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 glufosinate herbicide and resistant to Lepidoptera (*2mepsps*, modified *bar*, modified *cry1Ac*, modified *cry2Ab*, *Gossypium hirsutum* L.) (GHB614×LLCotton25×15985, OECD UI: BCS-GHØØ2-5×ACS-GHØØ1-3×MON-15985-7) [including the progeny lines isolated from the cotton lines, GHB614, LLCotton25 and 15985, that contain a combination of any of the transferred genes in the individual cotton lines (except those already granted an approval regarding Type 1 Use Regulation)]
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	-

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

1. Information concerning preparation of living modified organisms

This stack cotton line has properties of GHB614, LLCotton25 and 15985. Thus, information on preparation of the individual parent lines and others is described below.

The cotton 15985 was developed by newly transferring the modified *cry2Ab* gene derived from *Bacillus thuringiensis* ssp. *kurstaki* to the recombinant cotton DP50B which was developed by repeated crossing of the cotton 531 (531, OECD UI: MON-ØØ531-6) (resistant to Lepidoptera due to the transferred modified *cry1Ac* gene derived from *B. thuringiensis* ssp. *kurstaki* (modified *cry1Ac*, *Gossypium hirsutum* L.) and the non-recombinant cotton cultivar DP50. Therefore, information on preparation of the cotton 531 and others is also described below.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Component elements of the donor nucleic acid used for the development of GHB614, LLCotton25, 531 and 15985 are shown in Table 1 to Table 4 (p.3 - p.7) respectively. In addition, composition of the donor nucleic acid is shown in Figure 1 to Figure 4 (p.15 - p.18).

Table 1 Component elements of the donor nucleic acid used for the development of GHB614

Component elements	Position in vector	Size (bp)	Function
2mepsps gene expression cassette			
Ph4a748At	0026-1036	1011	A sequence including the promoter region of the histone H4 gene derived from *Arabidopsis thaliana* (Reference 10). This sequence constitutively initiates the transcription of the *2mepsps* gene in plant tissues.
intron1 h3At	1037-1553	517	A sequence including the first intron of the histone H3.3 II gene derived from *A. thaliana* (Reference 11).
TPotp C	1554-1926	373	A sequence developed based on the coding region of plastid transit peptide derived from RuBisCo small subunit genes of sunflower (*Helianthus annuus*) and maize (*Zea mays*) (Reference 37). This sequence transits the mature 2mEPSPS protein to the plastids.
2mepsps	1927-3264	1338	A gene encoding the double-mutant 5-enol-pyruvyl-shikimate-3-phosphate synthase (2mEPSPS protein), and resulting from two point mutations in 5-enol-pyruvyl-shikimate-3-phosphate synthase gene (*epsps* gene) derived from maize (*Z. mays*) (Reference 38). This gene confers the tolerance to glyphosate herbicide. In the 2mEPSPS protein, the 102nd amino acid threonine and the 106th amino acid proline in the wild-type EPSPS protein have been substituted by isoleucine and serine, respectively. The sequence encoding the plastid membrane transit peptide has been removed from the *epsps* gene.
3'histonAt	3265-4007	743	A sequence including the 3' untranslated region of the histone H4 gene derived from *A. thaliana* (Reference 10). This sequence terminates the transcription and causes the 3' polyadenylation.
Other component elements			
LB	0001-0025	25	A Left border repetitive sequence of T-DNA derived from *Rizobium radiobacter* (*Agrobacterium tumefaciens*) (Reference 75).
RB	4008-4032	25	A Right border repetitive sequence of T-DNA derived from *R. radiobacter* (*A. tumefaciens*) (Reference 75).
-	4033-4224	192	A fragment derived from plasmid pTiAch5 in the right border repetitive sequence (Reference 77).
*npt*I fragment	4225-4935	711	A fragment of transposon Tn903-derived *npt I* gene that codes for neomycin phosphotransferase (Reference 47). This sequence does not function, because it is a fragment.
ORI ColE1	4936-6108	1173	A sequence including the replication origin of plasmid pBR322 derived from *Escherichia coli* (Reference 7).

ORI pVS1	6109-9879	3771	A sequence including the replication origin (Reference 32) of plasmid vector pVS1 derived from *Pseudomonas* (Reference 29).
*aad*A	9880-11648	1769	A sequence including *E. coli*-derived gene that confers the tolerance to aminoglycoside derivative antibiotics (Reference 20).
-	11649-11953	305	A fragment derived from plasmid pTiAch5 in the left border repetitive sequence (Reference 77).

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

Table 2 Component elements of the donor nucleic acid used for the development of LLCotton25

Component elements	Position in vector	Size (bp)	Function
Modified *bar* gene expression cassette			
P35S3	250-1634	1385	The promoter region of cauliflower mosaic virus 35S transcript gene. This region initiates the transcription (Reference 44).
Modified *bar*	1635-2186	552	Bialaphos resistance (*bar*) gene derived from *Streptomyces hygroscopicus*. It confers the tolerance to glufosinate herbicide (Reference 65). Two codons in the N-terminal of the wild *bar* gene, or GTG and AGC, have been replaced with ATG and GAC, respectively. Amino acid has changed from serine to asparagine for the replacement of AGC with GAC, while remaining methionine for the replacement of GTG with ATG.
3'nos	2206-2465	260	The 3' untranslated region of nopaline synthase gene derived from T-DNA of pTiT37. This region terminates the transcription and causes the 3' polyadenylation (Reference 16).
Other component elements			
RB	198-222	25	A right border repetitive sequence derived from T-DNA of pTiB6S3 (Reference 25).
LB	2520-2544	25	A left border repetitive sequence derived from T-DNA of pTiB6S3 (Reference 25).
*aad*A	2545-4618	2074	A sequence including the streptomycin/spectinomycin-resistance gene derived from the transposon Tn7 (Reference 39).
pVS1ori	4619-8389	3771	The replication origin of plasmid pVS1 derived from *Pseudomonas* (Reference 32).
ColE1	8390-9555	1166	A sequence including ColE1 ori, which is the replication origin of the plasmid pBR322 (Reference 7).

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Table 3 Component elements of the donor nucleic acid used for the development of 531

Component elements	Function
Modified *cry1Ac* gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 35), from cauliflower mosaic virus (CaMV) (Reference 44).
Modified *cry1Ac*	A gene that encodes the modified Cry1Ac protein that exhibits insecticidal activity against order Lepidoptera such as Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm] (*Helicoverpa zea*), the major insect pests for cotton cultivation. Though it encodes the protein which shows 99.4% of amino acid sequence homology with the wild-type Cry1Ac protein produced by *Bacillus thuringiensis* ssp. *kurstaki*, the core protein contains the identical amino acid sequence to that of the wild-type Cry1Ac protein (Reference 1).
7S 3'	3' untranslated region of soybean *β*-conglycinin gene. Contains a signal for the polyadenylation of mRNA (Reference 56), and functions to terminate transcription of the target gene.
nptII gene expression cassette	
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (References 55; 56).
nptII	A gene derived from *E. coli* transposon Tn5 (Reference 4). Encodes neomycin phosphotransferase type II (*npt II*) and confers resistance to kanamycin. Used as marker to select the transgenic plant during the gene transfer (Reference 22).
NOS3'	3' untranslated region of nopaline synthase (NOS) gene derived from *R. radiobacter* (*A. tumefaciens*) (References 6; 16). It terminates transcription and induces polyadenylation.
Other component elements	
Right border sequence (RB)	A DNA sequence containing right border sequence (24bp) of nopaline type T-DNA derived from Ti plasmid pTiT37. RB (right border sequence) is used as the initiation point of T-DNA transfer from *R. radiobacter* (*A. tumefaciens*) to plant genome (References 6; 16).
*aad*A	Bacteria promoter, code region and terminator for the 3'(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin/streptomycin (Reference 20).
ori-V	The replication origin derived from the broad-host range plasmid RK2. Permits autonomous replication of vectors in *R. radiobacter* (*A. tumefaciens*) ABI strain (Reference 60).
ori322/rop	The replication origin derived from *E. coli* plasmid pBR322. Permits autonomous replication of vectors in *E. coli*. This region contains not only replication origin, but also *rop* region that is involved in the regulation of the replication initiation, and *oriT* sequence that is necessary for conjugal transfer of DNA from *E. coli* to *R. radiobacter* (*A. tumefaciens*) (References 7; 63).

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5

Table 4 Component elements of the donor nucleic acid used for the development of 15985

Component elements	Function
uidA gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 35), from cauliflower mosaic virus (CaMV) (Reference 44).
Modified *uidA*	*uidA* gene derived from *E. coli* plasmid pUC19. Encodes GUS (*β*-D-glucuronidase) protein (Reference 26). GUS protein is an enzyme that hydrolyzes the β-glucuronide, a condensate of glucuronic acid and various aglycone. It is used as the quantitative visual marker during transformation.
NOS'3	3' untranslated region of nopaline synthase (NOS) gene derived from *R. radiobacter* (*A. tumefaciens*) (References 6; 16). It terminates transcription and induces polyadenylation.
Modified *cry2Ab* gene expression cassette	
P-E35S	Promoter with duplicated enhancer (Reference 35), from cauliflower mosaic virus (CaMV) (Reference 44).
PetHSP70 leader	5' untranslated region of hsp70 (heat shock protein) of *Petunia hybrida*.
AEPSPS/CTP2	The sequence that encodes the N-terminal chloroplast transit peptide sequence derived from *A. thaliana* EPSPS gene (Reference 66).
Modified *cry2Ab*	It is a gene derived from *Bacillus thuringinensis* ssp. *kurstaki*, and 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] (*Helicoverpa zea*), which are the major pest insects of order Lepidoptera for cotton cultivation (Reference 73). The Cry2Ab protein exhibits insecticidal activity also against insects of order Lepidoptera: for example, Fall Armyworm (*Spodoptera frugiperda*), Beet Armyworm (*Spodoptera exigua*) and Soybean Looper (*Pseudoplusia includens*), which are the pest insects of order Lepidoptera for cotton cultivation.
NOS3'	3' untranslated region of nopaline synthase (NOS) gene derived from *R. radiobacter* (*A. tumefaciens*) (References 6; 16). It terminates transcription and induces polyadenylation.

