a nonselective herbicide, is the active ingredient of Roundup® agricultural herbicides, 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 (Reference 15; Reference 16). As a result, sensitive plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and consequently die. The modified *cp4 epsps* target gene, expresses the modified CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The activity of the modified CP4 EPSPS protein is not inhibited even under the presence of glyphosate, thus the recombinant plants that express this protein have normal functions of shikimate pathway and can grow.

EPSPS is located in chloroplasts or plastids in plants (Reference 17). The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants (Reference 18; Reference 16). This pathway is regulated by 3-deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It is extremely unlikely that the stages from DAHP to the synthesis of chorismic acid through the production of 5-enol-pyruvylshikimate-3-phosphate (EPSP), which is catalyzed by the EPSPS, are inhibited or suppressed by metabolic intermediates or end products of this pathway (Reference 19; Reference 20). This suggests that EPSPS is not a rate-determining enzyme in this pathway, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids,

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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 (Reference 21). In addition, Monsanto Co. examined amino acid content in the seeds of the glyphosate-tolerant recombinant crops in the process of food/feed safety assessment (soybean, canola, cotton, and maize),, and confirmed that there is no difference in the aromatic amino acid content between the original non-recombinant plants and recombinant plants. These facts support the theory that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS catalyzes a reversible reaction to produce EPSP and inorganic phosphate (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 22), and it is known to specifically react with these substrates (Reference 23). 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 thus unlikely that shikimate exerts any significant influence on the metabolic system of the plants.

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

[Target gene used for the development of 531 and 15985]

Functions of the genetic elements of the donor nucleic acid used for the development of 531 are shown in Table 2. Functions of the genetic elements of the donor nucleic acid used for the development of this recombinant cotton (15985) are shown in Table 3.

(Modified *cry1Ac* gene)

1) The modified *cry1Ac* gene has been created by modifying amino acid sequence as follows: The amino acids in the region from the N-terminal to 466th are derived from Cry1Ab protein which is produced by *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain, and the amino acids in the region from 467th to 1178th at the C-terminal are derived from Cry1Ac protein which is also produced by the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain. The

amino acid sequence of modified Cry1Ac protein which contains the above-mentioned two regions differs from that of wild-type Cry1Ac protein at 7 sites, and its homology is 99.4%. In addition, amino acid sequence of the core protein which possesses insecticidal activity is identical to that of wild-type Cry1Ac protein. The Cry1Ac protein expressed in this recombinant cotton is hereinafter referred to as "modified Cry1Ac protein." Cry1Ac protein, including the modified type, exhibits insecticidal activity against 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 USA 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 amino acid sequence of the core protein remains unchanged. Therefore, the modified Cry1Ac protein is considered to have 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 major 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 (Reference 24). *B.t.* proteins which are produced by the bacterium *B.t.* 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 (Reference 25; Reference 26). Also, the core protein, i.e., the active side of the modified Cry1Ac protein produced in this recombinant cotton is identical to the core protein of the Cry1Ac protein in B.t. preparation, which is a commercialized microbial agricultural insecticide. In the USA, European countries and Japan, B.t. preparation, which contains Cry1Ac protein, has been safely used for crops and trees as an insecticide to control order Lepidoptera.

2) 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, GenBank/EMBL). As a result, the modified Cry1Ac protein did not share structurally related homologous sequences with any of the known allergens examined.

(Modified *cry2Ab* gene)

1) Modified Cry2Ab protein which is encoded by the modified *cry2Ab* gene is derived from *Bacillus thuringiensis* subsp. *kurstaki*, a gram-positive bacterium, universally exists in soil. The protein is also called Cry2Ab2, CryIIB, CryB2 or CryIIAb. (Reference 27; Reference 28; Reference 29). 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 USA 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. The modified Cry2Ab protein was produced by modifying amino acid sequence only for the N-terminal sequence of the wild-type Cry2Ab protein in order to enhance its expression level in plants, and the amino acid sequence of the core protein remains unchanged. Therefore, the modified Cry2Ab protein is considered to have insecticidal activity against Lepidoptera as high as the wild-type Cry2Ab protein.

2) In order to investigate whether the modified Cry2Ab protein shares functionally important amino acid sequences with known contact allergens, the modified Cry2Ab protein was compared with allergens in the database (SwissProt, GenPept, PIR, GenBank/EMBL). As a result, the modified Cry2Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

(Modified *cry1Ac* gene＋modified *cry2Ab* gene)

Since modified Cry2Ab protein is newly produced in 15985, as well as modified Cry1Ac protein derived from 531, it can control Fall Armyworm, Beet Armyworm, and Soybean Looper, which have not been fully controlled by 531 (Reference 30, Reference 31).

In addition, 15985 produces modified Cry1Ac protein and modified Cry2Ab protein, which have a relatively overlapping insecticidal spectrum. Therefore, insects of order Lepidoptera, which show sensitivity against both B.t. proteins, could not become resistant without obtaining individual resistance for each of the B.t. proteins. Based on

the above, it is expected that 15985 can lower the probability of the occurrence of insects of order Lepidoptera that shows resistance against both Bt proteins, as compared with 531 that expresses only the modified Cry1Ac protein.

Table 1 Component elements of the vector PV-GHGT35 used for the development of MON 88913[1]

Component elements	Origin and Function
Modified *cp4 epsps* gene expression cassette which is regulated by P-FMV/TSF1	
P-FMV/TSF1	A chimeric promoter which was prepared by attaching *Arabidopsis thaliana* TSF1 promoter and enhancer sequence of *Figwort Mosaic Virus* (FMV) 35S promoter (Reference 32; Reference 33). Involved in the constitutive expression of introduced gene in reproductive organs as well as in vegetative organs. It has not been reported that viruses of genus *Caulimovirus*, which FMV belongs to, infect plants of genus *Gossypium*. Therefore, it is considered extremely unlikely that new types of virus will be created as a result of this recombination (Annex 4 of Biological Diversity Risk Assessment Report for MON 88913).
L-TSF1	Leader sequence (exon 1) of *Arabidopsis thaliana* TSF1 gene that encodes translation elongation factor EF-1 alpha (Reference 32). Enhances the expression of introduced gene.
I-TSF1	Intron sequence of *Arabidopsis thaliana* TSF1 gene that encodes translation elongation factor EF-1 alpha (Reference 32). Enhances the expression of introduced gene.
TS-*ctp2*	A sequence that encodes a chloroplast transit peptide derived from *Arabidopsis thaliana* EPSPS (Reference 34). It directs the CP4 EPSPS protein to the chloroplast, where aromatic amino acids are synthesized.
CR-*cp4 epsps* (Modified *cp4 epsps*)	Coding sequence for 5-enol-pyruvyl-shikimate-3-phosphate synthase derived from *Agrobacterium* strain CP4 (Reference 35; Reference 36). A modification is given to the nucleotide sequence to enhance its expression in plants without changing the function of the CP4 EPSPS protein. Only a single modification is introduced to the amino sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine.
T-E9	3' untranslated region of pea ribulose-1, 5-bisphosphate carboxylase E9 gene (Reference 37). Terminates transcription of mRNA and induces polyadenylation.
Modified *cp4 epsps* gene expression cassette which is regulated by P-35S/ACT8	
P-35S/ACT8	A chimeric promoter which was prepared by attaching *Arabidopsis thaliana* ACT8 promoter and enhancer sequence of cauliflower mosaic virus (CaMV) 35S promoter (Reference 38; Reference 39). Involved in the constant expression of the introduced gene in vegetative organs. It has not been reported that viruses of genus *Caulimovirus*, which CaMV belongs to, infect plants of genus *Gossypium*. Therefore, it is considered extremely unlikely that new types of virus will be created as a result of this recombination (Annex 4 of Biological Diversity Risk Assessment Report for MON 88913).
L-ACT8	Leader sequence of *Arabidopsis thaliana* ACT8 gene. Enhances the expression of the introduced gene (Reference 38).
I-ACT8	Intron sequence and nearby exon sequence of *Arabidopsis thaliana* ACT8 gene (Reference 38). Enhances the expression of the introduced gene.

