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

Name: Dow Chemical Japan Ltd.
Monty Bayer, Representative director
(Seal)
Address: 2-24 Higashi Shinagawa 2-chome,
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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<br>（*cry1F, cry1Ac*, *pat, Gossypium hirsutum* L.）<br>（281×3006, OECD UI : DAS-24236-5×DAS-21Ø23-5） |
| 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

This recombinant cotton was developed by cross-breeding Cotton 281 and Cotton 3006. Then the information concerning preparation of living modified organisms is explained individually in the following sections for Cotton 281 and Cotton 3006. Use of Cotton 281 and Cotton 3006 for provision as commercial lines is not planned.

1) Composition and origins of component elements

Composition of donor nucleic acid that was used for developing Cotton 281 and Cotton 3006, and origins of component elements are shown in Table 1 and Table 2.

The modified *cry1F* gene and the modified *cry1Ac* gene have been synthesized based on the *cry1F* gene derived from *Bacillus thuringiensis* (*B. t.*) var. *aizawai* and the *cry1Ac* gene derived from *B. t. kurstaki* respectively in order to enhance the insecticidal activity and maximize the expression in plants, and the modified Cry1F protein and the modified Cry1Ac protein, which are encoded by their corresponding genes, differ from the wild type in size and amino acid sequence. In addition, *pat* gene has been synthesized based on the *pat* gene derived from *Streptomyces viridochromogenes* to maximize its expression in plants, though the PAT protein, which is encoded by this gene, remains unchanged in the amino acid sequence.

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

The modified Cry1F protein exhibits higher insecticidal activity particularly against Beet Armyworm (*Spodoptera exigua*) and Soybean Looper (*Pseudoplusia includens*), which damage the leaves of cotton, and the modified Cry1Ac protein exhibits higher insecticidal activity particularly against Cotton bollworm (*Heliocoverpa zea*) and Pink bollworm (*Pectinophora gossypiella*), which damage the bolls of cottonseeds (Table 3). Then, in order to enhance the expression level of the modified Cry1F protein in leaves, (4ocs)DeltaMas2' was used as a promoter for the modified *cry1F* gene for its characteristics that it offers higher expression level in leaves. In addition, in order to enhance the expression level of the modified Cry1Ac protein in the cottonseed bolls, UbiZm1 was used as a promoter for the modified *cry1Ac* gene for its characteristics that it provides higher expression level in cottonseed bolls. Functions of component elements of donor nucleic acid that was used for the development of Cotton 281 and Cotton 3006 are shown in Table 1 and Table 2, respectively.

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

| Component elements | Origin and Function |
|---|---|
| Modified *cry1F* cassette | |
| (4ocs)DeltaMas 2' | A mannopine synthase promoter derived from pTi15955 (Barker *et al*. 1983) (GenBank Locus ATACH5, Accession X00491), which contains 4 copies of the octopine synthase (OCS) enhancer derived from pTiAch5 (Ellis *et al*. 1987) (GenBank Accession Numbers I05704 to I05712), providing an advantage of higher expression level in leaves of cotton. |
| Modified *cry1F* | It was synthesized based on *cry1F* gene derived from *B.t.* var. *aizawai*. This gene encodes modified Cry1F protein which shows insecticidal activity to major harmful insects for cotton cultivation including Tobacco budworm (*Heliothis virescens*), Beet Armyworm (*Spodoptera exigua*), and Cotton bollworm (*Helicoverpa zea*). |
| ORF25 polyA | A bi-directional terminator derived from *R.radiobacter* (*A.tumefaciens*) pTi15955 (Barker *et al.* 1983) (GenBank Locus ATACH5, Accession X00491). |
| *pat* cassette | |
| UbiZm1 (intron) | A ubiquitin 1 promoter of maize (*Zea mays*) including the first exon (untranslated enhancer) and the first intron, providing an advantage of higher expression level in cottonseed bolls. (Christensen *et al.* 1992) (US Patent No.5614199, GenBank Accession I18571) |
| *pat* | 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 *S. viridochromogenes* (Eckes *et al*. 1989). A gene which encodes PAT protein used as a selectable marker. |
| ORF25 polyA | A bi-directional terminator derived from *R.radiobacter* (*A.tumefaciens*) pTi15955 (Barker *et al.* 1983) (GenBank Locus ATACH5, Accession X00491). |