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2) Function of component elements

(a) Functions of individual component elements of donor nucleic acid, including target gene, expression regulatory region, localization signal, and selectable marker

Functions of the component elements of the donor nucleic acid that was used for the development of GHB614, LLCotton25, 531 and 15985 are respectively shown in Table 1 to Table 4 (p. 3 - p. 7).

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

2mEPSPS protein

The 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) protein (EC 2.5.1.19), one of the enzymes that catalyze the shikimate pathway, a biosynthetic pathway of aromatic amino acids specific to plants and microorganisms, catalyzes reversible reaction that synthesizes 5-enol-pyruvyl-shikimate-3-phosphate (EPSP) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P). The EPSPS protein binds to both PEP and S3P to construct a three-component enzyme-substrate complex intermediate. However, the activity of EPSPS is competitively inhibited by glyphosate herbicide binding reversibly to the PEP-binding site (Reference 8). As a result, the plants become unable to synthesize aromatic amino acids, which are essential for protein synthesis, to die after a while.

In the *2mepsps* gene transferred to GHB614, two nucleotides are changed by point mutation, compared with the *epsps* gene, which encodes the EPSPS protein cloned from maize (*Z. mays*). Concerning amino acid sequence of the 2mEPSPS protein, which is expressed from the *2mepsps* gene, the 102nd amino acid threonine and the 106th amino acid proline in the wild-type EPSPS protein have been substituted by isoleucine and serine, respectively. Consequently, the 2mEPSPS protein has lower binding affinity for glyphosate, able to promote the shikimate synthesis without being inactivated by glyphosate; therefore, GHB614 can survive even in the presence of glyphosate.

In addition, in 2008, amino acid sequence homology of the 2mEPSPS protein was explored inclusively in proteins that had been registered to various databases (Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD, GenPept and AllergenOnline). As a result, this protein did not show any homology to the known allergens.

Modified PAT protein

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. In detoxification of the produced ammonia, glutamine synthase plays a pivotal role. However, if sprayed to plants, glufosinate herbicide inhibits the glutamine synthase to allow the produced ammonia to accumulate, resulting in death of the plants.

In the N-terminal of the modified *bar* gene, two codons have been modified as follows: GTG codon has been replaced with ATG to become suitable codon for plants, and AGC codon has been replaced with GAC to increase translation efficiency. In translation for the replacement of GTG with ATG, the amino acid methionine remains unchanged, though for the replacement of AGC with GAC, serine has been changed to aspartic acid.

The modified PAT protein, the product expressed from the modified *bar* gene, can acetylate glufosinate to make N-acetyl-glufosinate, inactivating the inhibitory action of the glufosinate on glutamine synthase. This mechanism would prevent ammonia from accumulating, resulting in survival of plants, even if glufosinate herbicide were sprayed to the plants.

In 2009, amino acid sequence homology of the modified PAT protein was explored inclusively in proteins that had been registered to various databases (Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD, GenPept and AllergenOnline). As a result, this protein did not show any homology with the known allergens.

Modified Cry1Ac protein

The modified *cry1Ac* gene has been produced by applying the silent mutation to the nucleotide sequence to enhance its expression level in plants and also by combining the first 1,398 bases of the *cry1Ab* gene (1st to 466th in the amino acid sequence), which shows very high homology with the amino acid sequence in the Cry1Ac protein (Reference 51), and the 1,399th to 3,534th bases of the *cry1Ac* gene (467th to 1,178th in the amino acid sequence) (References 1; 21). The 1,399th to 3,534th bases of the *cry1Ac* gene used for the development of this protein were combined with the *cry1Ab* gene, following the new transferring of silent mutation to the nucleotide sequence to enhance expression level in plant body. The modified Cry1Ac protein expressed from the modified *cry1Ac* gene has seven (7) different amino acids modified compared to the wild-type Cry1Ac protein, which is produced from *B. thuringiensis* ssp. *kurstaki* HD-73. However, these substitutions are identical to those in the modified Cry1Ac protein expressed in the cotton resistant to Lepidoptera (*cry1Ac*, *Gossypium hirsutum* L.) (531, OECD UI: MON-ØØ531-6) (hereinafter referred to as

"531"), which has been already granted an approval for Type 1 Use (November 22, 2004). Of the 7 amino acid differences, six (6) differences exist within 466th in the amino acid sequence, which is attributed to the differences in the amino acid sequence between the Cry1Ab protein, the derivation of the first half amino acid sequence in the modified Cry1Ac protein, and the wild-type Cry1Ac protein. In addition, the difference in the 766th amino acid results from the diversity of the Cry1Ac protein from *B. thuringiensis*, and it was considered to reflect the amino acid mutation, which the strain used for the cloning of gene originally possessed. As a result, the homology between the deduced amino acid sequence in the modified Cry1Ac protein expressed in this stack cotton line and the deduced amino acid sequence in the wild-type Cry1Ac protein produced from the *B. thuringiensis* ssp. *Kurstaki* HD-73 strain (Reference 1; Genbank accession M11068) is found 99.4%.

It is generally known that the Cry1A protein exhibits insecticidal activity only against the order Lepidoptera (Reference 13). In addition, it is also known that the proteins classified as the Cry1Ac protein possess diversity in the range of 95% homology (Reference 13) and some mutant forms exist in the Cry1Ac protein identified from *B. thuringiensis* (Reference 69). As mentioned above, the homology between the modified Cry1Ac protein expressed in this stack cotton line and the wild-type Cry1Ac protein produced by the *B. thuringiensis* ssp. *kurstaki* HD-73 strain is 99.4% and it is found to fall within the range of 95% or higher homology which is inherent in the Cry1Ac protein. Based on the findings, it is considered that the modified Cry1Ac protein possesses the insecticidal spectrum against the order Lepidoptera at similar level as exhibited by the Cry1Ac protein, which exists in the nature.

In order to investigate whether the modified Cry1Ac protein shares functionally important amino acid sequences with known contact allergens, the Cry1Ac protein was compared with allergens in the database (AD_2010[1]). The results showed the Cry1Ac protein did not share structurally related sequences with any of the known allergens examined.

Modified Cry2Ab protein

The modified Cry2Ab protein, which is encoded by the modified *cry2Ab* gene, is derived from *B. thruingiensis* ssp. *kurstaki*, a gram-positive bacteria existing normally in soil and also known as Cry2Ab2, CryIIB, CryB2 or CryIIAb (References 13; 40; 73). The Cry2Ab protein exhibits insecticidal activity similarly as the Cry1Ac protein against Tobacco budworm (*Heliothis virescens*), Pink bollworm (*Pectinophora gossypiella*) and Cotton bollworm [also called Corn earworm (*Helicoverpa zea*)], the major insect pests of the order Lepidoptera for cotton cultivation in the US and Australia. It exhibits

insecticidal activity also against Fall Armyworm (*Spodoptera frugiperda*), Beet Armyworm (*Spodoptera exigua*), Soybean Looper (*Pseudoplusia includens*) and other insect pests of the order Lepidoptera, which do not show much sensitivity against the 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 by leaving the amino acid sequence for the core protein remaining unchanged. Thus, it is considered comparable to the wild-type Cry2Ab protein in terms of the activity against pest insects of the order Lepidoptera.

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 (AD_2010[1]). The results showed the modified Cry2Ab protein did not share structurally related homologous sequences with any of the known allergens examined.

Modified *cry1Ac* gene＋Modified *cry2Ab* gene

The modified Cry2Ab protein is newly expressed in 15985, as well as the modified Cry1Ac protein derived from 531. Thus, pest control of a family of armyworms (Fall Armyworm, Beet Armyworm) and a family of budworms (Soybean Looper), against which 531 was not able to provide pest control effects, would be attainable (References 2; 61).

As mentioned above, the pest insects of the order Lepidoptera, which show sensitivity against the modified Cry1Ac protein and the modified Cry2Ab protein, overlap each other. Then, for the insect pests of the order Lepidoptera to acquire the resistance to 15985, the Lepidopteran insects must obtain the resistance to each of the Bt proteins. Therefore, compared to 531 which expresses only the modified Cry1Ac protein, 15985 is expected to further decrease the probability that the target insect pests of the order Lepidoptera, which shows sensitivity against the both Bt proteins, could obtain the resistance.

Modified GUS protein

The β-glucuronidase (GUS) protein derived from *E. coli*, which is encoded by the *uidA* gene, is an enzyme that hydrolyzes the β-glucuronide, a condensate of glucuronic acid and various aglycone (Reference 49). For histochemical tests, 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) is used as the substrate. This substrate becomes dimerized due to the hydrolysis

[1] FARRP (Food Allergy Research and Resource Program): Database holding the sequences registered in the Allergen Online database (FARRP, 2010) as of January 2010.

by GUS and produces indigotin blue-colored dye and then, the *uidA* gene is used as the quantitative visual marker in the process of plant transformation (Reference 33).

In the Reference 26 commenting on the safety in plants in a wide variety of aspects, the GUS protein derived from *E.coli* and used as a selective marker in plants is evaluated as safe for use as food (Reference 26). In addition, GUS-like activity has been detected in many tissues such as embryo, fruit, seed coat and endosperm of 50 or more plants (Reference 31). The plants include apple, bean, beet, cabbage, carrot, celery, corn, cucumber, lettuce, pear, pepper, radish, soybean, spinach, tomato, and other many food crops.

NPTII protein

The NPTII protein is an enzyme that catalyzes the reaction to phosphorylate the hydroxyl group of aminoglycoside contained in the aminoglycoside antibiotics (Reference 57). The NPTII protein is reported to only take part in the phosphorylation of a limited number of aminoglycoside antibiotics such as neomycin, kanamycin, paromomycin, ribostamycin, and butirosin (References 14; 15; 50).