[1] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

TS-*ctp2*	A sequence that encodes a chloroplast transit peptide derived from *Arabidopsis thaliana* EPSPS, which directs the CP4 EPSPS protein to the chloroplast, where aromatic amino acids are synthesized (Reference 34).
CR-*cp4 epsps* (Modified *cp4 epsps*)	Coding sequence for 5-enol-pyruvyl-shikimate-3-phosphate synthase derived from *Agrobacterium* strain CP4 (Reference 35; Reference 36). A modification is given to the nucleotide sequence to enhance its expression in plants without changing the function of the CP4 EPSPS protein. Only a single modification is introduced to the amino sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine.
T-E9	3' untranslated region of pea ribulose-1, 5-bisphosphate carboxylase E9 gene. Terminates transcription of mRNA and induces polyadenylation (Reference 37).

Table 1 Component elements of the vector PV-GHGT35 used for the development of MON 88913 (Continued)[2]

Component elements	Origin and Function
Components of plasmid backbone	
B-Left Border (Left Border Sequence)	A DNA sequence containing the left border sequence (25 bp) derived from Ti plasmid pTiA6. Defines the T-DNA transferred from *Agrobacterium tumefaciens* to the plant genome (Reference 40).
OR-ORI V	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* (Reference 41).
CR-rop	A coding sequence to repress primer protein to maintain the number of copies of plasmids in *E. coli* (Reference 42).
OR-ORI-PBR322	Replication origin region isolated from pBR322, a plasmid derived from *E. coli*. Permits autonomous replication of vectors in *E. coli* (Reference 43).
CR-*aad*	The gene that encodes aminoglycoside adenyltransferase (AAD) derived from *E. coli* transposon Tn7. Confers resistance to spectinomycin or streptomycin (Reference 44).
B-Right Border (Right Border Sequence)	A DNA sequence containing right border sequence (25bp) of nopaline type T-DNA derived from Ti plasmid pTiT37. Defines the T-DNA transferred from *Agrobacterium tumefaciens* to plant genome (Reference 45).

[2] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 2 Component elements of the vector PV-GHBK04 used for the development of 531[3]

Component elements	Origin and Function
Modified *cry1Ac* gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 39), from cauliflower mosaic virus (CaMV) (Reference 46).
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*). Its homology of amino acid sequence with the wild-type Cry1Ac protein produced by *Bacillus thuringiensis* subsp. *kurstaki* is 99.4%, though the amino acid sequence of the core protein is identical to that of the wild-type Cry1Ac protein (Reference 47).
7S 3'	3' untranslated region of soybean *β-conglycinin* gene. Contains a signal for the polyadenylation of mRNA (Reference 48), and functions to terminate transcription of the target gene.
nptII gene expression cassette	
P-35S	35S promoter region of cauliflower mosaic virus (CaMV) (Reference 49; Reference 50).
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 (Reference 51).
NOS3'	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* (Reference 45; Reference 52). 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 (Reference 45; Reference 52).
aad	A gene encoding 3"(9)-0-aminoglycoside adenyltransferase (AAD) derived from *Staphylococcus aureus.* In sutable bacteria, it confers resistance to spectinomycin and streptomycin (Reference 44).
ori-V	The replication origin derived from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* ABI strain (Reference 41).
ori322/rop	The replication origin derived from *E. coli* plasmid pBR322. Permits autonomous replication of vectors in *E. coli.* This region contains not only the origin of replication, but also the *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* (Reference 53; Reference 43).

[3] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 3 Component elements of the vector PV-GHBK11L used for the development of 15985[4]

Component elements	Function
uidA gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 39) from cauliflower mosaic virus (CaMV) (Reference 46).
uidA	A *uidA* gene derived from *E.coli* plasmid pUC19. Encodes GUS (β-D-glucuronidase) protein (Reference 54).
NOS3'	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* (Reference 45; Reference 52). It terminates transcription and induces polyadenylation.
Modified *cry2Ab* gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 39), from cauliflower mosaic virus (CaMV) (Reference 46).
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 (Reference 55).
Modified *cry2Ab*	A gene that encodes the modified 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 (Reference 28). The modified 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 in cultivation.
NOS3'	3' untranslated region of nopaline synthase (NOS) gene derived from *Agrobacterium tumefaciens* (Reference 45; Reference 52). It terminates transcription and induces polyadenylation.

[4] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

2. Information concerning vector

(1) Name and origin

The plasmid vectors used to create MON 88913, 531 and 15985 are all derived from pBR322, which is a synthetic plasmid derived from *E.coli*.

(2) Properties

The total number of base pairs of PV-GHGT35 used to create MON 88913 is 13,741bp. Component elements in the vector are detailed in Table 1. The nucleotide sequence is shown in Annex 2 of Biological Diversity Risk Assessment Report for MON 88913.

The total number of base pairs of PV-GHBK04 used to create 531 is 11,407bp. Component elements in the vector are detailed in Table 2. The nucleotide sequence is shown in Annex 1 of Biological Diversity Risk Assessment Report for 531.

The total number of base pairs of PV-GHBK11 used to create 15985 is 8,718bp. Component elements in the vector are detailed in Table 3. The nucleotide sequence is shown in Annex 1 of Biological Diversity Risk Assessment Report for 15985.

3. Method of preparing living modified organisms

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

The location and orientation of component elements of donor nucleic acid in vector used for production of MON 88913 and the sections produced by restriction enzymes are shown in Figure 1.

The location and orientation of component elements of donor nucleic acid in vector used for the production of 531 and the sections produced by restriction enzymes are shown in Figure 2.

The location and orientation of component elements of donor nucleic acid in vector used for the production of 15985 and the sections produced by restriction enzymes are shown in Figure 3. To introduce gene to plant cells, PV-GHBK11 was treated by the restriction enzyme *Kpn*I, and a resulting linear DNA fragment, PV-GHBK11L, composed of the *uidA* gene expression cassette ([P-e35S]-[*uidA*]-[NOS 3']) and modified *cry2Ab* gene expression cassette ([P-e35S]-[PetHSP70

leader]-[AEPSPS/CTP2]-[*cry2Ab*]-[NOS3']) were used.

