

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

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#### Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton resistant to Lepidoptera, and tolerant to glufosinate herbicide and glyphosate herbicide ( <i>cryIF</i> , <i>cryIAC</i> , <i>pat</i> , <i>cp4 epsps</i> , <i>Gossypium hirsutum</i> L.)(281 × 3006 × 1445, OECD UI : DAS-24236-5 × DAS-21023-5 × MON- 01445-2)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

## Outline of the Biological Diversity Risk Assessment Report

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

#### 1. Information concerning preparation of living modified organisms

##### (1) Information concerning donor nucleic acid

Cotton 281 and Cotton 3006 were developed by Dow AgroSciences LLC in the US, and Cotton 1445 was developed by Monsanto Company in the US.

This stack line cotton, developed by cross-breeding between Cotton 281/3006 prepared by crossing Cotton 281 and Cotton 3006, and Cotton 1445, possesses the characteristics of these three recombinant cotton lines, Cotton 281, Cotton 3006, and Cotton 1445. Then the information concerning preparation of Cotton 281, Cotton 3006, and Cotton 1445 is explained individually in the following sections. The specific information for Cotton 1445 is derived from the information developed by Monsanto Company; therefore the outline is described based on the published analytical results and reference materials (Web sites offering the Outline of Biological Diversity Risk Assessment Report: Japan Biosafety Clearing-House (BCH) at [http://www.bch.biodic.go.jp/download/lmo/public\\_comment/15985\\_1445ap.pdf](http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf), and Web site of United States Department of Agriculture (USDA) at [http://www.usda.gov/brs/aphisdocs/95\\_04501p.pdf](http://www.usda.gov/brs/aphisdocs/95_04501p.pdf)).

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

The composition of donor nucleic acid used for the production of Cotton 281 and the origins of component elements are shown in Table 1, the composition of donor nucleic acid used for the production of Cotton 3006 and the origins of component elements are shown in Table 2, and the composition of nucleic acid used for the production of Cotton 1445 and the origins of component elements are shown in Table 3.

##### 2) Functions of component elements

###### (a) Functions of target genes, expression-regulating regions, localization signals, selectable markers and other component elements of donor nucleic acid

Functions of individual component elements of donor nucleic acid used for the production of Cotton 281 are shown in Table 1. Functions of individual component elements of donor nucleic acid used for the production of Cotton 3006 are shown in Table 2. Functions of individual component elements of donor nucleic acid used for the production of Cotton 1445 are shown in Table 3.

**Table 1 Donor nucleic acid used for developing Cotton 281**

Component elements	Origin and Function
Modified <i>cryIF</i> cassette	
(4ocs)DeltaMas 2'	A mannopine synthase promoter derived from pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491), which contains 4 copies of the octopine synthase (OCS) enhancer derived from pTiAch5 (Ellis <i>et al.</i> 1987) (GenBank Accession Numbers I05704 to I05712), providing an advantage of higher expression level in leaves of cotton.
Modified <i>cryIF</i>	It was synthesized based on <i>cryIF</i> gene derived from <i>B.t.</i> var. <i>aizawai</i> . This gene encodes modified Cry1F protein which shows insecticidal activity to major harmful insects for cotton cultivation including Tobacco budworm ( <i>Heliothis virescens</i> ), Beet armyworm ( <i>Spodoptera exigua</i> ), and Cotton bollworm ( <i>Helicoverpa zea</i> ).
ORF25 polyA	A bi-directional terminator derived from <i>R.radiobacter</i> ( <i>A.tumefaciens</i> ) pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491).
<i>pat</i> cassette	
UbiZm1 (intron)	A ubiquitin 1 promoter of maize ( <i>Zea mays</i> ) including exon 1 (untranslated enhancer) and intron 1, providing an advantage of higher expression level in cottonseed bolls. (Christensen <i>et al.</i> 1992) (US Patent No.5614199, GenBank Accession I18571)
<i>pat</i>	A synthetic gene tolerant to glufosinate, which was optimized to activate the expression in plant body, based on the gene sequence of phosphinothricin acetyltransferase derived from <i>S. viridochromogenes</i> (Eckes <i>et al.</i> 1989). A gene which encodes PAT protein used as a selectable marker.
ORF25 polyA	A bi-directional terminator derived from <i>R.radiobacter</i> ( <i>A.tumefaciens</i> ) pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491).

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**Table 2 Donor nucleic acid used for developing Cotton 3006**

Component elements	Origin and Function
Modified <i>cryIAC</i> cassette	
Ubi Zm1(intron)	A ubiquitin 1 promoter of maize ( <i>Zea mays</i> ) including exon 1 and intron 1, providing an advantage of higher expression level in cottonseed bolls. (Christensen <i>et al.</i> 1992) (US Patent No.5614199, GenBank Accession I18571)
Modified <i>cryIAC</i>	It was synthesized based on <i>cryIF</i> gene derived from <i>B.t.</i> var. <i>kurstaki</i> . This gene encodes modified Cry1F protein which shows insecticidal activity to major harmful insects for cotton cultivation including Tobacco budworm ( <i>Heliothis virescens</i> ), Beet armyworm ( <i>Spodoptera exigua</i> ), Cotton bollworm [also called Corn earworm ( <i>Helicoverpa zea</i> )], and Pink bollworm ( <i>Pectinophora gossypiella</i> ).
ORF25 polyA	A bi-directional terminator derived from <i>R.radiobacter</i> ( <i>A.tumefaciens</i> ) pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491).
<i>pat</i> cassette	
(4ocs)DeltaMas 2'	A mannopine synthase promoter derived from pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491), which contains 4 copies of the octopine synthase (OCS) enhancer derived from pTiAch5 (Ellis <i>et al.</i> 1987) (GenBank Accession Numbers I05704 to I05712), providing an advantage of higher expression level in leaves of cotton.
<i>pat</i>	A synthetic gene tolerant to glufosinate, which was optimized to activate the expression in plant body, based on the gene sequence of phosphinothricin acetyltransferase derived from <i>S. viridochromogenes</i> (Eckes <i>et al.</i> 1989). A gene which encodes PAT protein used as a selectable marker.
ORF25 polyA	A bi-directional terminator derived from <i>R.radiobacter</i> ( <i>A.tumefaciens</i> ) pTi15955 (Barker <i>et al.</i> 1983) (GenBank Locus ATACH5, Accession X00491).