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

2mEPSPS protein

The EPSPS protein, which is functionally identical to the 2mEPSPS protein, is an enzyme that catalyzes the shikimate pathway for biosynthesis of aromatic amino acids; however, it is not a rate-determining enzyme in the pathway and it is considered that the concentration of aromatic amino acids, the final product of this pathway, would not be increased even if the activity of EPSPS is increased (References 30; 71). In fact, it has been reported that cultured plant cells producing a 40-times or more amount of the EPSPS protein cannot excessively produce aromatic amino acids as a final product (Reference 58). In addition, there were no statistically significant differences in the content of aromatic amino acids (phenylalanine, tryptophan, and tyrosine) between GHB614 seeds, which express the 2mEPSPS protein, and seeds of the recipient cultivar.

In addition, the EPSPS protein is known to react also with shikimate, which is an analogue of S3P, as well as PEP and S3P. Nevertheless, the EPSPS protein has low reactivity with shikimate (Reference 27) and thus possesses high substrate specificity.

Based on the above understanding, due to the high substrate specificity, the 2mEPSPS protein is considered low to affect the metabolic system of the recipient organism.

Modified PAT protein

The product expressed from the modified *bar* gene, or the modified PAT protein, has high affinity for glufosinate. While glufosinate is classified into L-amino acid, the modified PAT protein cannot transfer the acetyl group to various amino acids. In addition, although glutamic acid is similar to glufosinate in structure, the modified PAT protein has little affinity for glutamic acid and it cannot cause substantial transfer reaction with glutamic acid *in vivo* (Reference 65). Furthermore, it has been reported that the modified PAT protein is not prevented from transferring the acetyl group to glufosinate even in the excessive presence of various amino acids (Reference 70). Thus, the modified PAT protein has high substrate specificity for glufosinate and it is considered unlikely to affect the metabolic system of the recipient organism.

Modified Cry1Ac protein and Modified Cry2Ab protein

There is no report that Bt protein exhibits any enzyme activity, and the modified Cry1Ac protein and the modified Cry2Ab protein are considered to function independently from the metabolic system of plants. Therefore, it is considered unlikely that these proteins could affect the metabolic system of the recipient organism.

Modified GUS protein

The substrate for the GUS protein, or glucuronide is synthesized by the action of UDP glucuronosyltransferase with the UDP glucuronate acting as the donor of glucuronyl group in the living body of vertebrate animals. This reaction means detoxification from the physiological viewpoint, in which steroid, bilirubin, aniline, benzoate and others are synthesized to glucuronide in the liver and then excreted in urine. It has been little known that β-glucuronide is present in plants, but it has been revealed to date that saponin-glucuronide (Reference 74), quercetin-glucuronide and flavonoid-glucuronide (Reference 43) are present in plants. Physiological activity of the β-glucuronide in plants has been little clarified and it has also been unknown whether they could become the substrate for the GUS protein, which is derived from *E. coli* and expressed in transgenic plants, and the gut GUS protein. However, it is known that glucuronide is removed from the primary metabolism through excretion to vacuole or apoplast as the readily water-soluble secondary metabolite (Reference 41).

In addition, based on the findings that no significant difference was observed between the recombinant control mother plant DP50B (531) and the non-recombinant control cotton DP50 as a result of analysis of component elements in 15985, that no difference was observed in morphological and growth characteristics as a result of environmental safety tests conducted in

the US and Japan, and that GUS-like activity has been recognized in food crops, it is considered unlikely that the expression of GUS protein can have significant effects on the metabolic pathway of plants.

NPTII protein

As mentioned in (b) above, it has been reported that the NPTII protein takes part in the phosphorylation of a limited number of aminoglycoside antibiotics such as neomycin, kanamycin, paromomycin, ribostamycin, and butirosin (References 14; 15; 50). In addition, as a result of investigation on the structure-activity relationship of the NPTII protein, it has been suggested that the NPTII protein becomes unable to utilize the aminoglycoside antibiotics for substrate due to any minute changes in the structure of aminoglycoside of aminoglycoside antibiotics (e.g., deletion of hydroxyl group, modification of amino group, etc.) (Reference 50). Consequently, it is considered unlikely that expression of the NTPII protein in cotton would create any new metabolic systems or new metabolites.

(2) Information concerning vector

1) Name and origin

The vector used for the development of GHB614 is pTEM2 derived from pGSC1700 (Reference 12), which was constructed based on the plasmid pBR322 derived from *E. coli* and the plasmid pVS1 derived from *Pseudomonas* (Figure 1, p. 15).

The vector used for the development of LLCotton25 is pGSV71 constructed based on the plasmid pBR322 derived from *E. coli* and the plasmid pVS1 derived from *Pseudomonas* (Figure 2, p. 16).

The plasmid vectors PV-GHBK04 and PV-GHBK11 used for the development of 531 and 15985 are both derived from pBR322. The pBR322 is a synthetic plasmid derived from *E. coli* (Figure 3, p. 17; Figure 4, p. 18).

2) Properties

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

GHB614: pTEM2; 11,953 bp
LLCotton25: pGSV71; 9,555 bp
531: PV-GHBK04; 11,407 bp
15985: PV-GHBK11; 8,710 bp

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

the functions

Component elements of pTEM2, pGS71, PV-GHBK04 and PV-GHBK11 are shown in Table 1 to Table 4 (p. 3 - p. 7) respectively.

(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

The infectivity of pTEM2, pGSV71, PV-GHBK04 and PV-GHBK11 is not known.

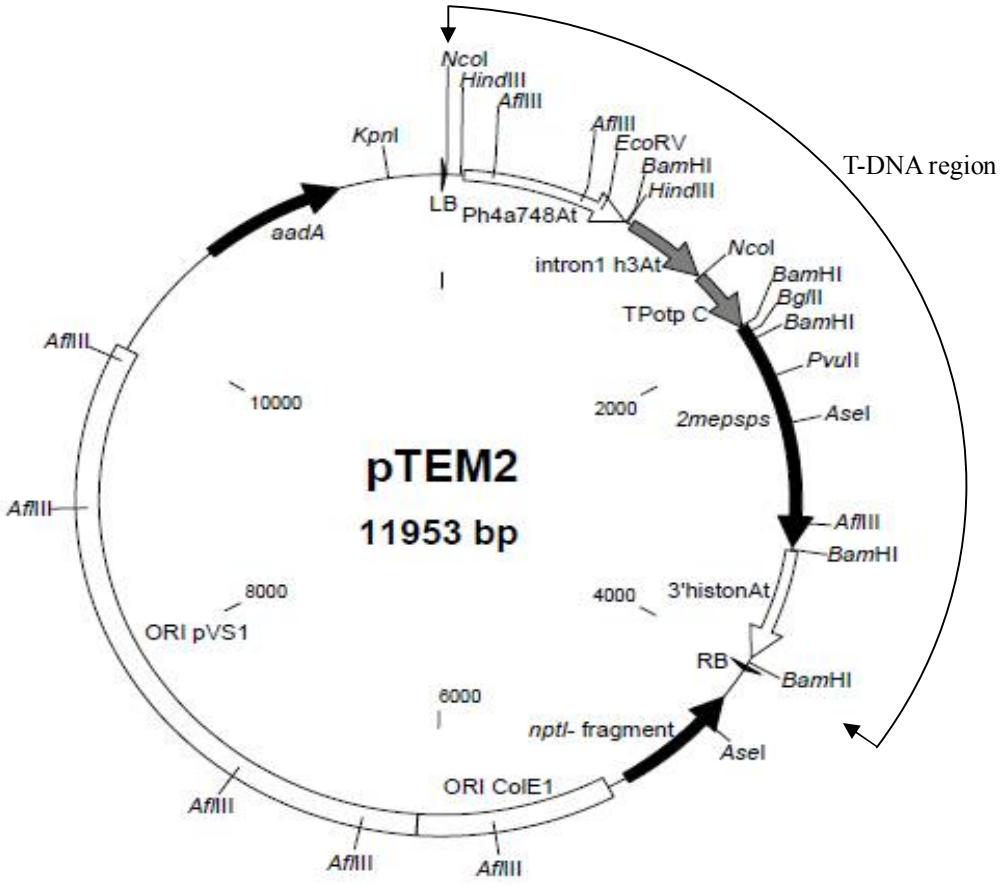

Figure 1 Map of the plasmid pTEM2 used for the development of GHB614 and the restriction enzymes cleavage sites