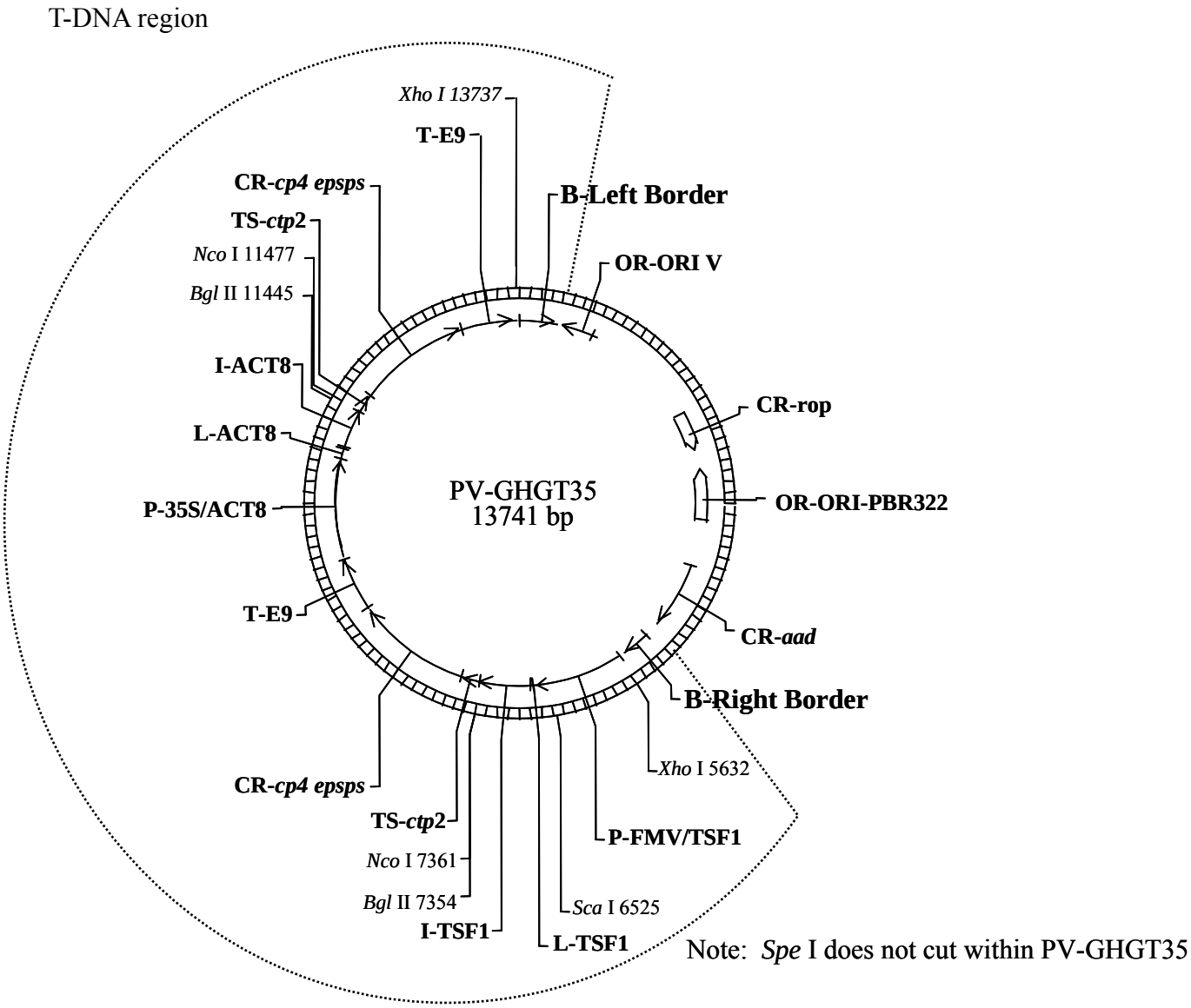

Figure 1 Map of the plasmid PV-GHGT35 used to create glyphosate-tolerant cotton, MON 88913 [5]

[5] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

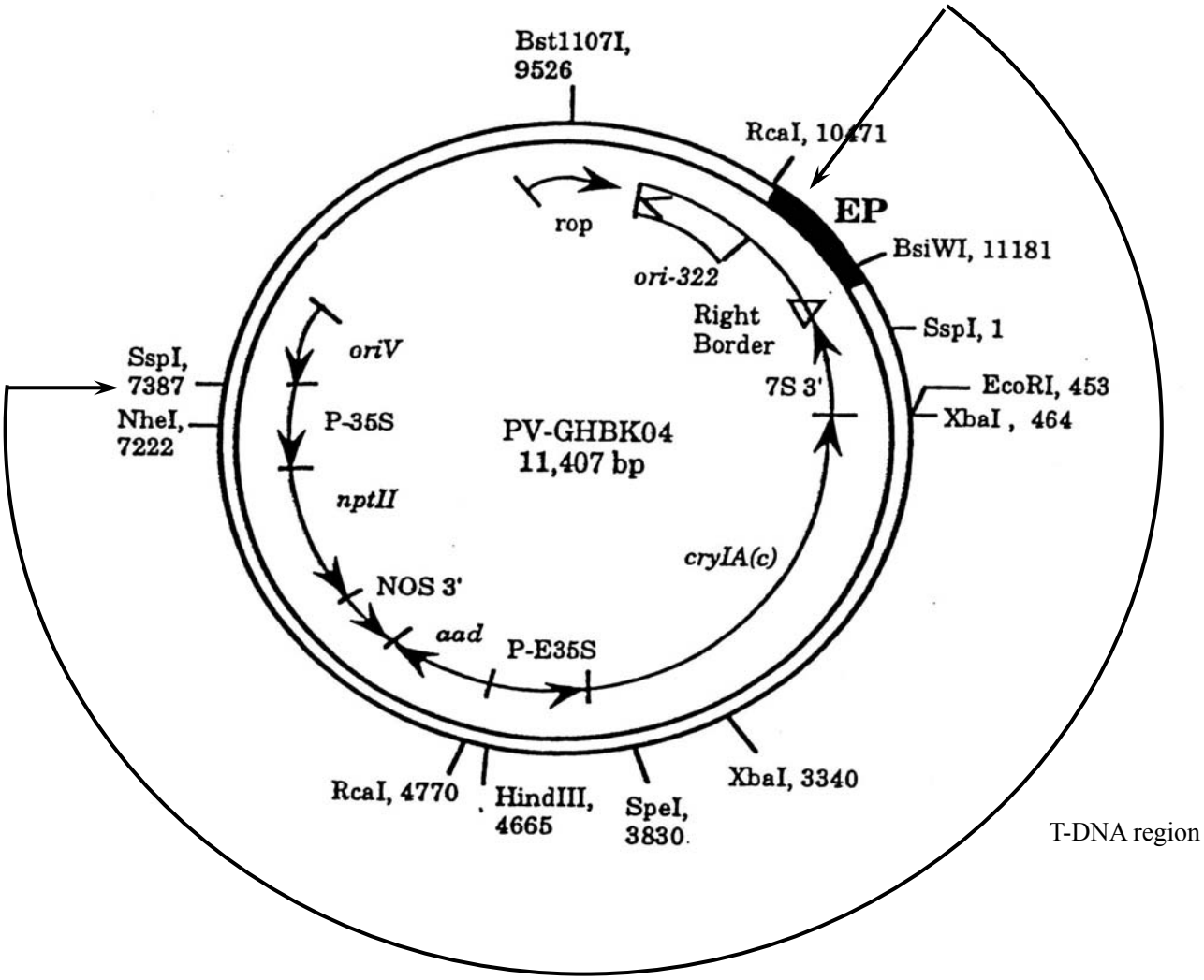

Figure 2 Map of the plasmid PV-GHBK04 used to create Lepidoptera-resistant cotton, 531[6]