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**Table 3 Donor nucleic acid used for developing Cotton 1445**

Component elements	Origin and Function
<i>Cp4 epsps</i> cassette	
CMoVb	35S promoter of figwort mosaic virus. Involved in the constitutive expression of the target gene in all tissues.
ctp2	A sequence in the <i>epsps</i> gene of <i>Arabidopsis thaliana</i> that encodes chloroplast transit peptide located at N-terminal region of EPSPS protein. Transports CP4 EPSPS protein to the chloroplast where aromatic amino acids are synthesized.
<i>Cp4 epsps</i>	5-enol-pyruvyl-shikimate-3-phosphate synthase gene derived from <i>Agrobacterium</i> CP4 strain.
E9 3'	3' untranslated region of pea ribulose-1, 5-bisphosphate carboxylase E9 gene. Terminates transcription of mRNA and induces polyadenylation.
<i>NptII</i> cassette	
35S	35S promoter region of cauliflower mosaic virus (CaMV). Involved in the constitutive expression of the target gene in all tissues.
<i>NptII</i> (Kan)	A gene derived from Tn5 transposon of <i>E. coli</i> . Encodes neomycin phosphotransferase type II ( <i>NPT II</i> ) enzyme protein and confers resistance to kanamycin. Utilized as a marker to select recombinant plant body in introducing genes.
NOS 3'	3'untranslated region of nopaline synthase (NOS) gene from <i>Agrobacterium tumefaciens</i> . Terminates transcription and induces polyadenylation.
<i>Gox</i> cassette (Not introduced in Cotton 1445)	
CMOVb	35S promoter of figwort mosaic virus. Involved in the constitutive expression of the target gene in all tissues.
Ctp1	A sequence that encodes chloroplast transit peptide located at N-terminal region of small subunit 1A of rubisco derived from <i>A. thaliana</i> . Transports GOX protein to chloroplast where aromatic amino acids are synthesized.
<i>Gox</i>	A sequence that encodes C-terminal of v247, a variant derived from glyphosate oxidoreductase ( <i>gox</i> ) of <i>Achromobacter</i> sp. strain LBAA. GOX protein dissolves glyphosate.
NOS 3'	3'untranslated region of nopaline synthase (NOS) gene from T-DNA of <i>Agrobacterium tumefaciens</i> . Terminates transcription of mRNA and induces polyadenylation.

Component elements	Origin and Function
Other component elements	
Ori-V	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in <i>Agrobacterium tumefaciens</i> ABI strain.
<i>Aad</i>	A gene encoding 3'' (9)-O-aminoglycoside adenyl transferase (AAD) derived from <i>Staphylococcus aureus</i> . Confers resistance to spectinomycin and streptomycin.
Right border sequence (Right Border)	A DNA sequence containing right border sequence (24bp) of nopaline type T-DNA derived from Ti plasmid pTiT37. Used as the initiation point of T-DNA transfer from <i>Agrobacterium tumefaciens</i> to plant genome.
<i>Ori322</i>	Replication origin region isolated from pBR322, a plasmid derived from <i>E. coli</i> . Permits autonomous replication of vectors in <i>E. coli</i> .
<i>Rop</i>	Derived from <i>E. coli</i> . Regulates the copy number of plasmids to be replicated in <i>E. coli</i> .

(Excerpt from [http://www.bch.biodic.go.jp/download/lmo/public\\_comment/15985\\_1445ap.pdf](http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf))

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

### **Modified Cry1F protein and modified Cry1Ac protein**

Much of the delta-endotoxins (protoxins) produced in the *B. t.* var. *aizawai* and *B.t.* var. *kurstaki* are proteins of about 120 to 140kDa (Schnepf *et al.* 1998). When protoxins are fed by target insects, the C-terminal and N-terminal are digested by the protease in the intestinal tract, and only the insecticidal core toxins remain. The core toxins have molecular weights of 65 to 70kDa, and penetrate into cellular membranes of target insects by binding to the specific receptors on the midgut epithelium of target insects due to the changed conformation. In addition, this protein forms oligomer, which creates in turn pores in the midgut cellular membranes. As a result, destruction of cells is induced, causing insects to die. The core protein, the active site of the modified Cry1F protein and modified Cry1Ac protein expressed in Cotton 281/3006, is identical to the core protein of the Cry1F protein and Cry1Ac protein of the wild-type bacterium *B.t.*. The Bt preparations using the wild-type bacterium *B.t.* have been long applied for control of Lepidoptera insects in the US, Europe, Japan and other countries.

It is known that the Cry1 protein including the Cry1F protein and Cry1Ac protein exhibits the insecticidal activity only against Lepidoptera insects (Prieto-Samsónov *et al.* 1997). The modified Cry1F protein exhibits the insecticidal activity against Lepidoptera insects, including Tobacco budworm (*Heliothis virescens*), Beet armyworm (*Spodoptera exigua*), Cotton bollworm (*Helicoverpa zea*), and Soybean looper (*Psuedoplusia includens*), which damage cotton. In addition, the modified Cry1Ac protein exhibits the insecticidal activity against Tobacco budworm, Beet armyworm, Cotton bollworm, Soybean looper, and Pink bollworm (*Pectinophora gossypiella*). Cotton 281/3006 expresses the both Cry proteins, the modified Cry1F protein and modified Cry1Ac protein, and thus it possesses the insecticidal activities from the both.

Furthermore, the modified Cry1F protein and modified Cry1Ac protein have been verified to exert no adverse effects on the non-target organisms including water flea (*Daphnia magna*), lacewing fly (*Chrysopa carnea*), ladybug (*Coccinellidae*), honeybees, *Nasonia vitripennis*, earthworm, and rainbow trout (*Oncorhynchus mykiss*) within the range of the doses applied.

### **PAT protein**

The PAT protein, the phosphinothricin acetyltransferase, specifically acetylates the glufosinate herbicide to transform it into non-toxic acetyl-glufosinate and extinguish the weed-killing activity; therefore plants expressing the PAT protein show the tolerance to glufosinate herbicide. It is reported that the PAT protein possesses very high substrate specificity against *L*-glufosinate, an active ingredient of glufosinate herbicide and it does not select other *L*-amino acids and *D*-glufosinate, an optical isomer of *L*-glufosinate, for substrate

(OECD 1999).

### **CP4 EPSPS protein**

Glyphosate inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), one of the enzymes in the shikimate synthesis pathway for aromatic amino acid biosynthesis, by specifically binding to the enzyme. As a result, plants treated with glyphosate cannot synthesize aromatic amino acid essential for protein synthesis due to the inhibition of EPSPS, and die. The activity of the CP4 EPSPS protein produced by the *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus the recombinant plants that express this protein have normal functions of shikimate synthesis pathway and can grow. EPSPS protein is the enzyme that specifically reacts with phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) and it is suggested not as the rate-determining enzyme in the shikimate pathway for aromatic amino acid biosynthesis. In addition, other than these substrates, shikimate is known to react with EPSPS protein, though the reactivity is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as a substrate in any living organisms.