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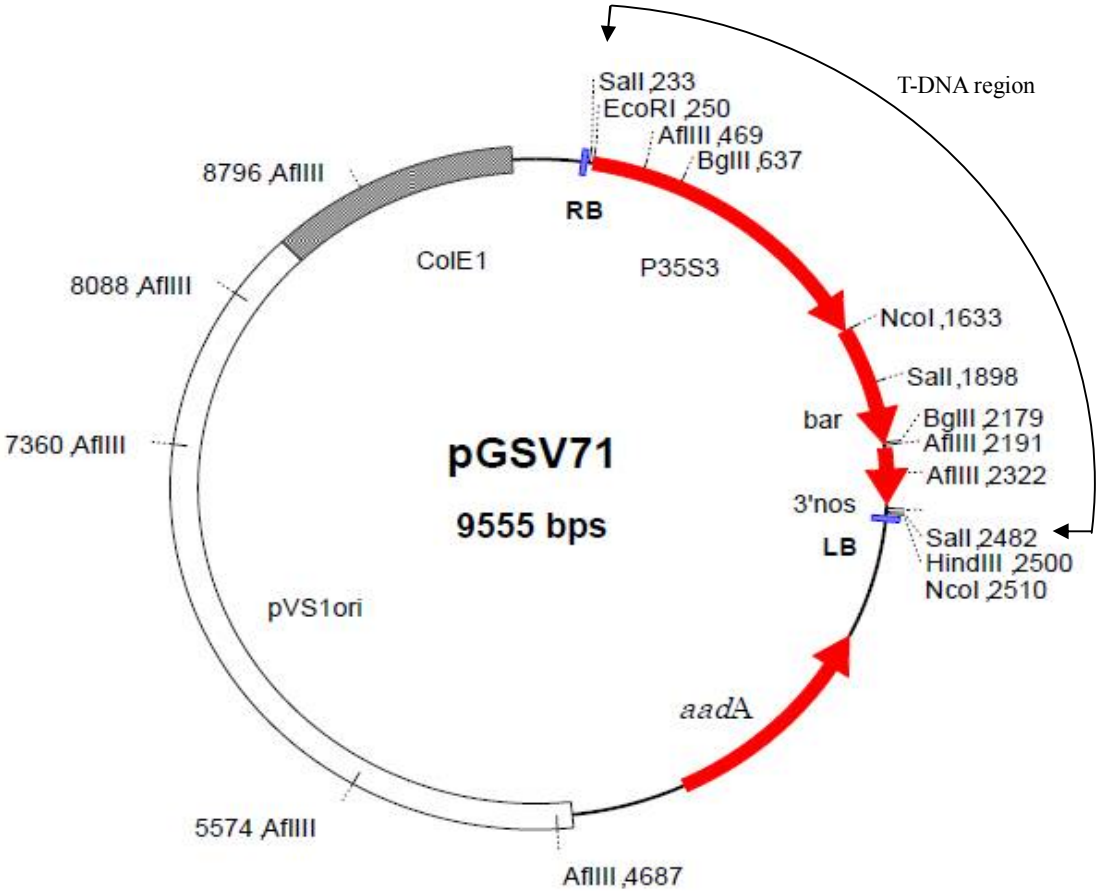

Figure 2 Map of the plasmid pGSV71 used for the development of LLCotton25 and the restriction enzymes cleavage sites

The "bar" refers to the modified *bar* gene.

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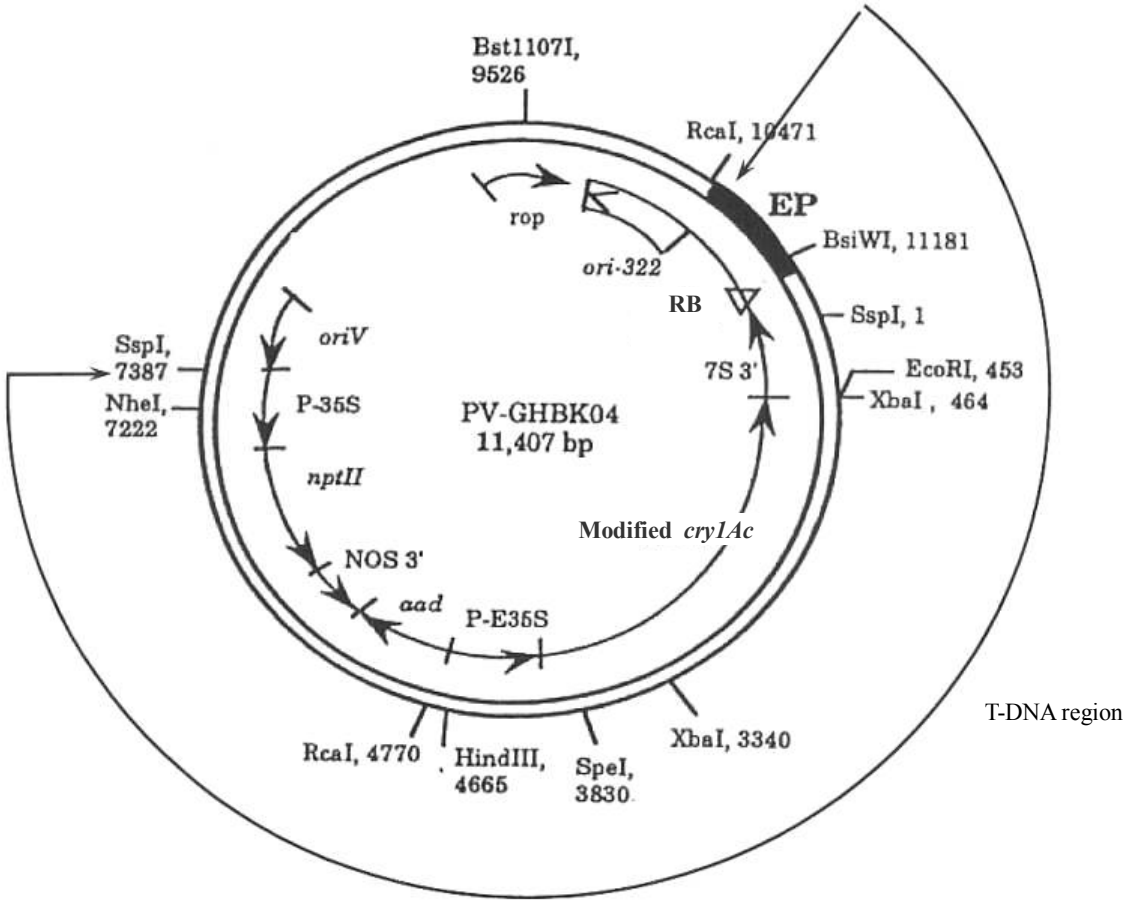

Figure 3 Map of the plasmid PV-GHBK04 used for the development of 531 and the restriction enzymes cleavage sites

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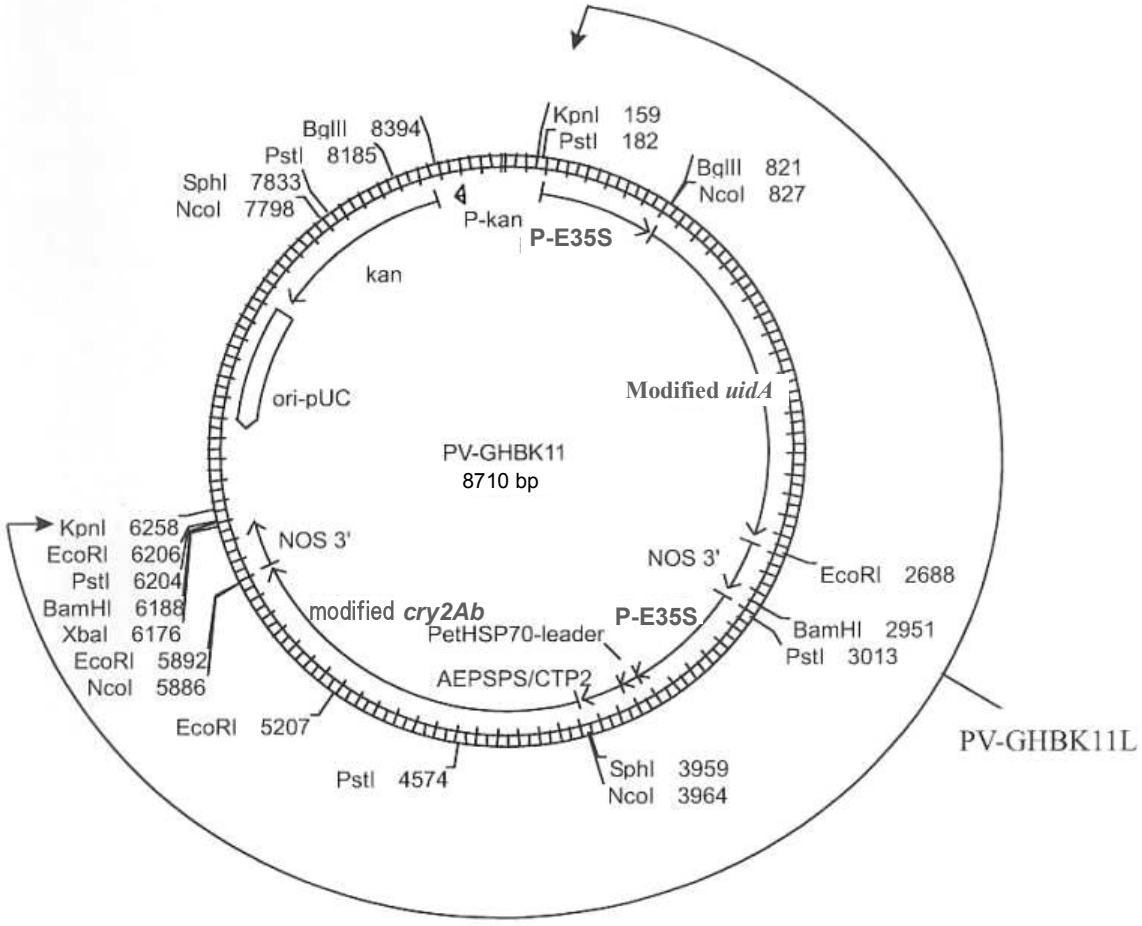

Figure 4 Map of the plasmid PV-GHBK11 used for the development of 15985 and the restriction enzymes cleavage sites

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(3) Method of preparing living modified organisms

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

For the development of GHB614 and LLCotton25, the T-DNA region of pTEM2 (Figure 1, p. 15) and pGSV71 (Figure 2, p. 16) were transferred into the recipient organism, respectively.

The positions and directions of the component elements of the donor nucleic acid and the restriction enzyme cleavage sites in the vectors used for the development of 531 are shown in Figure 3 (p. 17).

The positions and directions of the component elements of the donor nucleic acid and the restriction enzyme cleavage sites in the vectors used for the development of 15985 are shown in Figure 4 (p. 18). For transferring genes into plant cells, the PV-GHBK11 was treated by the restriction enzyme *Kpn*I, and the linear DNA fragment PV-GHBK11L composed of the modified *uidA* gene expression cassette ([P-e35S]-[modified *uidA*]-[NOS3']) and the modified *cry2Ab* gene expression cassette ([P-e35S]-[PetHSP70 leader]-[AEPSPS/CTP2]-[modified *cry2Ab*]-[NOS3']) was used.