[6] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

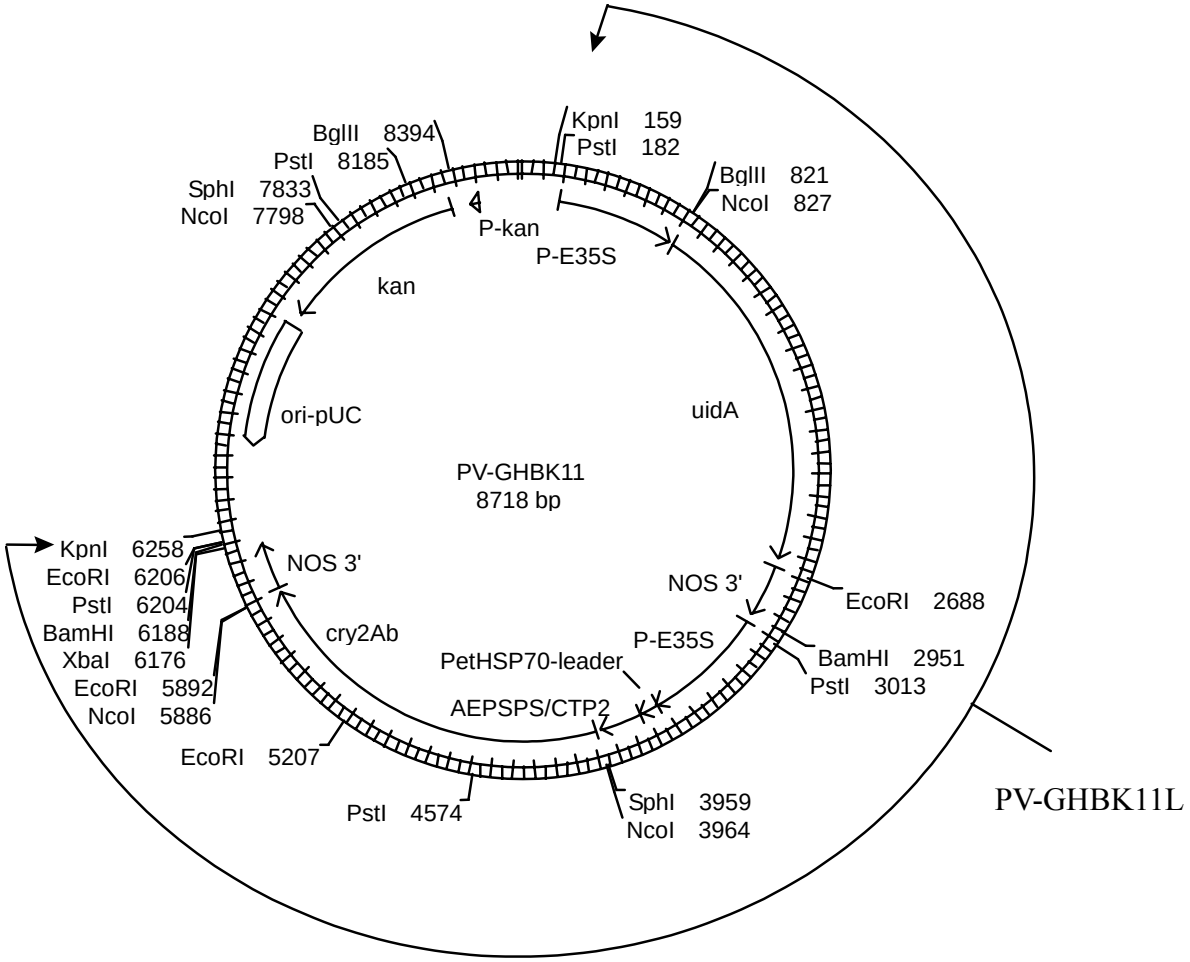

Figure 3 Map of the plasmid PV-GHBK11 used to create Lepidoptera-resistant cotton 15985 [7]

[7] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

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

To create MON 88913, the T-DNA region of the plasmid vector PV-GHGT35 was introduced to a conventional cotton cultivar, Coker 312, by the *Agrobacterium* method.

To create 531, the T-DNA region of the plasmid vector PV-GHBK04 was introduced to a conventional cotton cultivar, Coker 312, by the *Agrobacterium* method.

To create 15985, the linear DNA vector PV-GHBK11L was introduced to the recombinant cotton cultivar, DP50B, by particle gun bombardment. DP50B is a commercialized cotton cultivar generated by repeated crossing of 531 with the non-recombinant conventional cotton cultivar DP50.

(3) Processes of rearing of living modified organisms

[Processes of rearing of MON 88913]

1) The T-DNA of plasmid vector PV-GHGT35 was introduced into tissue sections of Coker 312 by the *Agrobacterium* method, and then regenerated individuals were obtained by culturing them in media containing glyphosate.

2) The plant tissue was cultivated in media containing carbenicillin and cefotaxime, and then it was cultivated in regenerating media containing no antibiotics to confirm that there was no remaining *Agrobacterium* (Reference 56).

3) For the individual regenerated plants, further selection was carried out based on the analysis of the inserted genes and production of the CP4 EPSPS protein. Tests in a climate chamber and greenhouse were then conducted. Glyphosate tolerance and agronomic characteristics (morphological and growth characteristics, characteristics regarding yield, sensitivity to pest insects, etc.) were examined in outdoor field tests. MON 88913 was selected based on the comprehensive evaluation of these results (the generations used in the tests are shown in Figure 4).

The following shows the approvals received from organizations in Japan.

November, 2004: Application for approval for safety of use as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries. Examination is in progress at present.

February, 2005: Application was submitted to the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for approval of Type I Use under the provisions of the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Law) (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them). Examination is in progress at present, and the examination by the Overall Review Board of the Committee for Review on the Biological Diversity Risk Assessment held on June 9 was ended.

April, 2005: Approval for safety of use as food was received by the Ministry of Health, Labor and Welfare.

[Development of 531]

1) T-DNA region of the plasmid vector PV-GHBK04 was introduced into the hypocotyls of Coker 312 by the *Agrobacterium* method, and then regenerated individuals were obtained by culturing them in media containing kanamycin.

2) In order to eliminate *Agrobacterium* from the regenerated plant, the regenerated plant was cultivated in media containing carbenicillin, and then it was cultivated in regenerating media containing no antibiotics to confirm that there was no remaining *Agrobacterium* (Reference 56).

3) Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes and production level of the modified Cry1Ac protein. Tests in a climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characteristics (morphological and growth characteristics, characteristics regarding yield, sensitivity to pest insects, etc.) were examined in outdoor field tests. 531 was selected based on the comprehensive evaluation of these results (the generations used in the tests are shown in Figure 5).

The following shows the approvals received from organizations in Japan.

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

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

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

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

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

November, 2004: Approval was received by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use under the provisions of the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Law) (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them).

[Development of 15985]

1) The recombinant cotton cultivar DP50B was used as the recipient plant, and PV-GHBK11L was introduced into its shoot apex cells by the particle gun

bombardment. Selection of regenerated individuals was carried out by the histochemical staining method detecting the GUS protein.

2) Regarding the regenerated plant individuals, further selection was carried out based on the analysis of inserted genes derived from PV-GHBK11L and the levels of the modified 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 generations used in the tests are shown in Figure 6).

The following shows the approvals received from organizations in Japan.

July, 2001: Based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", compliance with the guideline regarding recombinant being imported to Japan (used for processing and feed) was certified by the Ministry of Agriculture, Forestry and Fisheries.

October, 2002: The Ministry of Health, Labor and Welfare ensured the safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques".

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

December, 2004: Approval was received by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use under the provisions of the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Law) (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them).

[Development of MON 88913×15985]

This stack line cotton was developed by crossing the two recombinant cotton products, MON 88913 and 15985, with the use of conventional breeding methods (Figure 7).

Not made available or disclosed to unauthorized person

Figure 4　Development of the glyphosate herbicide-tolerant cotton, MON 88913

Not made available or disclosed to unauthorized person

Figure 5　Development of the Lepidoptera-resistant cotton, 531

Not made available or disclosed to unauthorized person

Figure 6　Development of the Lepidoptera-resistant cotton 15985

Not made available or disclosed to unauthorized person

Figure 7　Development of the stack line cotton MON 88913×15985

4. Stability **of the inserted DNA in the plant cells and stability of expression of traits caused by the inserted DNA.**

[State of existence of nucleic acid transferred in MON 88913 and stability of expression of traits]

(1) Location of copies of the introduced nucleic acids

On the cotton chromosome

(2) Number of copies of the introduced nucleic acids and inter-generational inheritance stability of copies of the introduced nucleic acids

Based on Southern blot analyses, it was confirmed that one copy of the T-DNA region was inserted at a site in the genome of MON 88913 (Figure 8). The plasmid backbone region outside of the T-DNA region was not inserted (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913), and the two intact modified *cp4 epsps* gene expression cassettes inside the T-DNA region were inserted as expected. (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913). Furthermore, Southern blotting analyses on multiple generations indicated that inserted genes were stably inherited in offspring. (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913).

(3) Inter-individual or inter-generational expression stability under natural conditions

We examined the levels of the modified CP4 EPSPS protein using samples obtained at four different fields in the USA (Alabama, California, Georgia and Texas) by ELISA analysis. As a result, it was confirmed that the modified CP4 EPSPS protein was produced in every plant organ examined. (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913). Furthermore, the inter-generational expression stability of the modified CP4 EPSPS protein was evaluated based on tolerance to herbicide glyphosate across several generations.

(4) Existence of transmission routes and its scale, if it is possible that nucleic acids introduced via viral infection or other routes might be transmitted to wild animals and plants

The plasmid PV-GHGT35 can only sustain autonomous replication in Gram-negative bacteria, such as *E.coli* and *A. tumefaciens*. Therefore, there is no possibility that the introduced nucleic acids might be transmitted to wild animals and plants that are not sexually compatable with cotton under natural conditions.

[Stability of the inserted DNA in 531 and stability of expression of traits]

(1) Location of copies of the introduced nucleic acids

On the cotton chromosome

(2) Number of copies of the introduced nucleic acids and inter-generational inheritance stability of copies of the introduced nucleic acids

The inserted genes were analyzed by Southern blotting analysis, cosmid cloning technique and genome walking method. As a result, gene insertion was found in the following 3 inserts in the 531 genomic DNA: the 1st insert consists of modified *cry1Ac* gene expression cassette, *nptII* gene expression cassette, and *aad* gene expression cassette; the 2nd insert 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 insert, in the reverse direction; and the 3rd insert consists of a 7S 3' terminator fragment of 245bp (Figure 9).