### **NPTII protein**

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

### **Allergic property**

In order to investigate whether the modified Cry1F, modified Cry1Ac, PAT, CP4 EPSPS and NPTII proteins share functionally important amino acid sequence with known allergens, they were compared with allergens in the allergen database (Swiss-Prot, PIR, GenRept, FARRP Protein Allergen Database, Genbank, EMBL, NRL3D). As a result, these proteins did not share structurally related homologous sequence with any of the known allergens examined.

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

The Cry protein is not an enzyme, the PAT protein and EPSPS protein possess high substrate specificities and the NPTII protein is an enzyme to phosphorylate aminoglycoside antibiotics, thus, it is considered that these proteins do not have any effects on the recipient organism's metabolic systems.



## (2) Information concerning vector

### 1) Name and origin

The expression vector pAGM281 used to generate the Cotton 281 and the expression vector pMYC3006 used to generate the Cotton 3006 were assembled from the broad-recipient range plasmid RK2. On the other hand, the expression vector PV-GHGT07 used to generate the Cotton 1445 is a synthetic plasmid vector assembled from pBR322 which is derived from *E.coli*.

### 2) Properties

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

The number of base pairs of expression vector pAGM281 is 14,950bp. The number of base pairs of expression vector pMYC3006 is 15,337bp. The number of base pairs of expression vector PV-GHGT07 is 12,032bp. The nucleotide sequences of the component elements of these expression vectors have become clear.

#### (b) Types of any nucleotide sequence having specific functions

Erythromycin resistance by the *ery<sup>R</sup>* gene was used for selection of the expression vectors pAGM281 and pMYC3006. The *ery<sup>R</sup>* gene is located outside the T-DNA region and then this gene has not been introduced in the Cotton 281 and Cotton 3006. The *nptII* gene which confers tolerance to kanamycin and neomycin and the *aad* gene which confers tolerance to spectinomycin in PV-GHGT07 were introduced in Cotton 1445. However, the *aad* gene does not express in Cotton 1445 since the *aad* gene does not possess promoter to function in plant. In actual measurement, the level of the AAD protein produced by the *aad* gene showed lower than the measurable limit in ELISA analysis (the fresh weight of seeds and leaves : 0.025ng/1mg, 0.013ng/1mg, respectively).

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

The pAGM281, pMYC3006 and PV-GHGT07 are known to offer no infectivity.

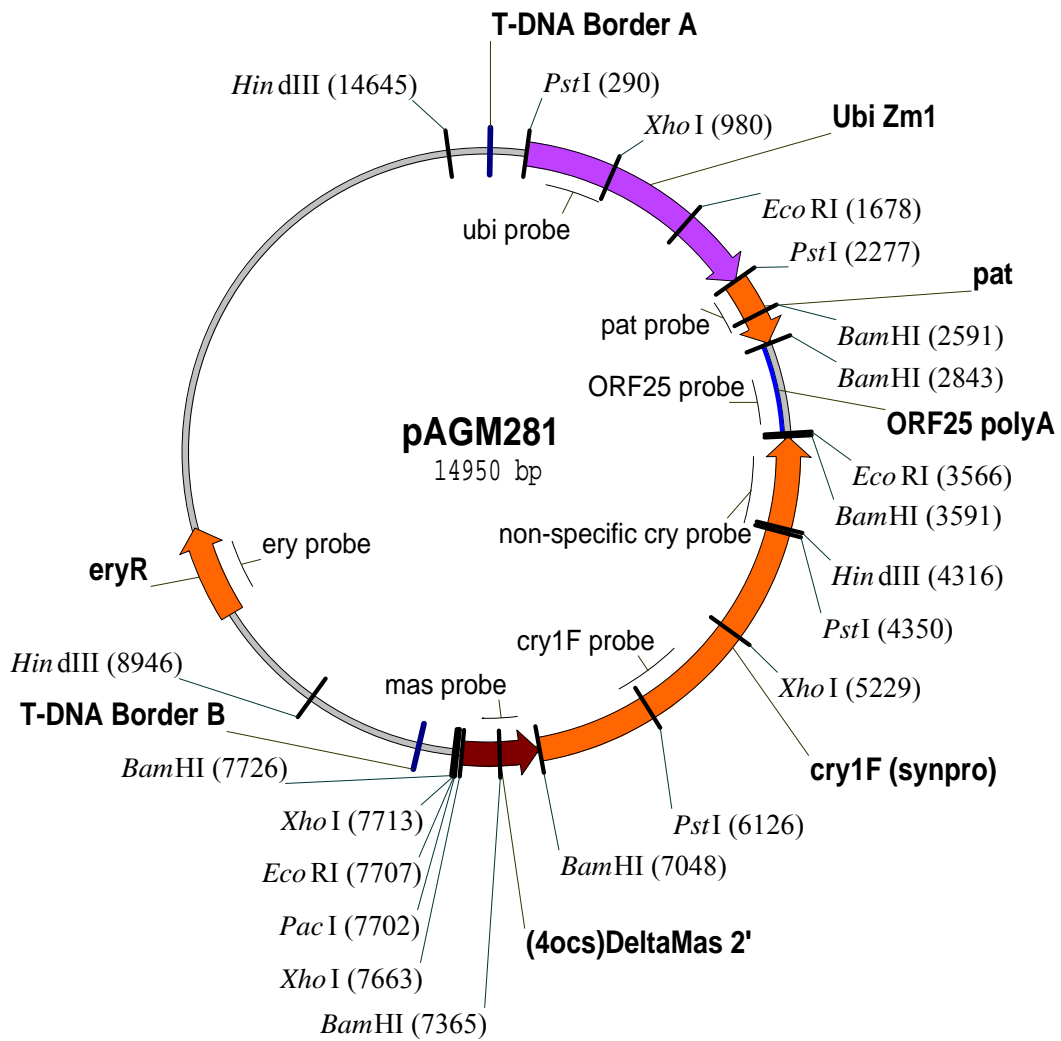
## (3) Method of preparing living modified organisms

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

The location and orientation of component elements of donor nucleic acid in the expression vector pAGM281 used for the production of Cotton 281, and the section digested by restriction enzyme are shown in Figure 1.

The location and orientation of component elements of donor nucleic acid in the expression vector pMYC3006 used for the production of Cotton 3006, and the section digested by restriction enzyme are shown in Figure 2.