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

GHB614:	*Agrobacterium* method
LLCotton25:	*Agrobacterium* method
531:	*Agrobacterium* method
15985:	Particle gun bombardment

3) Processes of rearing of living modified organisms

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

Selection of nucleic acid-transferred cells was made based on the following methods.

GHB614:	Culture on the medium containing glyphosate
LLCotton25:	Culture on the medium containing glufosinate
531:	Culture on the medium containing kanamycin
15985:	Histochemical staining using the modified GUS protein

(b) Presence or absence of remaining *Agrobacterium* in case of using *Agrobacterium* method for transferring nucleic acid

Either GHB614 or LLCotton25 cells, into which each nucleic acid was transferred, was cultured on the media that contained Claforan at 500 mg/L.

Agrobacterium bacteria used for the transformation were removed from the media. Moreover, these cells were cultured in the media that did not contain Claforan. It was confirmed that no *Agrobacterium* bacteria used remained in this media.

For 531, in order to eliminate *Agrobacterium* bacterial from the transgenic plant, the transgenic plant was cultured in the media containing Carbenicillin, and then it was cultured in regenerating media that did not contain this antibiotic. It was confirmed that no *Agrobacterium* used remained.

For 15985 which was developed based on the particle gun bombardment, this item is not applicable.

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

For either GHB614 or LLCotton25, regenerated individuals were transplanted into pots, and grown in greenhouse to obtain the original transformants (T0 generation). Then, elite lines were selected, considering the desired traits, or tolerance to glyphosate herbicide for GHB614 and tolerance to glufosinate herbicide for LLCotton25, the agronomic traits, and others.

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

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

This stack cotton line was developed by crossing the three cotton products, GHB614, LLCotton25 and 15985, after backcrossing of these products with

the same commercially available cultivar. Process of rearing of this stack cotton line is shown in Figure 5.

This application includes the cross generation (F1 generation) of this stack cotton line that was developed by crossing between the inbred progeny of F1 generation developed by crossing of GHB614 and LLCotton25, and 15985, and the progeny of this generation. Information on approval of GHB614, LLCotton25 and 15985 and this stack cotton line in Japan is summarized in Table 5.

Table 5 Status of approval of GHB614, LLCotton25 and 15985 and this stack cotton line in Japan

	Environmental safety	Safety as food	Safety as feed
GHB614	June, 2010: Approved for Type 1 Use Regulation	January, 2010: Approved safety of use as food	June, 2010: Approved safety of use as feed
LLCotton25	February, 2006: Approved for Type 1 Use Regulation	June, 2004: Approved safety of use as food	February, 2006: Approved safety of use as feed
531	November, 2004: Approved for Type 1 Use Regulation	March, 2001: Approved safety of use as food	March, 2003: Approved safety of use as feed
15985	December, 2004: Approved for Type 1 Use Regulation	October, 2002: Approved safety of use as food	March, 2003: Approved safety of use as feed
This stack line	September, 2010: Pending application	June, 2010: Pending application	2010 Scheduled for application

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Confidential: Not made available or disclosed to unauthorized person

Figure 5 Process of rearing this stack cotton line

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

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

It has been confirmed in GHB614, LLCotton25, 531 and 15985 that the transferred nucleic acid exists on the cotton chromosome.

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

[GHB614 and LLCotton25]
It has been confirmed by Southern blotting analysis that one copy of the T-DNA region is transferred into both GHB614 and LLCotton25. In addition, the stability of inheritance of the transferred gene has been confirmed by Southern blotting analysis over multiple generations of the individual parent lines.

[531]
The transferred genes were analyzed in Southern blotting analysis, cosmid cloning technique and genome walking method. As a result, gene transferring was found in the following 3 regions in the genome DNA of 531: the 1st transferred gene consisting of the modified *cry1Ac* gene expression cassette, the *nptII* gene expression cassette, and the *aad*A gene expression cassette; the 2nd transferred gene that consists of a 3' region fragment of the modified *cry1Ac* gene and the 7S3' terminator, which are transferred next to the 5' terminal of the 1st transferred gene, in the reverse direction; and the 3rd transferred gene consisting of a 7S3' terminator fragment of 242 bp (Figure 6, p. 24).

Southern blotting analysis was conducted in combination of 7 varieties of probes and 6 varieties of restriction enzymes treatment.

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 transferred gene; the 3'-terminal flanking sequence of the 1st transferred gene; and the 3'- and 5'-terminal flanking sequences of the 3rd transferred gene. In order to conclusively analyze the structure of the 1st and 2nd transferred genes, a PCR analysis was performed with a primer designed based on the nucleotide sequence of the 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 transferred genes was determined.

In addition, as a result of Southern blotting analysis of the genome DNAs extracted from multiple inbred generations and backcross generations, it was

confirmed that the 1st and 2nd transferred genes were stably inherited in posterity. However, the backcross generations do not contain the 3rd transferred gene, the fragment of 7S3' sequence, in the genome DNA.

A possible reason is that the location of the 3rd transferred gene was, on the chromosome, distant from the 1st and 2nd transferred genes; therefore, the 3rd transferred gene may have been separated from the others during the backcrossing process. Namely, since the 3rd transferred gene was a fragment of the 7S3' sequence, which terminates transcription, it does not contribute to the resistance to Lepidoptera, the target trait of the cotton 531; therefore, it was considered that the 3rd transferred gene was not used for the target of selection during backcrossing breeding.

[15985]

As a result of analysis of transferred gene by Southern blotting analysis, it was confirmed that one copy of the transferred gene was transferred at one site in the genome of chromosome of 15985 (Figure 7, p. 24). Then, as a result to confirm the completeness of the modified c*ry2Ab* gene expression cassette and the modified *uidA* gene expression cassette by using the component elements of each gene expression cassette as probes, it was indicated that the modified *cry2Ab* gene expression cassette was transferred in complete condition; on the other hand, the modified *uidA* gene expression cassette was transferred in partially defective condition. It was confirmed that the defective part of this modified *uidA* gene expression cassette was about 279 bp at 5'-terminal side of P-E35S and polylinker from multi-cloning site of about 24 bp, as a result of genome walking analysis around neighboring sequence of the transferred gene.

In addition, the stable inheritance of the transferred gene has been confirmed by Southern blotting analysis in multiple generations.

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

This item is not applicable because there is one copy of transferred nucleic acid in all of GHB614, LLCotton25 and 15985.

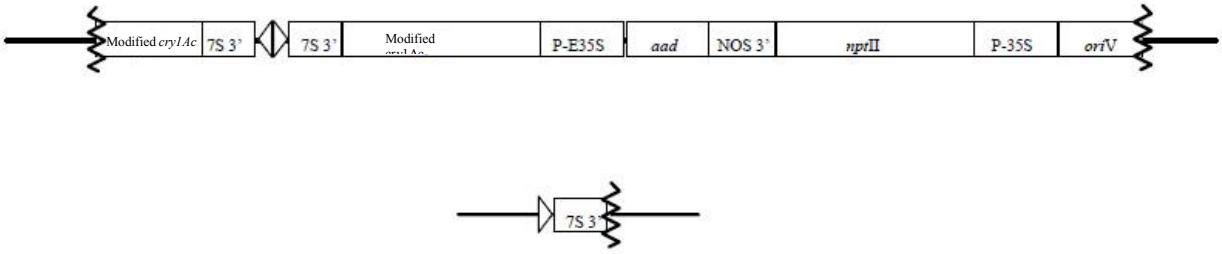

Figure 6 Map of the transferred gene to 531

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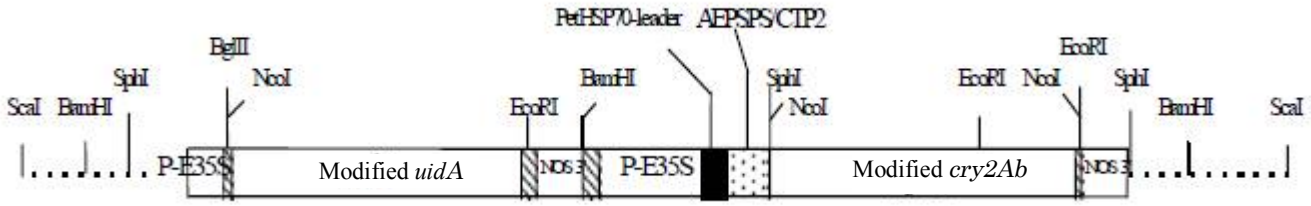

Figure 7 Map of the transferred gene to 15985

(All the rights pertinent to the information in the diagram above and the responsibility for the content remain with Monsanto Japan Limited.)

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

The stability of expression of proteins in the individual parent lines of this stack cotton line was identified based on the methods listed below.

GHB614: Confirming the stability of expression of the 2mEPSPS protein by ELISA method and glyphosate herbicide-spraying test

LLCotton25: Confirming the stability of expression of the modified PAT protein by ELISA method and glufosinate herbicide-spraying test

531: Confirming the stability of expression of the modified Cry1Ac protein by simple ELISA method

15985: Confirming the stability of expression of the modified Cry2Ab protein by Western blotting analysis

Regarding 15985, as a result of nucleotide sequence analysis of the transferred gene, the 1,490th base from 5'-terminal of the *uidA* gene changed from guanine (G) to adenine (A) in comparison with the *uidA* gene nucleotide sequence in plant expression plasmid to be transferred 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 structure 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 affect the active region and its three-dimensional structure of GUS protein; and (iii) as a result of examining whether GUSE377K shares amino acid sequence homology with known allergens or not with use of the protein database (SwissProt ver.30, PIR ver.41), GUSE377K shows no homology of sequence among known allergens.

In addition, the generation analyzed of the transferred 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 the *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 the *uidA* gene was brought by replication of

plasmid in *E. coli* for expression in plant or by gene transferring by particle gun method, not brought during transmission to progeny.

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

Neither the nucleic acid transferred into GHB614 nor LLCotton25 has transmission-associated DNA sequence, considered likely to be transmitted in natural environment to wild animals and plants, and others.