Southern blotting analysis was conducted in combination of seven different probes (Probe 1 thru Probe 6, and the 5'-terminal neighboring sequence of the inserted genes), (Annex 2 of Biological Diversity Risk Assessment Report for 531), and six different restriction enzyme treatments (*Ase*I + *Bst*Z17I, *Ssp*I, *Xmn*I, *Bam*HI, *Bam*HI + *Nde*I, *Bam*HI + *Pme*I) (Annex 2 of Biological Diversity Risk Assessment Report for 531).

Then, DNA fragments obtained in the cosmid cloning technique and genome walking method were analyzed so as to determine the 5' neighboring sequence of the 2nd insert (Annex 2 of Biological Diversity Risk Assessment Report for 531); 3' neighboring sequence of the 1st insert (Annex 2 of Biological Diversity Risk Assessment Report for 531); and both neighboring sequences of the 3rd insert (Annex 2 of Biological Diversity Risk Assessment Report for 531). In addition, in order to conclusively analyze the structure of the 1st and 2nd inserts, 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 (Annex 2 of Biological Diversity Risk Assessment Report for 531). Moreover, by analyzing the DNA sequence of these PCR products, the complete nucleotide sequence of the 1st and 2nd inserts was determined (Annex 2 of Biological Diversity Risk Assessment Report for 531).

As a result of Southern blotting analysis of the genome DNAs extracted from the recombinant in R5 and R6 generations and from two commercialized cultivars, it was confirmed that the 1st and 2nd inserts were stably inherited (Annex 2 of Biological Diversity Risk Assessment Report for 531). Incidentally, the genomic DNAs of the two commercialized cultivars do not contain the 3rd insert, i.e., the fragment of 7S3' sequence (Annex 2 of Biological Diversity Risk Assessment Report for 531).

A possible reason is that the location of 3rd insert was located on the chromosomes some distance from the 1st and 2nd inserts; therefore, the 3rd insert may have been separated from the other inserts through segregation during the back-cross breeding process. Besides, since the 3rd insert was a fragment of 7S3' sequence, which terminates transcription, it does not contribute to this recombinant cotton's resistance to Lepidoptera, the target trait of 531. Therefore, during back-cross breeding, the 3rd insert was not used as a selection target.

(3) Inter-individual or inter-generational expression stability under natural conditions

It was confirmed during the process of rearing 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.

(4) Existence of transmission routes and its scale, if it is possible that nucleic acids introduced via viral infection or other routes might be transmitted to wild animals and plants

The plasmid PV-GHBK04 can only sustain autonomous replication in Gram-negative bacteria, such as *E.coli* and *A. tumefaciens*. Therefore, there is no possibility that the introduced nucleic acids might be transmitted to wild animals and plants that are not sexually compatable with cotton under natural conditions.

[State of existence of nucleic acid transferred in 15985 and stability of expression of traits]

(1) Location of copies of the introduced nucleic acids

On the cotton chromosome

(2) Number of copies of the introduced nucleic acids and inter-generational inheritance stability of copies of the introduced nucleic acids

As a result of the analysis of inserted genes by Southern blotting analysis, it was confirmed that one copy of the inserted *cry2Ab* gene was inserted at one site in the genome (chromosomes) of 15985 (Annex 2 of Biological Diversity Risk Assessment Report for 15985). Then, to confirm the completeness of modified *cry2Ab* gene expression cassette and *uidA* gene expression cassette by using the component elements of each gene expression cassette as probes, it was determined that the modified *cry2Ab* gene expression cassette was inserted in complete condition (Annex 2 of Biological Diversity Risk Assessment Report for 15985); on the other hand, the *uidA* gene expression cassette was inserted in partial condition (Annex 2 of Biological Diversity Risk Assessment Report for 15985). It was confirmed that this partial part of the *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 of the neighboring sequence of the inserted gene (Annex 3 of Biological Diversity Risk Assessment Report for 15985). The map of the inserted DNA is shown in Figure 10.

(3) Inter-individual or inter-generational expression stability under natural conditions

As a result of western blotting analysis, the modified Cry2Ab protein showed stable expression in R1, R3, R4 and BC2F3 generations for 15985 (Annex 7 of Biological Diversity Risk Assessment Report for 15985).

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* (Annex 4 of Biological Diversity Risk Assessment Report for 15985). As a result, it emerged that the 377th amino acid residue from N terminal of amino acid sequence changed from glutamate (E) to lysine (K) (Annex 4 of Biological Diversity Risk Assessment Report for 15985) (this protein is hereinafter referred 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 the all GUS protein family expressed in plants, microorganisms and mammals (Annex 4 of Biological Diversity Risk Assessment Report for 15985); ii) this amino acid variation does not effect the active region and its three-dimensional structure of GUS protein (Annex 4 of Biological Diversity Risk Assessment Report for 15985); and iii) as a result of examining whether GUSE377K shares amino acid sequence with known allergens or not with use of the protein database (SwissProt ver.30, PIR ver.41), GUSE377K shows no homology of sequence between known allergens (Annex 4 of Biological Diversity Risk Assessment Report for 15985).

In addition, the molecularly analyzed generation is the R3 generation and the multiple BC2F3 generations derived from the R1 generation, that were evaluated by environmental safety tests in the USA (Figure 6). In all generations analyzed, the 1,490th base from 5' terminal of *uidA* gene appeared 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, and not occurring during transmission to progeny. Based on the above, it was suggested that GUSE377K is expressed also in the (R1 and R4) generations evaluated by environmental safety test in Japan.

The hereditary stability of 15985 was confirmed by Southern blotting analysis in multiple generations (R1, R2, R3 and R4, which are selfed lines from 15985; and BC2F3 generation developed by crossing with two (2) current cotton cultivars) (Annex 8 of Biological Diversity Risk Assessment Report for 15985).

(4) Existence of transmission routes and its scale, if it is possible that nucleic acids introduced via viral infection or other routes might be transmitted to wild animals and plants

The plasmid PV-GHBK11 can only sustain autonomous replication in Gram-negative bacteria, such as *E.coli* and *A. tumefaciens*. Therefore, there is no possibility that the introduced nucleic acids might be transmitted to wild animals and plants that are not sexually compatable under natural conditions.

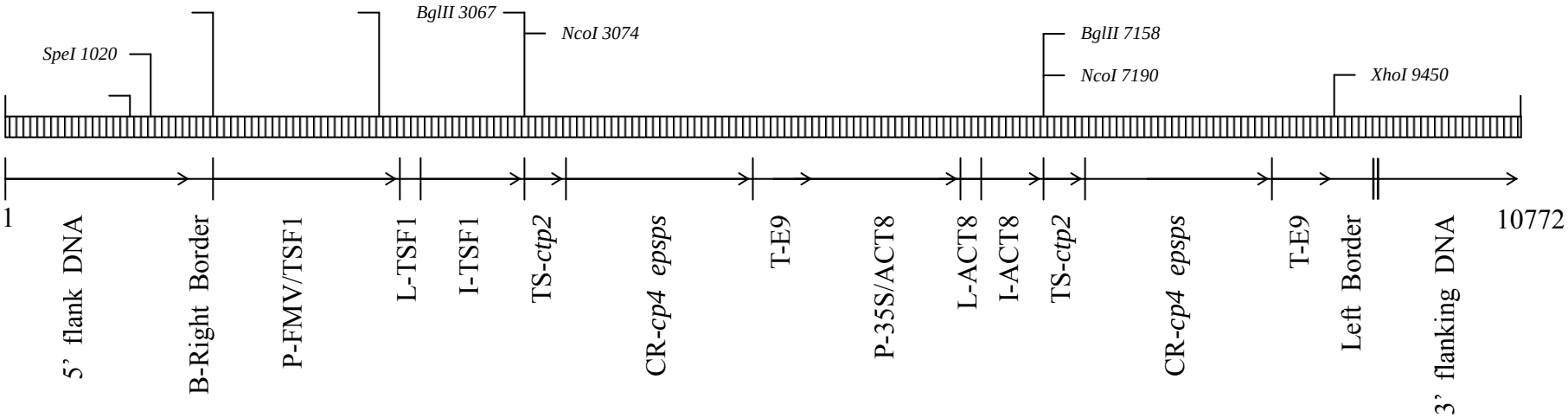

Figure 8 Genetic map of inserted genes in cotton tolerant to glyphosate herbicide, MON 88913 [8]