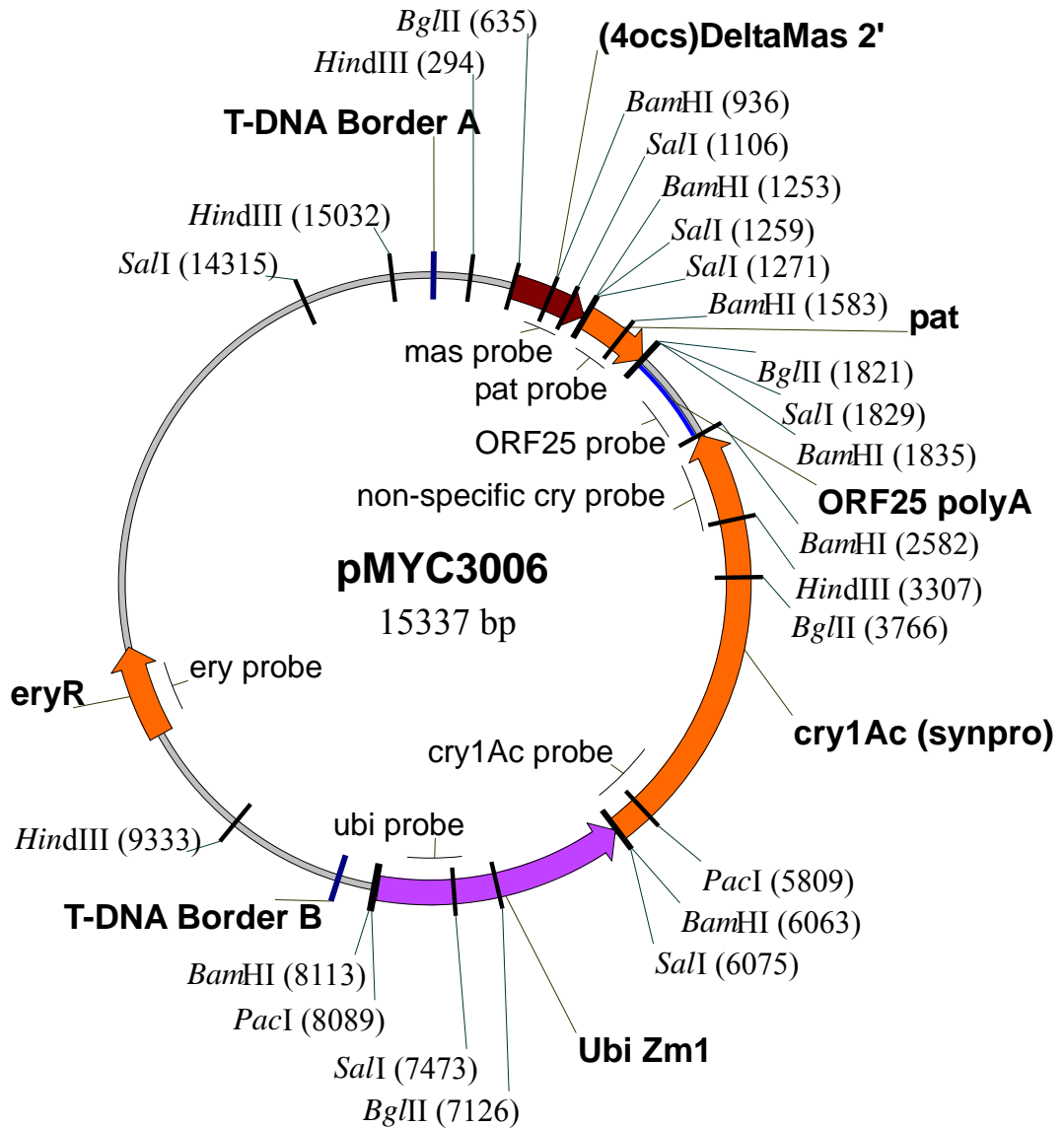
The location and orientation of component elements of donor nucleic acid in the expression vector PV-GHGT07 used for the production of Cotton 1445, and the section digested by restriction enzyme are shown in Figure 3.



T-DNA Border A : T-DNA left terminal  
T-DNA Border B : T-DNA right terminal

**Figure 1** Compositions of the expression vector pAGM281 used for the production of Cotton 281

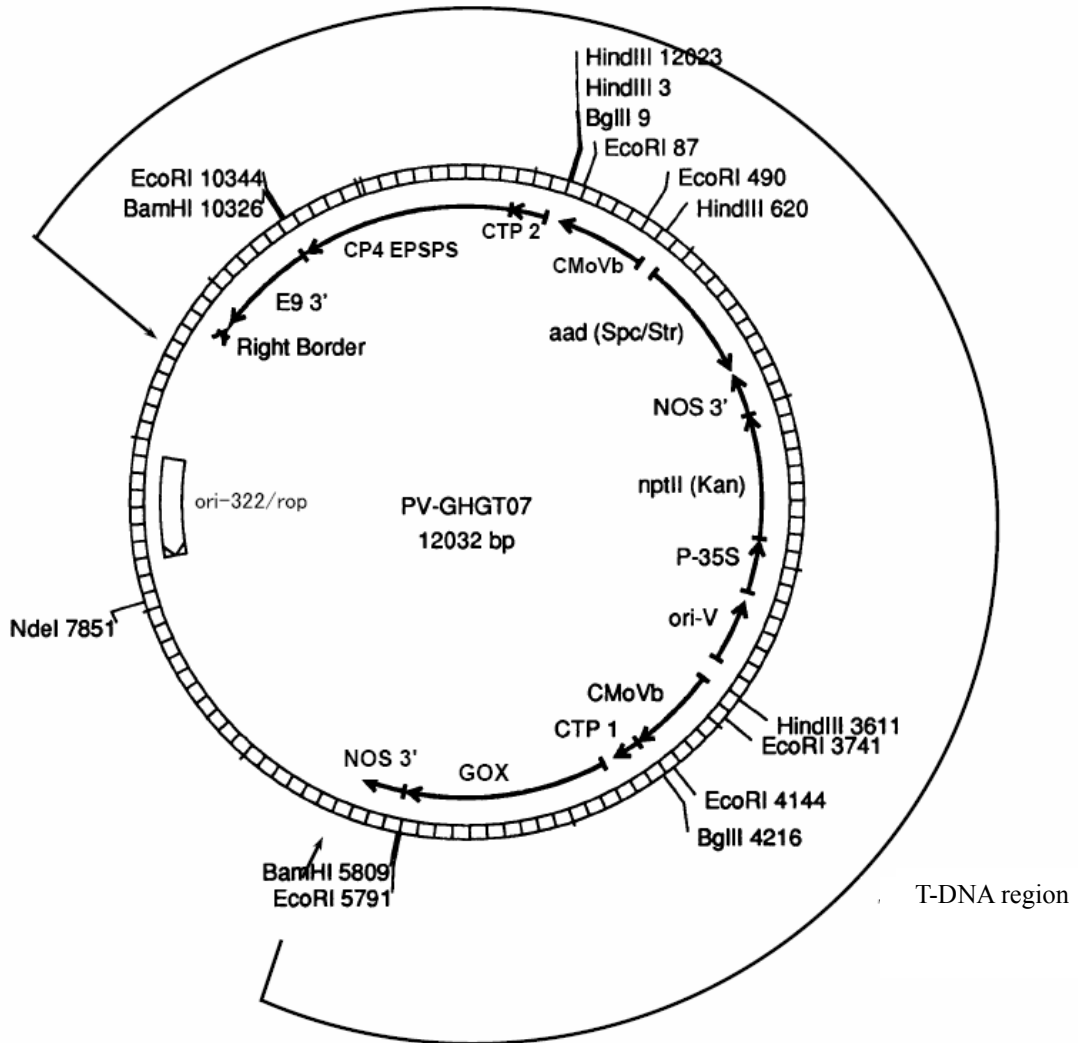
(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)