Since the recipient organisms allowing the autonomous replication of the plasmid PV-GHBK04 used for the development of 531 are limited to *E. coli* and some gram-negative bacteria such as *R. radiobacter* (*A. tumefaciens*), it cannot be considered that the transferred nucleic acid is transferred to wild animals and wild plants under natural conditions.

Since the recipient organisms allowing the autonomous replication of the plasmid PV-GHBK11 used for the development of 15985 are limited to *E. coli* and some gram-negative bacteria such as *R. radiobacter* (*A. tumefaciens*), it cannot be considered that the transferred nucleic acid is transferred to wild animals and wild plants under natural conditions.

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

GHB614 and LLCotton25 can be identified by PCR method that employs primers corresponding to its surrounding region of their transferred DNA sequence.

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

For the detection and identification of this stack cotton line, a single seed or a plant body must be analyzed by the above-mentioned methods, respectively.

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

1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of replication products of transferred nucleic acid

This stack cotton line has the following traits for each parent line:

GHB614: Glyphosate herbicide tolerance conferred by the *2mepsps* gene

LLCotton25: Glufosinate herbicide tolerance conferred by the modified *bar* gene

15985: Lepidoptera resistance conferred by the modified *cry1Ac* gene and the modified *cry2Ab* gene

15985 possesses the β-glucuronide hydrolysis conferred by the modified *uidA* gene and the kanamycin resistance conferred by the *nptII* gene, which were both used as selection markers for transformation. The modified *uidA* gene and the *nptII* gene encode the modified GUS protein and the NPTII protein, respectively. Although it has not been clarified whether the glucuronide, which can be the substrate for the modified GUS protein, is present in plant body, the glucuronide is removed from the primary metabolism through excretion to vacuole or apoplast in the form of readily water-soluble secondary metabolite (Reference 41). In addition, the NPTII protein is reported to take part in the phosphorylation of only a limited number of aminoglycoside antibiotics (References 14; 15; 50). Thus, it is considered unlikely that these proteins would affect the metabolic pathway of the recipient organism.

The 2mEPSPS protein, which is expressed in GHB614, is an enzyme that binds to both phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) to catalyze the reaction synthesizing 5-enol-pyruvyl-shikimate-3-phosphate (EPSP) in the shikimate pathway in the same way as EPSPS. It has been reported that, since EPSPS is not a rate-determining enzyme in the shikimate pathway, an increase in EPSPS activity leads to no excess production of the aromatic amino acid, which is the final product of this pathway (Reference 58). Although EPSPS is known to have reactivity with shikimic acid, which is an analog of S3P, this reactivity is low (Reference 27) and then EPSPS has high substrate specificity. Consequently, the 2mEPSPS protein has high substrate specificity and considered unlikely to affect the metabolic system of the recipient organism.

The modified PAT protein, which is expressed in LLCotton25, is an enzyme that can inactivate glufosinate by transferring the acetyl group to it. While glufosinate is classified into L-amino acid, the modified PAT protein cannot transfer the acetyl group to various amino acids. The modified PAT protein has little affinity for glutamic acid, which is similar to glufosinate especially in structure, and then it cannot cause substantial transfer reaction with glutamic acid *in vivo* (Reference 65). In addition, it has been reported that the modified PAT protein is not prevented from transferring the acetyl group to glufosinate even in the excessive presence of various amino acids (Reference 70). Thus, the modified PAT protein has high substrate specificity for glufosinate, considered unlikely to affect the metabolic system of the recipient organism.

There is no report that the Bt protein possesses any enzyme activity, and it is considered that the modified Cry1Ac protein and the modified Cry2Ab protein expressed in 15985 function independently from the metabolic system of plants.

Since these proteins work independently from each other based on different mechanisms of action, it was considered low that these proteins also expressed in this stack cotton line exhibit any functional interaction to affect the metabolic system of the recipient organism.

Actually, in order to confirm that there are no functional interactions between the 2mEPSPS protein, the modified PAT protein, the modified Cry1Ac protein and the modified Cry2Ab protein in this stack cotton line, herbicide-spraying tests were carried out in the US in 2009 and feeding tests to Lepidopteran insects were carried out in Belgium in the same year.

Glyphosate herbicide-spraying tests

After grown to the 2nd to 3rd true-leaf stage in the greenhouse in the US in 2009, ten (10) seedlings each of this stack cotton line, GHB614 and the non-recombinant cotton were sprayed with glyphosate herbicide at the standard concentration [active ingredient: 0.75 lb. (340 g)/acre], 8-time, 16-time and 32-time higher concentrations. On the 7th and 14th days after spraying, the severity of herbicide injury was investigated.

As a result, for the results of 16-time-concentration plot on the 7th day after spraying, a statistically significant difference was observed in the severity of herbicide injury between this stack cotton line and GHB614. However, the mean value of herbicide injury score was 2.10 for this stack cotton line and 2.80 for GHB614, showing a slight difference. For the results of the same plot on the 14th day after spraying, no statistically significant difference was observed between this stack cotton line and GHB614. In the standard-, 8-time-, and 32-time-concentration plots, no difference nor statistically significant difference was observed between this stack cotton line and GHB614 on both 7th and 14th days after spraying (Table 6, p. 30).

Consequently, it is considered that the glyphosate herbicide tolerance in this stack cotton line remains unchanged from the parent line.

Glufosinate herbicide-spraying tests

After grown to the 2nd to 3rd true-leaf stage in the greenhouse in the US in 2009, seedlings of this stack cotton line, LLCotton25 and the non-recombinant cotton were sprayed with glufosinate herbicide at the standard concentration [active ingredient: 0.75 lb. (236 g)/acre], 8-time, 16-time and 32-time higher concentrations. On the 7th and 14th days after spraying, the severity of herbicide injury was investigated.

As a result, for the results of 8-time-concentration plot on the 7th day after spraying, a statistically significant difference was observed in the severity of herbicide injury between this stack cotton line and LLCotton25. However, the mean value of herbicide injury score was 1.00 for this stack cotton line and 0.00 for LLCotton25, showing low levels of herbicide injury for the both lines. In addition, for the same plot on the 14th day after spraying, no statistically significant difference was observed between this stack cotton line and LLCotton25. In the standard-, 16-time-, and 32-time-concentration plots, no difference nor statistically significant difference was observed between this stack cotton line and LLCotton25 on both 7th and 14th days after spraying (Table 6, p. 30).

Consequently, it is considered that the glufosinate herbicide tolerance in this stack cotton line remains unchanged from the parent line.

Feeding tests to Lepidoptera

Eight (8) weeks (early squaring stage) and 11 weeks (mid squaring stage) after sowing, the bolls of this stack cotton line, 15985 and the non-recombinant cotton cultivated in the greenhouse in Belgium in 2009 were collected and fed to the third instar larvae of Cotton bollworm (*Helicoverpa armigera*) to investigate the mortality 3 and 6 days after feeding. As a result, 3 and 6 days after bolls at the early squaring stage were fed, no statistically significant difference was observed in the mortality between this stack cotton line plot and the cotton 15985 plot. On the other hand, the mortality 3 days after feeding of bolls at the mid squaring stage was found 34.26% for this stack cotton line plot and 50.00% for the 15985 plot, showing a statistically significant difference between the lines. However, the mortality 6 days after feeding was found 90% or more for the both lines plots, showing no statistically significant difference between the both lines. In addition, as a result of comparison of the total mortality combining the mortalities in the plots fed with bolls at the early squaring stage and mid squaring stage between this stack cotton line and 15985, the results both 3 and 6 days after feeding showed no statistically significant difference between the lines examined (Table 7, p. 32).

Consequently, it is considered that the Lepidoptera resistance in this stack cotton line remains unchanged from the parent lines.

Table 6 Comparison of injury levels induced by spraying of herbicides [1] (mean value ± standard deviation) [2]

Glyphosate herbicide				
	Standard concentration [3]	8-time concentration	16-time concentration	32-time concentration
7 days after spraying				
This stack line [5]	0.00 ± 0.00	0.60 ± 0.52	2.10 ± 0.74	3.50 ± 0.53
GHB614	0.00 ± 0.00	0.80 ± 0.42	2.80 ± 0.42	3.70 ± 0.48
Significance test [6]	–	ns	s	ns
Non-recombinant control cotton	0.80 ± 0.42	3.40 ± 0.52	4.70 ± 0.48	5.00 ± 0.00
14 days after spraying				
This stack line	0.00 ± 0.00	0.50 ± 0.53	3.00 ± 0.47	5.00 ± 0.00
GHB614	0.00 ± 0.00	1.00 ± 0.00	3.50 ± 0.53	5.00 ± 0.00
Significant difference	–	ns	ns	–
Non-recombinant control cotton	1.30 ± 0.48	4.90 ± 0.32	5.00 ± 0.00	5.00 ± 0.00
Glufosinate herbicide				
	Standard concentration [4]	8-time concentration	16-time concentration	32-time concentration
7 days after spraying				
This stack line	0.00 ± 0.00	1.00 ± 0.00	2.00 ± 0.00	3.60 ± 0.52
LLCotton25	0.00 ± 0.00	0.00 ± 0.00	2.20 ± 0.63	3.70 ± 0.48
Significance test	–	s	ns	ns
Non-recombinant control cotton	4.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
14 days after spraying				
This stack line	0.00 ± 0.00	1.50 ± 0.53	2.70 ± 0.48	4.10 ± 0.32
LLCotton25	0.00 ± 0.00	1.10 ± 0.32	3.00 ± 0.67	4.10 ± 0.32
Significant difference	–	ns	ns	ns
Non-recombinant control cotton	4.60 ± 0.52	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00

[1]: Evaluation of injury levels (Visual evaluation)

0: Damage <10%; Trace amount of bronzing to the cuticle of cotyledons

1: Damage between 10 and 20%; Moderate bronzing of cotyledons, and trace etching of the true leaves

2: Damage between 21 and 40%; Moderate bronzing of cotyledons, and minor etching and curling of the true leaves

3: Damage between 41 and 60%; Moderate to severe bronzing and necrosis of cotyledons, and moderate etching and curling of the true leaves

4: Damage between 61 and 80%; Moderate to severe etching, curling and necrosis to the cotyledons and true leaves

5: Damage between 81 and 100%; Severe chlorosis, necrosis and leaf drop to cotyledons and true leaves

[2]: n=10

[3]: Active ingredient: 0.75 lb. (340 g)/acre

[4]: Active ingredient: 0.52 lb. (236 g)/acre

[5]: F4 generation

[6]: Mann-Whitney U test (significance level 5%). ns: There was no statistically significant difference between this stack cotton line and its parent lines. s: There was statistically significant difference between this stack cotton line and its parent lines. -: In this rating, as variance is not recognized, the statistical analysis was impossible in this test.