[8] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

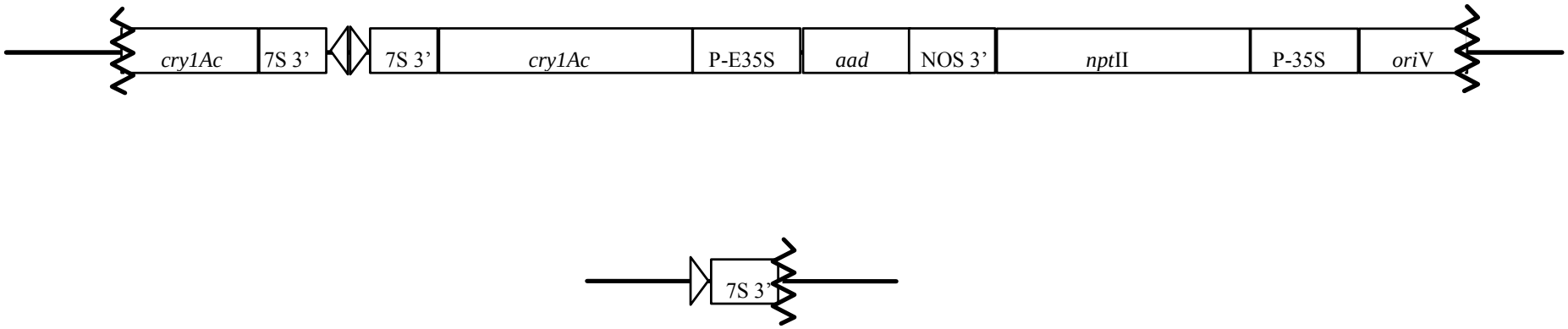

Figure 9 Genetic map of inserted genes in cotton resistant to Lepidoptera, 531 [9]

[9] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsanto Japan Limited.

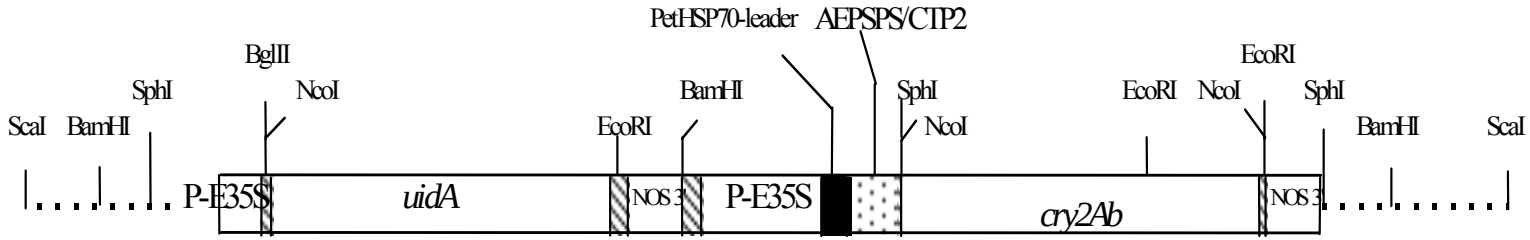

Figure 10　Genetic map of inserted genes in cotton resistance to Lepidoptera, 15985[10]

[10] All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon Monsant Japan Limited.

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

For the detection and identification of MON 88913, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and the nearby regions of the plant genome are used as primers. This method makes it possible to specifically detect MON 88913. This method is detailed in Annex 1 of Biological Diversity Risk Assessment Report for MON 88913.

For the detection and identification of 15985, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and the nearby regions of the plant genome are used as primers. This method makes it possible to specifically detect 15985. This method is detailed in Annex 3 of Biological Diversity Risk Assessment Report for 15985.

For the detection and identification of this stack line cotton, the above-mentioned two methods must be applied to each grain of cotton seeds.

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

It has been indirectly demonstrated that the modified CP4 EPSPS protein, modified Cry1Ac protein and modified Cry2Ab protein are expressed in the plant body of this stack cotton by the function of genes which were inserted in parent lines MON 88913 and 15985. As mentioned in I-1-(2), it is suggested that EPSPS protein, which possesses the same functions as the modified CP4 EPSPS protein, is not a rate-determining enzyme in the shikimate pathway. In addition, Monsanto Co. examined aromatic amino acid content 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 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 the metabolic pathway of recipient organisms. Furthermore, the modified CP4 EPSPS protein has high substrate specificity. The modified Cry1Ac protein and modified Cry2Ab protein do not possess enzyme activity and function independently of the metabolic system of recipient organism. Based on the above understanding, there is no reason to suspect that these three proteins would affect each other.

To confirm the above understanding in practice, regarding tolerance to glyphosate herbicide of this stack cotton, glyphosate-spraying tests were carried out for MON 88913, this stack line cotton (MON 88913×15985) and non-recombinant control cotton to examine the degree of plant necrosis by glyphosate. As a result, no statistically significant difference was observed in the degree of necrosis caused by the sprayed glyphosate between MON 88913 and this stack line cotton (Table 5).

Similarly, regarding resistance to Lepidoptera of this stack cotton, leaf tissues taken from 15985, this stack line cotton and non-recombinant control cotton were given to the 3rd instar larvae of tobacco budworm (TBW), the target insect, and 6 days later, mortality and fresh weight of larvae were examined. As a result, no statistically significant difference was observed in the mortality and fresh weight of larvae of TBW between 15985 and this stack line cotton (Table 6). This test was conduced also with the 2nd instar larvae which are more sensitive than the 3rd instar larvae. However, statistical analysis was not conducted since the average mortality on 6th day after giving the leaf tissue became 100% for both 15985 and MON 88913×15985.

Based on the above results, it was proved that the modified CP4 EPSPS protein modified Cry1Ac protein and modified Cry2Ab protein expressed in this stack line cotton function independently from each other. Then, for the information used in the Item-by-item assessment of Adverse Effects on Biological Diversity in II about the difference between this stack line cotton and the taxonomic cotton species to which the recipient organism belongs, the results of individual examinations for the various characteristics of MON 88913 and 15985 were attached as follows.

Table 5　Incidence of necrosis in MON 88913, this stack line cotton, and non-recombinant control cotton sprayed with glyphosate herbicide[11]

	Dose 1.125 lb ae/acre [c]	Dose 16.125 lb ae/acre
Test sample [b]	13th leaf stage	13th leaf stage (Standard error)
MON 88913	0.0	26.7 (1.67) a
MON 88913 x 15985	0.0	22.5 (1.71) a
Non-recombinant control cotton	10.0	81.7 (1.67) b

a, Incidence of necrosis (0-100%) was measured about 7 days after spraying of glyphosate. Any difference in the alphabetical letter following the numerical values in individual columns means statistically significant difference was observed in the degree of necrosis ($p < 0.05$). The sprayed amount of glyphosate of 1.125 lb ae/acre is equivalent to about 1.5 times higher dose than normal, and the sprayed amount of 16.125 lb ae/acre is equivalent to about 21 times higher does than normal. In this way, by spraying excessive amount of glyphosate, confirmation was made for possible increase in the tolerance to glyphosate between MON 88913 and this stack line cotton. As a result, no statistically significant difference was observed in the degree of growth inhibition due to necrosis between this stack line cotton and MON 88913. Consequently, it was indicated that the tolerance to glyphosate herbicide of this stack line cotton is comparable to that of MON 88913 which expresses the CP4 EPSPS protein alone.

b, The number of test sample plants used in the glyphosate spraying tests is listed below.