T-DNA Border A : T-DNA left terminal  
 T-DNA Border B : T-DNA right terminal

**Figure 2** Compositions of the expression vector pMYC3006 used for the production of Cotton 3006

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**Figure 3** Compositions of the expression vector PV-GHGT07 used for the production of Cotton 1445

(Excerpts from [http://www.bch.biodic.go.jp/download/lmo/public\\_comment/15985\\_1445ap.pdf](http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf))

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

The *Agrobacterium* transformation method was used to introduce the expression vectors pAGM281 and pMYC3006 into the experimental cotton GC510 line (*G. hirsutum*), the recipient organism of Cotton 281 and Cotton 3006, and also to introduce the expression vector PV-GHGT07 into a current cotton cultivar, Coker 312 (*G. hirsutum*), the recipient organism of Cotton 1445. In order to create Cotton 281 and Cotton 3006, the T-DNA region on the expression vectors pAGM281 and pMYC3006 was introduced into the slices of cotyledon of the GC510 line cotton. On the other hand, in order to create Cotton 1445, the T-DNA region of the expression vector PV-GHGT07 was introduced into the tissue section of Coker 312.

3) Processes of rearing of living modified organisms

(a) Method of selecting the cell into which nucleic acid is transferred

For the development of Cotton 281 and Cotton 3006, in the media containing glufosinate and antibiotics carbenicillin, callus was produced and then regenerated to plant using the regeneration media. The regenerated individuals were checked for the presence of transferred genes by Southern blotting analysis and then for the resistance to Tobacco budworm, the target pest, based on the bioassay of leaf disc.

For the development of Cotton 1445, nucleic acid was introduced by the *Agrobacterium* transformation method, and then regenerated individuals were obtained by culturing them in media containing kanamycin. The transformed plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics. It was confirmed that there was no *Agrobacterium* remained. Regarding the obtained regenerated individuals, further selection was carried out based on the analysis of inserted genes and the expression level of the CP4 EPSPS protein. Tests in climate chamber and greenhouse were then carried out, and actual tolerance to glyphosate and agricultural characteristics were examined in outdoor field tests. Cotton 1445 was selected upon the comprehensive evaluation of these results.

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

For the development of Cotton 281 and Cotton 3006, any remaining *Agrobacterium* was killed by adding the antibiotics carbenicillin in the process of callus induction, and it was confirmed there was no remaining *Agrobacterium*. On the other hand, for the development of Cotton 1445, the transformed plant was cultivated in media containing carbenicillin and paromomycin, and then it was cultivated in regenerating media containing no antibiotics. It was confirmed that there was no *Agrobacterium* remained.

(c) Process of rearing and genealogical tree

Cotton 281/3006 was bred based on the commonly used line breeding method by self-pollination. First, in the F2 generation, the events, which contain the

both genes, modified *cryIF* and modified *cryIAC*, were selected by the PCR method, and the events, which do not contain the both genes, were eliminated before the time of flower initiation. For the selected events which came into flower, self-pollination was applied to obtain the F3 generation. Then the F4 generation was obtained by repeating this process. On the other hand, Cotton 1445 was crossed with the truebreed cotton for selective breeding.

In addition, the F4 generation of Cotton 281/3006 and the BC3F1 generation of Cotton 1445 were crossed with each other based on the conventional breeding method to produce this stack line cotton. This stack line cotton was bred based on the commonly used breeding method by self-pollination. As a result of checking for the presence of the both genes, modified *cryIF* and modified *cryIAC*, by the PCR method and of spraying of glyphosate herbicide, this stack line cotton was selected. The process of rearing of this stack line cotton is shown in Figure 4.

Regarding Cotton 281, Cotton 3006, Cotton 281/3006 and Cotton 1445, the approvals received from organizations in Japan and the status of applications for approval are listed below. Cotton 281/3006 has been developed based on the traditional breeding method and thus, it is under no legal obligation to obtain a new approval for safety of use as feed.

### **Cotton 281**

- July, 2005: Application was filed to the Ministry of Agriculture, Forestry and Fisheries for approval for safety of use as feed in accordance with the "Safety Evaluation Criteria for Feed and Additives derived from Recombinant-DNA Techniques."
- September, 2005: Approval was obtained from the Ministry of Health, Labour and Welfare for safety of use as food in accordance with the "Safety Evaluation Criteria for Genetically-Modified Foods (Seed Plants)."

### **Cotton 3006**

- July, 2005: Application was filed to the Ministry of Agriculture, Forestry and Fisheries for approval for safety of use as feed in accordance with the "Safety Evaluation Criteria for Feed and Additives derived from Recombinant-DNA Techniques."
- September, 2005: Approval was obtained from the Ministry of Health, Labour and Welfare for safety of use as food in accordance with the "Safety Evaluation Criteria for Genetically-Modified Foods (Seed Plants)."

### **Cotton 281/3006**

- December, 2004: Application was filed to the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for approval of Type I Use (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them) under the provisions of the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms."
- October, 2005: Approval was obtained from the Ministry of Health, Labour and Welfare for safety of use as food in accordance with the "Safety Evaluation Criteria for Genetically-Modified Foods (Seed Plants)."

### **Cotton 1445**

- December, 1997: The Ministry of Agriculture, Forestry and Fisheries ensured the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed), based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries".
- December, 1997: The Ministry of Health, Labour and Welfare ensured the safety of use for food, based on the "Guideline for the conduct of Food Safety Assessment of Food and Additives derived from Recombinant-DNA Plants, Chapter 4".
- January, 1998: The safety of use of the cultivar for feed was ensured based on the "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)".
- March, 2001: The Ministry of Health, Labour 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, based on the "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques".
- November, 2004: Approval was obtained from the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Environment for Type I Use (Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them) under the provisions of the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified



Not made available or disclosed to unauthorized person

**Figure 4 Process of rearing of this stack line cotton**

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

Neither of Cotton 281 and Cotton 3006 will be commercialized individually. Only the Cotton 281/3006 produced by crossing Cotton 281 and Cotton 3006 will be commercialized and then, Assessment of Adverse Effect on Biological Diversity was conducted on the Cotton 281/3006. Therefore, the stability of expression of traits is discussed based on the Cotton 281/3006 and Cotton 1445.