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

Table 7 Feeding test to Lepidopteran insects (mortality % ± standard deviation) [1]

		This stack line [3]	15985	Significance test [4]	Non-recombinant control cotton
Early squaring stage (8 weeks after sowing)	3 days later	47.22±15.46	39.81±15.30	ns	0.93±1.61
	6 days later	92.59± 5.78	96.11± 4.19	ns	0.93±1.61
Mid squaring stage (11 weeks after sowing)	3 days later	34.26± 4.24	50.00± 7.35	s	0.93±1.61
	6 days later	96.29± 3.21	90.74± 3.20	ns	1.85±3.21
Early + Mid squaring stages [2]	3 days later	40.74±12.38	44.91±12.10	ns	0.93±1.44
	6 days later	94.44± 4.65	93.43± 4.45	ns	1.39±2.33

[1]: Tests were carried out in 3 repeats for each test plot with six (6) 6-wells plates defined as one repeat. One boll and one 3rd instar larva of *H. armigera* were put into each well on the 6-wells plate. Three (3) days and 6 days later, mortality per plate was determined. For 15985, since the sufficient number of bolls at the early squaring state could not be collected, tests were carried out with 5 larvae per plate in one of the three repeats.

[2]: Mean value of the total mortality at the early and mid squaring stages

[3]: F4 generation

[4]: Mann-Whitney U-test (significance level 5%). ns: There was no statistically significant difference between this stack cotton line and 15985. s: There was statistically significant difference between this stack cotton line and 15985.

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

As a result of the bioassays, with regard to herbicide tolerances, it is considered unlikely that there would occur any functional interaction between the 2mEPSPS protein and the modified PAT protein, between the 2mEPSPS protein and the modified Cry1Ac protein and modified Cry2Ab protein, and between the modified PAT protein and the modified Cry1Ac protein and the modified Cry2Ab protein. In addition, with regard to Lepidoptera resistance, it is considered

unlikely that there would occur any functional interaction between the modified Cry1Ac protein and the modified Cry2Ab protein, and the 2mEPSPS protein and the modified PAT protein. Consequently, it is considered that the traits that this stack cotton line obtained may have not changed through the crossing.

Thus, difference in physiological or ecological properties between this stack cotton line and the cotton, which is a taxonomical species to which the recipient organism belongs, will be estimated, based on the results of the individual examination of the parent lines, or GHB614, LLCotton25 and 15985.

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

GHB614: In 2008, isolated field tests were conducted at the National Institute for Agro-Environmental Sciences (Annex 1; Confidential: Not disclosed to any unauthorized person). In 2007, cold-tolerance at the early stage of growth was examined at a special screened greenhouse in Japan (Annex 2; Confidential: Not disclosed to any unauthorized person).

LLCotton25: In 2003, isolated field tests were carried out at the National Agricultural Research Center for Kyushu Okinawa Region, the National Agricultural Research Organization (today, the National Agricultural Research Center for Kyushu Okinawa Region, the National Agriculture and Food Research Organization) (Annex 3; Confidential: Not disclosed to any unauthorized person). In 2002, fertility and size of the pollen were evaluated in France (Annex 4; Confidential: Not disclosed to any unauthorized person).

15985: Isolated field tests of 15985, the recipient organism control cotton cultivar DP50B and the non-recombinant cotton DP50 were carried out in isolated fields of Kyushu National Agricultural Experiment Station and Kawachi Research Farm (KRF), Monsanto Japan Limited (Inashiki-gun, Ibaraki Prefecture), from May 2000 to March 2001 (Annex 5; Confidential: Not disclosed to any unauthorized person). The DP50B is the recombinant commercial cotton cultivar derived from repeated crossing of 531 and the DP50, which is the non-recombinant cotton cultivar.

(a) Morphological and growth characteristics

For the morphological and growth characteristics of GHB614, LLCotton25

and 15985 and their non-recombinant control cotton, an examination was conducted for the items listed in Table 8 (p.35).

For GHB614, a statistically significant difference from the non-recombinant control cotton was observed in the germination rate of the seeds used for the isolated field testing (hereinafter referred to as "seeds for field experiment") (Annex 1; Confidential: Not disclosed to any unauthorized person). This difference is considered attributed to different sampling locations of the two lines of seeds (GHB614 seeds sampled at Catalonia, Spain; and the non-recombinant control cotton seeds sampled at Andalusia, Spain). Also it is considered that the rainfall before harvesting at the sampling location of the non-recombinant control cotton might deteriorate seeds and affected germination rate. It was confirmed that the non-germinating seeds of the both lines had all decayed and died.

For LLCotton25, a statistically significant difference from the non-recombinant control cotton was observed in the stem length after 60 days of the sowing and in the number of nodes after 60 and 120 days of the seeding. However, no statistically significant difference was observed at the other evaluation days in the stem length and the number of nodes (Annex 3; Confidential: Not disclosed to any unauthorized person).

For 15985, no difference was observed in all the items examined in the tests conducted in the isolated fields at the Kawachi Research Farm (KRF) between 15985, the recipient organism control cotton DP50B and the non-recombinant cotton DP50 (Annex 5; Confidential: Not disclosed to any unauthorized person).

On the other hand, in the isolated field test in Kyushu National Agricultural Experiment Station, there was a statistically significant difference in leaf shape (leaf length) and the weight of under-ground parts. However, no difference was observed in other items (Annex 5; Confidential: Not disclosed to any unauthorized person).

Table 8 Evaluation items associated with morphological and growth characteristics of GHB614, LLCotton25 and 15985

Item	GHB614	LLCotton25	15985	
			Kawachi Research Farm (KRF)	Kyushu National Agricultural Experiment Station
Uniformity of germination	○	○	○	○
Flowering time	○	○	○	○
Boll opening time	○	○	○	○
Harvesting time	○	○	○	○
Leaf shape	○	○	○	○
Plant shape	○	○	○	○
Flower shape	○	○	○	○
Flower color	○	○	-	-
Boll shape	○	○	○	○
Lint color	○	○	○	○
Seed shape	○	○	-	-
Seed color	○	○	○	○
Germination rate	○*	○	○	○
Leaf length	○	○	○	○*
Leaf width	○	○	-	-
Stem length	○	○*	○	○
Number of appearing flower buds/Effective number of flower buds	○	○	○	○
Number of nodes	○	○*	-	-
Number of vegetative branches	○	-	-	-
Number of fruiting branches	○	○	○	○
Total number of branches	○	○	-	-
Number of harvested bolls per plant	○	○	○	○
Number of non-harvested bolls per plant	○	○	○	○
Total number of bolls per plant	○	○	-	-
Weight of aerial-ground parts	○	○	○	○
Weight of under-ground parts	○	○	○	○*
Boll length	○	○	-	-
Boll width	○	○	-	-
Boll weight [1)]	○	○	○	○
Number of segments of a	○	○	○	○

boll				
Number of seeds per boll segment	-	○	-	-
Number of seeds per boll	○	○	○	○
100-seed weight	○	-	-	-

○: Evaluation was conducted. -: No evaluation was conducted.
*: Some test plots showed statistically significant differences. For the detailed information, see I-6-2)-(a) (pp.33-34).
[1)] For GHB614 and LLCotton25, fresh weight was evaluated and for 15985, dry weight was evaluated.
(All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

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

Both the young plant bodies of GHB614 and LLCotton25 died under the low-temperature conditions (4 to 5℃) like the non-recombinant control cotton (Annex 2; Confidential: Not disclosed to any unauthorized person; Annex 3; Confidential: Not disclosed to any unauthorized person).

For 15985, cold-tolerance tests were not conducted at the early stage of growth in isolated fields. Instead, the observation of volunteer individuals up to the following spring was carried out in 22 isolated fields in the US. All of these field testes were conducted in famous regions for cotton cultivation in the South of the US. Besides, compared to average climate conditions in Japan, the winter coldness in these regions is relatively mild. Therefore, it is considered that these regions provide better climatic conditions for cotton growth than Japan (Reference 19).

As a result of observation, it was found that some seeds spilled on the field had germinated in fall after harvesting. However, 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 mature plant

Both GHB614 and LLCotton25 continued to be grown also after the harvest time at the isolated field in Japan, and were found dead when they were observed in February of the following year (Annex 1; Confidential: Not disclosed to any unauthorized person).

In addition, it was observed that the plants of 15985 were partly dead when the isolated field tests of 15985 were completed (harvested in October at Kyushu National Agricultural Experiment Station, and harvested in November and plowed in in November at the Kawachi Research Farm

(KRF)).

(d) Fertility and size of the pollen

GHB614 and LLCotton25 were compared with the non-recombinant control cotton in the fertility and size of the pollen; neither GHB614 nor LLCotton25 showed statistically significant difference or different points (Annex 1; Confidential: Not disclosed to any unauthorized person; Annex 4; Confidential: Not disclosed to any unauthorized person).

For 15985, the fertility and size of pollens were not examined. However, as mentioned in the next item (e), no statistically significant difference from the control cultivars was observed in the number of bolls per plant, the number of segments of a boll and the number of seeds per boll examined with regard to seed production; therefore, it is considered unlikely that the characteristics of pollens of 15985 significantly differ from those of the control cultivars.