1.125 lb ae/acre

MON 88913 ; 7 samples

MON 88913×MON 15985 ; 11 samples

Non-recombinant control cotton; 4 samples

16.125 lb ae/acre

MON 88913 ; 6 samples

MON 88913×MON 15985 ; 10 samples

Non-recombinant control cotton; 3 samples

[11] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

c, ae ; acid equivalent refers to the values in terms of acid. Glyphosate is contained in the Roundup preparation in the form of glyphosate isopropylamine salt. The active ingredient is glyphosate acid, and then the measurements were indicated in terms of acid as the unit for active ingredient. 1.125 b ae/acre is equivalent to a dose of 350 mL of undiluted solution of glyphosate herbicide per 10 a, and 16.125 lb ae/acre is equivalent to 5,000 mL of undiluted solution of glyphosate herbicide per 10 a.

Table 6 Mortality and weight of larvae of tobacco budworm (TBW) of order Lepidoptera given the leaf tissues of 15985, this stack line cotton and non-recombinant control cotton[12]

	TBW	
Test sample	% M[a] (Standard error)	Wt[2] (Standard error)
15985	80 (9.4) a	33.4(2.9) a
MON 88913 x 15985	85 (6.1) a	29.9 (2.5) a
Non-recombinant control cotton	0 (0.0) b	99.3 (3.1) b

To the 32-well plate where the 3rd instar larvae of TBW are present in each well, leaf tissues taken from 15985, this progeny variety, and non-recombinant control cotton are given and 6 days later, mortality and weight of larvae were measured. Any difference in the alphabetical letter following the numerical values in individual columns means statistically significant difference was observed in the degree of mortality ($p < 0.05$). 15985, which expresses the *Bt* proteins of both the modified Cry1Ac protein and modified Cry2Ab protein, exhibits a significantly higher level of expression, about 4 times on average compared to 531 which expresses modified Cry1Ac protein alone based on qualitative bioassay. Therefore, if either one of the modified *cry1Ac* gene or modified *cry2Ab* gene is separated, it could be immediately detected.

As a result of statistical analysis, no statistically significant difference was observed between 15985 and this stack line cotton in the mortality and weight of TBW. Consequently, it was proved that this stack line cotton is comparable to 15985 in the resistance to Lepidoptera.

[a] % M＝Average mortality of TBW on the 6th day after being given the leaf tissues (%). The parenthesized values refer to standard error.

[2]Wt＝Average weight of TBW which survived on the 6th day after being given the leaf tissues (mg). The parenthesized values refer to standard error.

[12] All the rights pertinent to the information in the table above and the responsibility for the content rest upon Monsanto Japan Limited.

(1) Regarding MON 88913, it was confirmed based on ELISA analysis that the modified CP4 EPSPS protein, which is encoded by the modified *cp4 epsps* gene to confer tolerance to herbicides, is expressed in young leaves, leaves, roots, seeds, and pollen (Table VI-1 on Page 64 of Annex 1 of Biological Diversity Risk Assessment Report for MON 88913).

Regarding 15985, it was confirmed based on ELISA analysis that the modified Cry1Ac protein, which is encoded by the modified *cry 1Ac* gene, and the modified Cry2Ab protein, which is encoded by the modified *cry2Ab* gene, are expressed, and the modified Cry2Ab protein is expressed in young leaves, leaves, seeds and plant body of 15985 (Annex 5 of Biological Diversity Risk Assessment Report for 15985). On the other hand, regarding modified Cry1Ac protein, the amount of expression in 15985, and its recombinant mother plant DP50B was examined based on ELISA analysis with the use of young leaves, leaves, seeds, plant body and pollen. As a result, no difference is found between 15985 and DP50B, regarding the amount of expression of modified Cry1Ac protein in each organ. Therefore, it was proved that modified Cry1Ac protein and modified Cry2Ab protein do not show interaction in 15985 (Annex 5 of Biological Diversity Risk Assessment Report for 15985). In addition, regarding NPTII protein, the amount of expression is also examined in 15985 and DP50B based on ELISA analysis with the use of leaves and seeds and as a result, no definite difference was found in the amount of expression (Annex 5 of Biological Diversity Risk Assessment Report for 15985).

Based on the above results, it is considered that modified Cry1Ac protein, modified Cry2Ab protein and modified CP4 EPSPS protein are expressed in leaves and seeds of this stack line cotton.

(2) Isolated field tests of [13]MON 88913 were carried out in isolated fields in Kawachi Research Farm (KRF) of Monsanto Japan Limited from May 2004 to February 2005 (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913). Cotton used in the isolated field tests is as follows: for the recombinant cotton, seeds (= R3 generation) harvested from the R2 generation of MON 88913 was used; and for the non-recombinant control cotton, glyphosate-sensitive Null type cotton was used which was obtained through the screening of the modified *cp4 epsps* gene in R2 generation carried out during the rearing process of MON 88913 (which is represented as

[13] All the rights pertinent to the information in the paragraphs (a) through (g) following this section and the responsibility for the contents rest upon Monsanto Japan Limited.

MON 88913(-) in Annex 1 of Biological Diversity Risk Assessment Report for MON 88913, though, hereinafter referred to as "Null-type cotton" in this Report) (Figure 4).

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

(a) Morphological and growth characteristics

[Morphological and growth characteristics of MON 88913]

Differences in morphological and growing characteristics were examined between this MON 88913 and the control Null-type cotton with respect to the following 18 items: the uniformity of germination; germination rate; plant type; plant height; the number of flowers; flower color; leaf shape; the number of effective flower buds; the number of bearing shoots; boll opening time; the shape of bolls; the color of fiber (lint); the number of bolls per plant; the number of segments of a boll; the number of seeds per boll; the color of seeds; the weight of a boll; and the weights of above and under-ground parts at the harvest time. (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913).

Measurement and statistical analysis were performed especially with respect to the following items: germination rate; plant height; the number of flowers; the number of effective flower buds; the number of bearing shoots; the number of bolls per plant; the weight of a boll; the number of segments of a boll; the number of seeds per boll; and the weights of an above and under-ground parts at the harvest time (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913). As a result, statistically significant differences ($p<0.05$) between this recombinant cotton and the control Null-type cotton were found in germination rate and plant height, but not in other items.

The germination rate, in which a statistical difference was observed, averaged 41.0% in this MON 88913, and 55.8% in the control Null-type cotton. For reference, in germination tests carried out in 2002 at three fields in the USA (California [CA], Georgia [GA], and Alabama [AL]), seeds from the MON 88913

and the control Null-type cotton collected in Georgia and Alabama showed low germination rates, as well, at less than 50% on average. (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913).

The plant height, in which a statistical difference was also observed, averaged 167.2 cm in this MON 88913, and 175.2 cm in the control Null-type cotton. For reference, in study carried out at 14 fields in the USA in 2002 to examine plant height 4 weeks (first plant height), 8 weeks (second plant height) and 12 weeks (third plant height) after seeding, no statistical differences were observed between MON 88913 and the control Null-type cotton. (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913).

[Morphological and growth characteristics of 15985]

Differences in the following 20 items of morphological and growth characteristics were examined between 15985, the recombinant parental control variety 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 below-ground parts at the harvest time. As for the analysis of bolls among these items, 2 bolls were selected from each individual plant. For characteristics concerning bolls, two bolls per plant were measured. With regard to the analysis of the following items, including uniformity of germination, germination rate, flowering time, boll opening time, and harvest time, all individuals were analyzed.

As a result, no difference was observed among 15985, the recombinant parental control variety, DP50B, and the non-recombinant control cotton DP50 in isolated field tests conducted in Kawachi Research Farm (KRF) using the R1 generation (Annex 8 of Biological Diversity Risk Assessment Report for MON 15985).

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 weight of the below-ground parts. However, no difference was observed in other items (Annex 8 of Biological Diversity Risk Assessment Report for 15985). Regarding leaf length, the significant difference from this recombinant cotton was observed both in the recombinant parental varity DP50B and the non-recombinant control cotton DP50. The average leaf length of 15985, the recombinant parental variety DP50B, and the non-recombinant control cotton DP50, were 16.5cm, 17.8cm and 17.9cm, respectively. Regarding the weight of the below-ground parts, the significant difference from this recombinant cotton was observed only in the non-recombinant control cotton DP50, and there was no difference between this recombinant cotton and the recombinant parental variety, DP50B. The average weight of the below-ground parts of 15985, the recombinant parental variey DP50B, and the non-recombinant control cotton DP50, were 163.3g, 156.7g and 133.3g, respectively.