1) Location of the copy of transferred nucleic acid

Examination was made to identify what ratio of segregation the traits introduced in the Cotton 281/3006 exhibits in the population of F1 and F2 generations. As a result, the segregation ratio was found as expected based on the Mendel's Law on the nuclear genes, and it was confirmed that the transferred genes have been successfully integrated into the genome. In addition, regarding the Cotton 1445, as a result of Southern blotting analysis on the transferred genes, it was confirmed that the transferred genes have been successfully integrated into the genome.

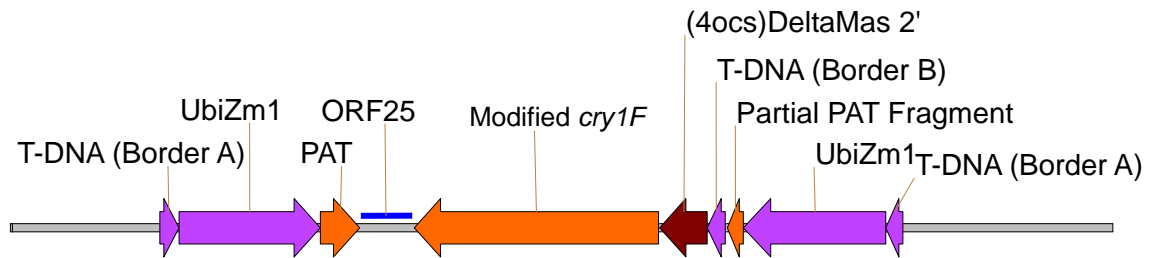
2) The number of copies of transferred nucleic acid and stability of its inheritance through multiple generations

The stability of the inheritance of nucleic acid transferred in the Cotton 281/3006 and Cotton 1445 through multiple generations has been already examined in the Assessment of Adverse Effect on Biological Diversity for the individual cotton lines.

For this stack line cotton, self-propagation has been repeated for 5 generations and those individuals, which have the homozygous genotype, have been selected.

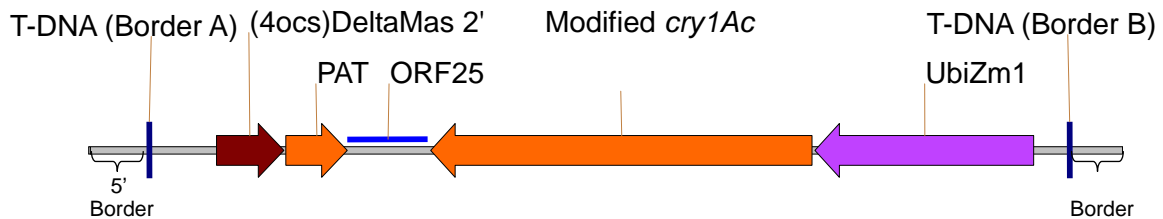
As a result of the analysis of introduced gene by Southern blotting analysis, it was confirmed that the introduced genes derived from Cotton 281/3006 and Cotton 1445 are stably present in this stack line cotton of the F4 generation (Green *et al.* . 2005). The analysis was conducted on the DNA extracted from the leaves of Cotton 281/3006, Cotton 1445 and this stack line cotton which were cultivated in a greenhouse. In order to examine the presence of introduced gene derived from Cotton 281/3006, Southern blotting analysis was conducted using the *cryIF*, *cryIAc*, or *pat* gene as a probe by digesting with the restriction enzyme *EcoRV*, *BamHI* or *XhoI*. In addition, in order to examine the presence of introduced gene derived from Cotton 1445, Southern blotting analysis was conducted using the *nptII*

gene as a probe by digesting with the restriction enzyme *HindIII*. As a result, one copy of the modified *cryIF* gene, one copy of the modified *cryIAC* gene, two copies of the *pat* gene and one copy of the partial *pat* gene derived from Cotton 281/3006, and one copy of *nptII* gene derived from Cotton 1445 were identified (Annex 1). In addition, as a result of Lateral Flow Strip test for the expression of the CP4 EPSPS protein in the leaves used in the Southern blotting analysis, the expression of the CP4 EPSPS protein was identified; therefore it is considered that the *cp4 epsps* gene is stably present in this stack line cotton.



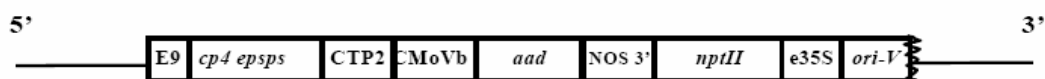
**Figure 5 Schematic representation of introduced gene of Cotton 281**

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)



**Figure 6 Schematic representation of introduced gene of Cotton 3006**

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)



**Figure 7 Schematic representation of introduced gene of Cotton 1445**

(Excerpts from [http://www.bch.biodic.go.jp/download/lmo/public\\_comment/15985\\_1445ap.pdf](http://www.bch.biodic.go.jp/download/lmo/public_comment/15985_1445ap.pdf))

- 3) Nearby or separate location of multiple copies, if present, on the chromosome

In the Cotton 281/3006, two copies of the *pat* gene are present, one copy derived from Cotton 281 and the other derived from Cotton 3006. The *pat* gene present in the Cotton 281 has been introduced as T-DNA region of the expression vector pAGM281 along with the modified *cryIF* gene. In addition, the *pat* gene present in the Cotton 3006 has been introduced as T-DNA region of the expression vector pMYC3006 along with the modified *cryIAC* gene. The modified *cryIF/pat* gene introduced in the Cotton 281 and the modified *cryIAC/pat* gene introduced in the Cotton 3006 are located on the genomes specific to the Cotton 281 and Cotton 3006, respectively.

Cotton 281/3006 has been produced by crossing between Cotton 281 and Cotton 3006 based on the cross-breeding method, and two independent nuclear genes have exhibited the segregation ratios following the Mendel's Law in the traits segregation tests on the F1 and F2 generations. In addition, also as a result of detection of Cotton 281/3006 by the PCR analysis using the nucleotide sequences specific to Cotton 281 and Cotton 3006, Cotton 281 and Cotton 3006 specific bands have been observed in the Cotton 281/3006.

Based on the above understanding, it is considered that the two copies of *pat* gene in the Cotton 281/3006 are located separate from each other and that the *pat* genes also in this stack line cotton produced by crossing between Cotton 281/3006 and Cotton 1445 are located separate from each other.

- 4) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid

The inter-individual or inter-generational expression stability of Cotton 281/3006 and Cotton 1445 has been confirmed based on the ELISA analysis and bioassay in the Assessment of Adverse Effect on Biological Diversity of the respective lines.