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

Regarding seed production, neither GHB614 nor LLCotton25 showed statistically significant differences from the control cultivars in the number of harvested bolls per plant, the total number of bolls per plant and the number of seeds per boll (Annex 1; Confidential: Not disclosed to any unauthorized person; Annex 3; Confidential: Not disclosed to any unauthorized person).

For 15985, the differences among 15985, the recipient organism cotton DP50B and the non-recombinant control cotton DP50 have been examined in 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 examined (Annex 5; Confidential: Not disclosed to any unauthorized person).

Regarding the shedding habit, as a result of observation of GHB614 and the control cultivar for shedding of the seed, GHB614 showed no shedding of the seeds like the control cultivar in the isolated field testing (Annex 1; Confidential: Not disclosed to any unauthorized person).

LLCotton25 was not examined for the shedding habit, though LLCotton25 is confirmed not to be different from the non-recombinant control cotton in the morphology and opening properties of bolls (Annex 3; Confidential: Not disclosed to any unauthorized person).

In both of 15985 and its non-recombinant control cotton, seeds are covered

with lint at harvest time; therefore, shedding habits of the seed under natural conditions were not observed.

GHB614 and LLCotton25 seeds harvested in the isolated field were used to evaluate their dormancy and germination rate. For GHB614, the just-harvested seeds and those stored under room temperature for 3 months after the harvesting were sown to examine the germination rate. As a result, there were no statistically significant differences; the 3-month-stored seeds showed a germination rate of 96% or more for both GHB614 and its non-recombinant control cotton (Annex 1; Confidential: Not disclosed to any unauthorized person).

For LLCotton25, the seeds stored under room temperature for approximately one month after the harvesting were compared for the germination rate with those of the non-recombinant control cotton. As a result, the seeds of the both lines showed a germination rate of 100% (Annex 3; Confidential: Not disclosed to any unauthorized person).

Regarding the germination rate of harvested seeds from 15985, in three (3) isolated fields in Texas (TX), South Carolina (SC) and Louisiana (LA) in the US in 1999, the germination rate of the seed was examined using the harvested seeds of 15985, the recipient organism cotton DP50B, the non-recombinant control cotton DP50, and 11 existing cultivars added for reference, under the different temperature conditions from 5 to 40°C. As a result, under some temperature conditions, a statistically significant difference ($p<0.05$) was observed between 15985 and the recipient organism control cotton DP50B. However, the difference was within the value range of 11 existing cultivars which were added for reference (Annex 6; Confidential: Not disclosed to any unauthorized person). On the other hand, in various temperature conditions, the seeds of 15985, the recipient organism cotton DP50B and 11 existing cultivars for reference were germinated, "Viable Firm Swollen" or degenerated, and no seed of "Viable Hard" was observed (Annex 6; Confidential: Not disclosed to any unauthorized person). Moreover, with regard to germination rate, as mentioned in "a) Morphological and growth characteristics," no difference was observed between 15985 and the recipient organism control cotton DP50B and the non-recombinant cotton DP50 (Annex 5; Confidential: Not disclosed to any unauthorized person).

(f) Crossability

In Japan, any related species that can cross with cotton have not been growing voluntarily. Thus, crossability of GHB614 and 15985 was not

assessed. For reference, however, for LLCotton25, 20 seeds of LLCotton25 and 180 seeds of the non-recombinant control cotton cultivated at a place of 1 m apart from LLCotton25 were sown to germinate and then sprayed with glufosinate herbicide in the isolated field testing. As a result, the seedlings from the seeds of LLCotton25 all exhibited tolerance to the herbicide, while the seedlings from the seeds of the non-recombinant control cotton all died. Within the scope of this test, no possibility that crossing occurred was observed (Annex 3; Confidential: Not disclosed to any unauthorized person).

(g) Productivity of harmful substances

GHB614, LLCotton25 and 15985 underwent the succeeding crop test, plow-in test, and soil microflora test. As a result, none of them showed statistically significant differences from the non-recombinant control cotton in various evaluation items concerning germination and growth of test plants in the succeeding crop test and plow-in test, and in the number of living soil microorganisms (Annex 1; Confidential: Not disclosed to any unauthorized person; Annex 3; Confidential: Not disclosed to any unauthorized person; Annex 5; Confidential: Not disclosed to any unauthorized person).

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

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

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

This stack cotton line was developed by the crossbreeding method of cotton tolerant to glyphosate herbicide (GHB614), cotton tolerant to glufosinate herbicide (LLCotton25) and cotton resistant to Lepidoptera (15985), 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 used in accordance with the Type 1 Use Regulation, which is to be applied also to this stack cotton line.

Bt proteins (the modified Cry1Ac protein and the modified Cry2Ab protein) have been reported not to possess any enzyme activity, thus considered to function independently from the metabolic system of plants. Both the 2mEPSPS protein and the modified PAT protein have high substrate specificity, considered unlikely to affect the metabolic system of the recipient organism, even if these proteins express in this stack cotton line. Thus, these proteins function independently from each other by their own action mechanism. Therefore, it was considered unlikely that the proteins expressed in this stack cotton line exhibit any functional interaction and additionally affect the metabolic system of the recipient organism.

In addition, for the traits conferred to this stack cotton line, or the glufosinate-herbicide tolerance, glyphosate-herbicide tolerance and Lepidoptera resistance, expressions of the traits are the same levels as in the parent lines. Then, it is considered low that these proteins derived from the individual parent lines could affect each other functionally in the plant body of this stack cotton line, and it is considered unlikely that notable changes in traits have occurred in this stack cotton line except for the traits it received from the parent lines.

(1) Competitiveness

Cotton (*Gossypium hirsutum* L.), the biological species to which the recipient organism belongs, has been imported to Japan and used for processing for a long time. However, there is no report that cotton has grown voluntarily in natural environment.

Various traits relating of the competitiveness of the parent lines (GHB614, LLCotton25 and 15985) of this stack cotton line were investigated. As a result, a statistically significant difference from the non-recombinant control cotton was observed in the germination rate of the seeds for field experiment for GHB614, and in the stem length and the number of nodes in a part of test plots for LLCotton25. For 15985, a statistically significant difference from the recombinant mother cotton DP50B was observed in the leaf length at one of the two test sites, and a statistically significant difference from the recombinant mother cotton DP50B and the non-recombinant control cotton in the weight of under-ground parts. However, for all the lines examined, regarding the other traits than those discussed above, no significant difference from the non-recombinant control cotton or from the recombinant mother cotton was observed. Thus, it was considered low that these differences in the above-mentioned traits increase the competitiveness.

This stack cotton line is given traits to be tolerant to both glyphosate and glufosinate herbicides. However, since under natural environmental condition the line may not be affected by spraying of these herbicides, these traits are considered unlikely to increase its competitiveness of this stack cotton line. Regarding the resistance to Lepidoptera, it is considered unlikely that only this trait would cause the cotton, a cultivation plant, to grow voluntarily under natural conditions and additionally increase its competitiveness, even though the survival rate of this stack cotton line may be increased temporarily compared to existing cotton for the less susceptibility to feeding damage by Lepidopteran insects. Consequently, it was considered unlikely that these traits increase the competitiveness of this stack cotton line.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack cotton line and the progeny lines of stack cotton line isolated from the parent lines GHB614, LLCotton25 and 15985 that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to competitiveness.

(2) Productivity of harmful substances

Cotton seeds contain gossypol, which exhibits toxicity against non-ruminant animals, and cyclopropene fatty acid, which causes discoloration and decreases hatchability in hen eggs by suppressing desaturation of the saturated fatty acids. However, there is no report that cotton seeds are eaten by wild animals. In addition, cotton seeds are not

known to produce allelopathic and other substances that could affect the inhabitation or growth of wild animals and wild plants.

It has been confirmed that the 2mEPSPS protein, the modified PAT protein, the modified Cry1Ac protein and the modified Cry2Ab protein, which are expressed in this stack cotton line, have no sequence homology with any known allergens.

Since the 2mEPSPS protein and the PAT protein have high substrate specificity, it is considered impossible that these proteins act on the metabolic system of the recipient organism to produce any harmful substances. For the Bt protein, there is no report that it has enzyme activity, and it is considered that the modified Cry1Ac protein and the modified Cry2Ab protein function independently from the metabolic system of the recipient organism. Thus, it is considered unlikely that these proteins could affect the metabolic system of the recipient organism and produce any harmful substances.

Actually, the succeeding crop test, soil microflora test, and plow-in test were performed to examine GHB614, LLCotton25 and 15985 for the ability to produce any harmful substances (substances that are secreted from roots to affect other plants and soil microorganisms, substances contained in plant bodies that affect other plants after their death), and compare these lines with the control in this ability. As a result, in all these tests, neither of these lines showed any statistically significant differences from the non-recombinant cotton, considered likely to have newly obtained the ability to produce any harmful substances.

In addition, due to the expression of the modified Cry1Ac protein and the modified Cry2Ab protein in this stack cotton line, there was a concern about the possibility that this stack cotton line would affect the survival of Lepidopteran insects, which eat plant body or pollen of this stack cotton line. In Japan, however, this stack cotton line is not commercially cultivated and thus, such effect could occur only when the seeds imported for processing grew after spilled during transportation. However, there has been no report that cotton seeds spilled during transportation grew and became self-seeding in the natural environment in Japan. Moreover, this stack cotton line has not obtained any traits that favor the growth in the natural environment. Therefore, it was considered extremely low similarly as in the case of existing cotton imported to date that the spilled seeds could grow or become self-seeding and become eaten by Lepidopteran insects.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack cotton line and the progeny lines of stack cotton line isolated from the parent lines GHB614, LLCotton25 and 15985 that contain a combination of any of the transferred genes in the individual parent lines, would pose no risk of Adverse Effect on Biological Diversity that is attributable to productivity of harmful substances.

(3) Crossability

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stack cotton line and the progeny lines of stack cotton line isolated from the parent lines of this stack cotton line, GHB614, LLCotton25 and 15985, that contain a combination of any of the transferred genes in the individual parent lines, in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.