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

[Cold-tolerance and heat-tolerance at the early stage of growth of MON 88913]

The seedlings of MON 88913 and the control Null-type cotton were set under cold (5℃) conditions. As a result, they had both died almost completely by the 24th day. No difference was observed between them (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913).

[Cold-tolerance and heat-tolerance at the early stage of growth of 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 USA. All of these fields are located in common regions for cotton cultivation in the South of the USA. The winter in these regions is relatively mild compared to the average climatic conditions of Japan. Therefore, it is considered that these regions provide better climatic conditions for cotton growth than Japan (Reference 57).

As a result of observation, it was found that some seeds split on the field had germinated in fall after harvesting. However, it was reported that all of them had died by the following spring. Based on the above understanding, it was judged that

cold-tolerance of 15985 is as low as that of the non-recombinant control cotton at the early stage of its growth.

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

Basically cotton is a perennial plant, but only in tropical regions. In other cotton cultivation regions in the world, including Japan, cotton naturally dies during winter after bearing-fruit. In practice, it was observed that the plants were partly dead when the isolated field tests of MON 88913 and 15985 were completed. Based on the above, the wintering ability test was not conducted for adult plants of MON 88913 and 15985.

(d) Fertility and size of the pollen

In Japan, there are no plans for MON 88913 and 15985 to be commercialized, or cultivated for commercial use (Reference 5). Therefore, if MON 88913 and 15985 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 displace other plants from the area. However, the fertility and the size of pollens were not examined for MON 88913 and 15985, because there have been no reports of seeds spilled during transportation, or that they have grown or become self-seeding under natural conditions in Japan. Additionally, pollen would not be formed until the spilled cotton seeds germinate, establish themselves, grow to maturity, and flower.

(e) Production, shedding habit, dormancy and germination rate of the seed

[Production, shedding habit, dormancy and germination rate of the seed of MON 88913]

A comparison was made for the number of bolls per plant and the number of seeds per boll between MON 88913 and the control Null-type cotton. As a result, no statistically significant difference was observed between them (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913).

Low shedding habit was expected in both MON 88913 and the control Null-type

cotton because the fiber adhering to cotton seeds does not allow them to shed.

As for dormancy and germination rate, it was found that the germination rates of the seeds from MON 88913 and the control Null-type cotton were low, at 58.9% and 54.4%, respectively. No statistically significant differences were observed between them (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913).

For reference, here are presented the results of germination tests conducted in the USA in 2002, using seeds from MON 88913 and the control Null-type cotton collected at 3 fields in California (CA), Georgia (GA) and Alabama (AL), as well as from additional 6 conventional cultivars included for reference. Seeds from the recombinant cotton and the control Null-type cotton collected in Georgia and Alabama showed low germination rates, as well, at less than 50% on average. In addition, all the seeds were found to be in the condition of germination, death or viable firm swollen, and none of them was in the condition of dormancy viable hard (Annex 1 of Biological Diversity Risk Assessment Report for MON 88913).

[Production, shedding habit, dormancy and germination rate of the seed of MON 15985]

Regarding seed production, the differences among 15985, the recombinant parental variety 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, and the number of seeds per boll. As a result, no statistically significant difference was observed in all items for both R1 and R4 generations (Annex 8 of Biological Diversity Risk Assessment Report for 15985).

Regarding shedding habit, in both of 15985 and the non-recombinant control cotton, seeds are covered with down and lint at harvest time. Therefore, shedding habit of the seed under natural conditions was not observed.

To evaluate dormancy, in 3 isolated fields in Texas (TX), South Carolina (SC) and Louisiana (LA) in the US in 1999, germination rate of the seeds was examined with use of the harvested seeds of 15985, the recombinant parental variety DP50B, the non-recombinant control cotton DP50 and additional 11 current commercial varieties included for reference, under the different temperature conditions from 5 to 40℃ (Annex 9 of Biological Diversity Risk Assessment Report for 15985).

As a result, under some temperature conditions, statistically significant difference was observed between 15985 and the recombinant mother plant DP50B ($p<0.05$).

However, the difference was within the value range of 11 current commercial varieties which were added for reference (Annex 9 of Biological Diversity Risk Assessment Report for 15985). On the other hand, in various temperature conditions, the seeds of 15985, the recombinant mother plant DP50B and 11 current cultivars were germinated, viable firm swollen or degenerated, and no seed of viable hard was observed (Annex 9 of Biological Diversity Risk Assessment Report for 15985).

Germination rate was examined in "(a) Morphological and growth characteristics". As a result, no difference was observed between the recombinant parental variety DP50B, and the non-recombinant control cotton DP50 in both R1 and R4 generations. (Annex 8 of Biological Diversity Risk Assessment Report for 15985).

(f) Crossability

In Japan, no wild relatives exist that belong to *Gossypium* which could sexually cross with the tetraploid cotton species, *Gossypium hirsutum*,, to which MON 88913 and 15985 belong (Reference 3, Reference 4). Thus, the outcrossing ability of MON 88913 and 15985 was not assessed.

(g) Productivity of harmful substances

[Productivity of harmful substances of MON 88913]

As a result of a plow-in test, a successive cropping test, and soil microflora test, no statistically significant difference was observed in all items examined between MON 88913 and the control Null-type cotton (Annex 3 of Biological Diversity Risk Assessment Report for MON 88913).

[Productivity of harmful substances of 15985]

As a result of a plow-in test, a successive cropping test, and soil microflora test by using R1 generation and R4 generation plants, no statistically significant difference was observed in all items examined between 15985, the recombinant parental variety DP50B, and the non-recombinant control cotton DP50 (Annex 8 of Biological Diversity Risk Assessment Report for 15985).

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

A review was made by persons with specialized knowledge and experience concerning Adverse Effects on Biological Diversity (called Experts) for possible Adverse Effects 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 developed by traditional crossbreeding method of cotton tolerant to glyphosate herbicide (MON-88913-8) and cotton resistant 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 for the Type I Use same as this stack line cotton.

It is reported that the modified CP4 EPSPS protein, which is encoded by the modified *cp4 epsps* (gene tolerant to glyphosate) derived from MON-88913-8, has high substrate specificity and that the modified Cry1Ac protein and modified Cry2Ab protein, which are encoded by the modified *cry1Ac* and modified *cry2Ab* (genes resistant to Lepidoptera) derived from MON-15985-7 do not possess enzyme activity. Therefore, it is considered that the characteristics conferred by modified *cp4 epsps,* modified *cry1Ac,* and modified *cry2Ab* do not interact with each other.

It was confirmed, based on the various herbicide-spraying tests and the bioassay using the tobacco budworm (*Heliothis virescens*) respectively, that the tolerance to glyphosate herbicide, and the resistance to Lepidoptera are properly expressed in this stack line cotton.

Based on the above understanding, it is considered that there is no specific change in the characteristics 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

This stack line cotton possesses the glyphosate herbicide tolerance derived from MON-88913-8 and the Lepidoptera resistance derived from MON-15985-7. However, it is considered that the glyphosate does not exert pressure for selection under a natural

environment in Japan, and that the insect damage by Lepidoptera is not the major cause to make the cotton difficult to grow in the natural environment in Japan. Consequently, it is considered that these characteristics do not increase the competitiveness and thus 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 modified CP4 EPSPS protein derived from MON 88913 and the modified Cry1Ac protein and modified Cry2Ab protein derived from 15985. It is confirmed that the modified Cry1Ac and modified Cry2Ab proteins possess the insecticidal activity against insects of the order Lepidoptera, though the modified CP4 EPSPS protein is not harmful to animals and plants. Thus, it is considered that the production of harmful substances of this stack line cotton would not become higher than that of the parent lines, even if this stack line cotton contains both of these proteins.

Therefore, the conclusion that the use of this recombinant cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to the production of harmful substances, which was made by the applicant, is valid.

(3) Crossability

In the Japanese natural environment, there are no wild species which cross with cotton. Therefore, it was judged that there are no specific wild plants or wild animals that are possibly affected by this recombinant cotton, and that the use of such cotton poses no risk of Adverse Effect on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by applicant is valid.

2. Conclusion based on the Biological Diversity Risk Assessment Report

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.

[Bibliography]

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