This stack line cotton exhibited satisfactory pest control activity similarly as Cotton 281/3006 in the field tests in the US conducted in 2004 against Lepidoptera insects which damage cotton. Thus, it was considered that the modified Cry1F protein and modified Cry1Ac protein are stably produced also in this stack line cotton (Huckaba *et al.* 2004, P.19. Table 4 and Table 5). In addition, in the field tests conducted at 5 sites in the US in 2004, comparison was made for the expression level of modified Cry1F and modified Cry1Ac proteins in the leaves and squares of Cotton 281/3006 and this stack line cotton. As a result, the expression level of the modified Cry1F protein (ng/mg in terms of weight of dry tissue) was found 8.1 to 32.5 in this stack line cotton and 19.2 to 39.2 in the Cotton 281/3006 in leaves, and 3.9 to 5.3 in this stack line cotton and 4.5 to 5.0 in the Cotton 281/3006 in squares. In addition, the expression level of the modified Cry1Ac protein (ng/mg in terms of weight of dry tissue) was 1.6 to 2.6 in this stack line cotton and 2.0 to 2.5 in the Cotton 281/3006 in leaves, and 1.2 to 2.1 in this stack line cotton and 1.1 to 2.2 in the Cotton 281/3006 in squares. Consequently, it was considered that the modified Cry1F protein and modified Cry1Ac proteins are stably expressed also in this stack line cotton similarly as the Cotton 281/3006 even in various

environmental conditions in the typical cotton cultivation areas (Table 6). In addition, the expression level of the PAT protein (ng/mg in terms of weight of bio-tissue) in seeds was 0.28 to 0.79 in this stack line cotton and 0.33 to 0.71 in the Cotton 281/3006. Consequently, it was confirmed that the PAT protein is stably expressed also in this stack line cotton similarly as the Cotton 281/3006 (Table 7).

Then, the tolerance to glyphosate herbicide in this stack line cotton derived from Cotton 1445 was examined based on the glyphosate spraying tests. This stack line cotton, Cotton 1445 and Cotton 281/3006 were sprayed with glyphosate of normal dose (870g ae/ha) or 20 times higher dose than normal (17,400g ae/ha) to determine the incidence of necrosis in the plant body due to glyphosate. As a result, no difference was observed between this stack line cotton and Cotton 1445 in the incidence of necrosis due to the spraying of glyphosate (Table 8).

**Table 4 Control effect for Lepidoptera insects<sup>1)</sup>** (Blackbill, North Carolina, in 2004)

	Damage rate of square (%) <sup>2)</sup>	Damage rate of cotton boll (%) <sup>3)</sup>
This stack line cotton	2.0 b	0.0 b
Cotton 281/3006	3.0 b	2.0 b
Cotton 1445	42.0 a	34.0 a

1) Spontaneous generation : Cotton bollworm (81%), Tobacco budworm (19%). The rate of spontaneous generation was calculated from the number of insects captured by a pheromone trap that was installed in a field.

2) It was examined in 76<sup>th</sup> day after sowing regarding to 25 squares/repeat (4 repeats).

3) It was examined in 82<sup>nd</sup> day after sowing regarding to 25 bolls/repeat (4 repeats).

\* The same alphabetical letters indicate that there is no significant difference between the relevant measurements in the Tukey's HSD tests (P>0.05).

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)

**Table 5 Control effect for Cotton bollworm<sup>1)</sup>** (Jamesbill, North Carolina, in 2004)

	Damage rate of square (%) <sup>2)</sup>	Damage rate of cotton boll (%) <sup>3)</sup>
This stack line cotton	7.0 b	7.5 b
Cotton 281/3006	11.3 b	5.8 b
Cotton 1445	65.8 a	56.3 a

1) Spontaneous generation

2) It was examined in 90<sup>th</sup> day after sowing regarding to 40 squares/repeat (4 repeats).

3) It was examined in 90<sup>th</sup> day after sowing regarding to 40 bolls/repeat (4 repeats).

\* The same alphabetical letters indicate that there is no significant difference between the relevant measurements in the Tukey's HSD tests (P>0.05).

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)

**Table 6 Average expression level of the modified Cry1F and modified Cry1Ac proteins in the leaves and squares of this stack line cotton and Cotton 281/3006**

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**Table 7 Average expression level of the PAT protein in the seeds of this stack line cotton and Cotton 281/3006**

Not made available or disclosed to unauthorized person

**Table 8 Glyphosate spraying tests for this stack line cotton, Cotton 1445 and Cotton 281/3006**

Test sample <sup>2)</sup>	Incidence of necrosis (%) <sup>1)</sup>	
	× 1 (870g ae/ha) <sup>3)</sup>	× 20 (17,400g ae/ha) <sup>3)</sup>
This stack line cotton	0	37
Cotton 1445	3	43
Cotton 281/3006	70	90

- 1) Incidence of necrosis (0%: No necrosis to 100%: Withering) was measured 7<sup>th</sup> day after spraying of glyphosate onto the plant body at 2<sup>nd</sup> leaf stage which was raised in a greenhouse at Dow AgroSciences LLC in the US.
- 2) Average value of 3 plants/area in 3 repeats was determined.
- 3) ae (acid equivalent): The preparation containing the glyphosate in the form of potassium salt was used. In order to indicate the measurement in terms of glyphosate acid, the active ingredient, acid equivalent was used. 870g ae/ha is equivalent to a dose of 1.6 l/ha of undiluted solution of glyphosate herbicide, and 17,400g ae/ha is equivalent to a dose of 32 l/ha of undiluted solution of glyphosate herbicide.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Dow Chemical Japan Ltd.)

- 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

This stack line cotton does not contain any sequence allowing transmission; therefore there is no possibility that the genes transferred to this stack line cotton might be transmitted to wild animals and wild plants.

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

For the detection and identification of Cotton 281/3006, the PCR method has been developed where the nucleotide sequences specific to Cotton 281 and Cotton 3006 respectively are used as primers. This method makes it possible to specifically detect Cotton 281/3006. In addition, for the detection and identification of Cotton 1445, the qualitative PCR method has been developed where the DNA sequences of the

introduced genes and neighboring areas of plant genome are used as primers. This method makes it possible to specifically detect Cotton 1445. For the detection and identification of this stack line cotton, a single seed is analyzed by the above-mentioned two methods. When the seed is found positive by the both methods, it refers to the detection and identification of this stack line cotton.

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

The modified Cry1F protein and modified Cry1Ac protein do not possess any enzyme activity and thus, it is considered that they will not have any effects on the recipient organism's metabolic systems. In addition, it became evident in the tests for pest control effects conducted in the US that the modified Cry1F protein and modified Cry1Ac protein do not interact with each other. The PAT protein is the enzyme that acetylates glufosinate very specifically; therefore the protein which becomes substrate in plant body is limited to glufosinate. Consequently, it is unlikely that the PAT protein might affect any other metabolic systems. In addition, it is suggested that the EPSPS protein, which possesses the similar functions as the CP4 EPSPS protein, is not a rate-determining enzyme in the shikimate pathway; the CP4 EPSPS protein has high substrate specificity and thus it is unlikely to exert any influence on the other metabolic systems. Therefore, it is hard to consider that these proteins might interact with each other. Furthermore, as mentioned in I-1-(4)-4), it was confirmed as a result of the tests for traits expressed in this stack line cotton that the traits expressed by Cotton 281/3006 and Cotton 1445 do not affect each other.

Based on the above understanding, for the difference between this stack line cotton and the taxonomic cotton species to which the recipient organism belongs, estimation was made based on the results of individual examinations for the various traits of Cotton 281/3006 and Cotton 1445.

- 1) Details of physiological or ecological properties conferred as a result of the expression of copies of the introduced nucleic acid (including the contents, if expressed specifically in specific tissue or at specific growth stage)

By the function of genes which were introduced into the parent lines of this stack line cotton, Cotton 281/3006 and Cotton 1445, the modified Cry1F, modified Cry1Ac, PAT and CP4 EPSPS proteins are expressed in this stack line cotton. Resistance to Lepidoptera insects is conferred with the expression of modified Cry1F and modified Cry1Ac proteins, and tolerance to glyphosate herbicide is conferred with the expression of CP4 EPSPS protein. In addition, the PAT protein is used as a selective marker in the production of Cotton 281 and Cotton 3006, and tolerance to glufosinate herbicide is conferred.

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

The isolated field tests were conducted in Japan in 2003 for Cotton 281/3006 and in 1997 for Cotton 1445 to identify any difference from non-recombinant cotton.

(a) Morphological and growth characteristics

Regarding Cotton 281/3006 and Cotton 1445, differences in morphological and growth characteristics were examined between the recombinant cotton plants and the non-recombinant control cotton with respect to the following items: the uniformity of germination; germination rate; plant type; plant height (height of main stem); time of flower initiation, flower color; leaf shape; the number of effective flower squares; the number of bearing shoots; boll opening time; the color of fiber (lint); the shape of bolls (fruit of cotton); the number of bolls per plant (measured separately for harvested and non-harvested bolls for Cotton 281/3006); the number of segments of a boll; the number of seeds per boll; the color of seeds; harvest time; the weight of a boll (dry weight for Cotton 1445); and the weights of above and under-ground parts at the harvest time. As a result, there was no such difference observed between the recombinant cotton plants and the non-recombinant control cotton to cause Adverse Effect on Biological Diversity. Therefore, it is considered that this stack line cotton does not involve any difference which could cause Adverse Effect on Biological Diversity.

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

Cold-tolerance test at the early stage of growth of Cotton 281/3006 in climate chamber, and examinations for germination rate in winter and wintering ability in fields for Cotton 1445 were conducted. As a result, it was confirmed that the both did not grow during winter season. Consequently, it is considered that this stack line cotton does not offer cold-tolerance at the early stage of growth.

(c) Wintering ability of the matured plant

As a result of observation of the plants of Cotton 281/3006 and Cotton 1445 cultivated in their respective isolated field tests, it was confirmed that the plants of Cotton 281/3006 died completely due to the exposure to low temperatures and frosting before the end of December and also that Cotton 1445 had started to die partially on which this isolated field test was completed; thus, it was considered that the matured plants are hard to overwinter. Consequently, it is considered that the matured plants of this stack line cotton are hard to overwinter.

(d) Fertility and size of the pollen

In Japan, there are no plans for seed of this stack line cotton to be commercialized, and the cotton is not cultivated for commercial use. Therefore, if this stack line cotton caused Adverse Effect on Biological Diversity in Japan, it would be in the following way: cotton seeds imported for oil extraction and feed are spilled during transportation, into Japanese natural environment; then the spilled seeds grow or become self-seeding, and expel other plants from the area. However, pollens are not formed until spilled cotton seeds germinate, grow or become self-seeding, and become adult; and there have been no reports that seeds spilled during transportation grow or become self-seeding under natural conditions in Japan. Consequently, the

fertility and size of pollens were not examined for this stack line cotton.

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

Regarding seed production, the differences between this recombinant cotton and the non-recombinant control cotton have been examined in the number of bolls per plant, the number of segments of a boll, the number of seeds per boll, and the weight of boll. As a result, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton. Low shedding habit was expected because fiber twining around seeds does not allow them to shed. It is known that the level of seed dormancy of cotton is extremely low and the seeds germinate under certain conditions including the humidity and temperature (14°C or more) in soil. In addition, it was considered that this recombinant cotton seeds have extremely poor survival ability in low temperature and extremely low wintering ability and fail to maintain the germinating ability under low-temperature conditions in winter season in Japan, so the test concerning dormancy was not conducted. Regarding the germination rate, there was a significant difference between the Cotton 1445 and the non-recombinant control cotton. However, it is considered that this result was due to the deterioration of seed samples by bursting of seed, and not causing Adverse Effect on Biological Diversity. Consequently, it is considered that there is no difference between this stack line cotton and the non-recombinant control cotton.

(f) Crossability

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

(g) Productivity of harmful substances

As mentioned in (d), if this stack line cotton caused Adverse Effect on Biological Diversity in Japan by producing any harmful substances, 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 productivity of harmful substances was not examined for this stack line cotton, because any harmful substances secreted from the roots and/or above-ground parts fail to become so enough in quantity to exert adverse effects until the spilled seeds germinate and grow to a certain degree, and there have been no reports of seeds spilled during transportation, or that they have germinated and grown or become self-seeding under natural conditions in Japan.



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

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

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

#### **(1) Competitiveness**

This stack line cotton possesses the Lepidoptera resistance and glufosinate herbicide tolerance derived from DAS-24236-5×DAS-21023-5 and the glyphosate herbicide tolerance derived from MON-01445-2. However, it is considered that the glufosinate and glyphosate do not exert pressure for selection under a natural environment in Japan, and 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 becomes competitive over the parent lines. Based on the above understanding, it was 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 Cry1F protein, modified Cry1Ac protein and PAT protein derived from DAS-24236-5×DAS-21023-5 and the CP4 EPSPS protein derived from MON-01445-2. The modified Cry1F and modified Cry1Ac proteins possess the insecticidal activity against Lepidoptera insects, though the PAT protein and modified CP4 EPSPS protein are 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 these proteins. Therefore, the conclusion that the use of this stack line 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 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 line cotton in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above is valid.

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**Annex List**

Annex 1: Southern blotting analysis on this stack line cotton

(Not made available or disclosed to unauthorized person)