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

| Component elements | Origin and Function |
|---|---|
| Modified *cry1Ac* cassette | |
| Ubi Zm1(intron) | A ubiquitin 1 promoter of maize (*Zea mays*) including the first exon and the first intron, providing an advantage of higher expression level in cottonseed bolls. (Christensen *et al*. 1992) (US Patent No.5614199, GenBank Accession I18571) |
| Modified *cry1Ac* | It was synthesized based on *cry1F gene* derived from *B.t*. var. *kurstaki.* This gene encodes modified Cry1F protein which shows insecticidal activity to major harmful insects for cotton cultivation including Tobacco budworm (*Heliothis virescens*), Beet Armyworm (*Spodoptera exigua*), Cotton bollworm [also called Corn earworm (*Helicoverpa zea*)], and Pink bollworm (*Pectinophora gossypiella*). |
| ORF25 polyA | A bi-directional terminator derived from *R.radiobacter* (*A.tumefaciens*) pTi15955 (Barker *et al.* 1983) (GenBank Locus ATACH5, Accession X00491). |
| *pat* cassette | |
| (4ocs)DeltaMas 2' | A mannopine synthase promoter derived from pTi15955 (Barker *et al*. 1983) (GenBank Locus ATACH5, Accession X00491), which contains 4 copies of the octopine synthase (OCS) enhancer derived from pTiAch5 (Ellis *et al*. 1987) (GenBank Accession Numbers I05704 to I05712), providing an advantage of higher expression level in leaves of cotton. |
| *pat* | 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 *S. viridochromogenes* (Eckes *et al*. 1989). A gene which encodes PAT protein used as a selectable marker. |
| ORF25 polyA | A bi-directional terminator derived from *R.radiobacter* (*A.tumefaciens*) pTi15955 (Barker *et al.* 1983) (GenBank Locus ATACH5, Accession X00491). |

(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 this recombinant cotton, 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 insects of the order Lepidoptera in the US, Europe, Japan and other courtiers.

It is known that the Cry1 protein including the Cry1F protein and Cry1Ac protein exhibits the insecticidal activity only against the insects of the order Lepidoptera (Prieto-Samsónov *et al*. 1997). The modified Cry1F protein exhibits the insecticidal activity against order Lepidoptera, 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*). This recombinant cotton expresses the both Cry proteins, the modified Cry1F protein and modified Cry1Ac protein, and thus it possesses the insecticidal activities from the both. Regarding the Cotton 281 which expresses the modified Cry1F protein, Cotton 3006 which expresses the modified Cry1Ac protein, and this recombinant cotton which expresses the both Cry proteins, the effects of control of order Lepidoptera are summarized in Table 3.

In addition, the modified Cry1F protein and the modified Cry1Ac protein have been verified as safe for non-target organisms including water flea (*Daphnia magna*), lacewing fly (*Chrysopa carnea*), ladybug, honey bees, *Nasonia vitripennis*, earthworm, and Rainbow trout (*Oncorhynchus mykiss*).

**Table 3　Pest control ability against Lepidopteran insects**

| Order Lepidoptera | Cotton 281 (Modified Cry1F protein) | Cotton 3006 (Modified Cry1Ac protein) | This recombinant cotton |
|---|---|---|---|
| Tobacco budworm (*Heliothis virescens*) | ○ | ○ | ○ |
| Beet Armyworm (*Spodoptera exigua*) | ○ | △ | ○ |
| Soybean Looper (*Psuedoplusia* | ○ | △ | ○ |
| Cotton bollworm (*Helicoverpa zea*) | △ | ○ | ○ |
| Pink bollworm (*Pectinophora* | × | ○ | ○ |

○ : Superior pest control achievable
△ : Pest control available
× : No pest control attained

**PAT protein**

The PAT protein, the phosphinothricin acetyltransfase, specifically acetylates the glufosinate herbicide to transform it into non-toxic acetyl-glufosinate and extinguish the weed-killing activity; therefore plants containing the *pat* gene exhibit the tolerance to glufosinate herbicide.

**Allergic property**

In order to investigate whether modified Cry1F protein, modified Cry1Ac protein and PAT protein share functionally important amino acid sequence with known allergens, these proteins were compared with allergens in the database (Swiss-Prot, PIR, GenRept, FARRP Protein Allergen Database). As a result, they did not share structurally related homologous sequences 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 and thus, it is considered that the modified Cry1F protein and the modified Cry1Ac protein will not have any effects on the recipient organism's metabolic systems. In addition, the PAT protein is the enzyme that acetylates glufosinate very specifically (Thompson *et al*. 1987); therefore the protein, which constitutes substrates in plant body, is limited to glufosinate. Consequently, it is unlikely that the PAT protein might affect any other metabolic systems.

(2) Information concerning vector

1) Name and origin

The expression vector pAGM281 used to generate the Cotton 281, one mother plant of this recombinant cotton, and the expression vector pMYC3006 used to generate the Cotton 3006, the other mother plant of this recombinant cotton, were assembled from the broad-recipient range plasmid RK2 (Schmidhauser *et al.* 1985).

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.

(b) Types of any nucleotide sequence having specific functions

Erythromycin tolerance by the $ery^R$ gene was used for selection of the expression vectors pAGM281 and pMYC3006. The $ery^R$ gene is located outside the T-DNA region and then this gene has not been introduced in the Cotton 281 and Cotton 3006.

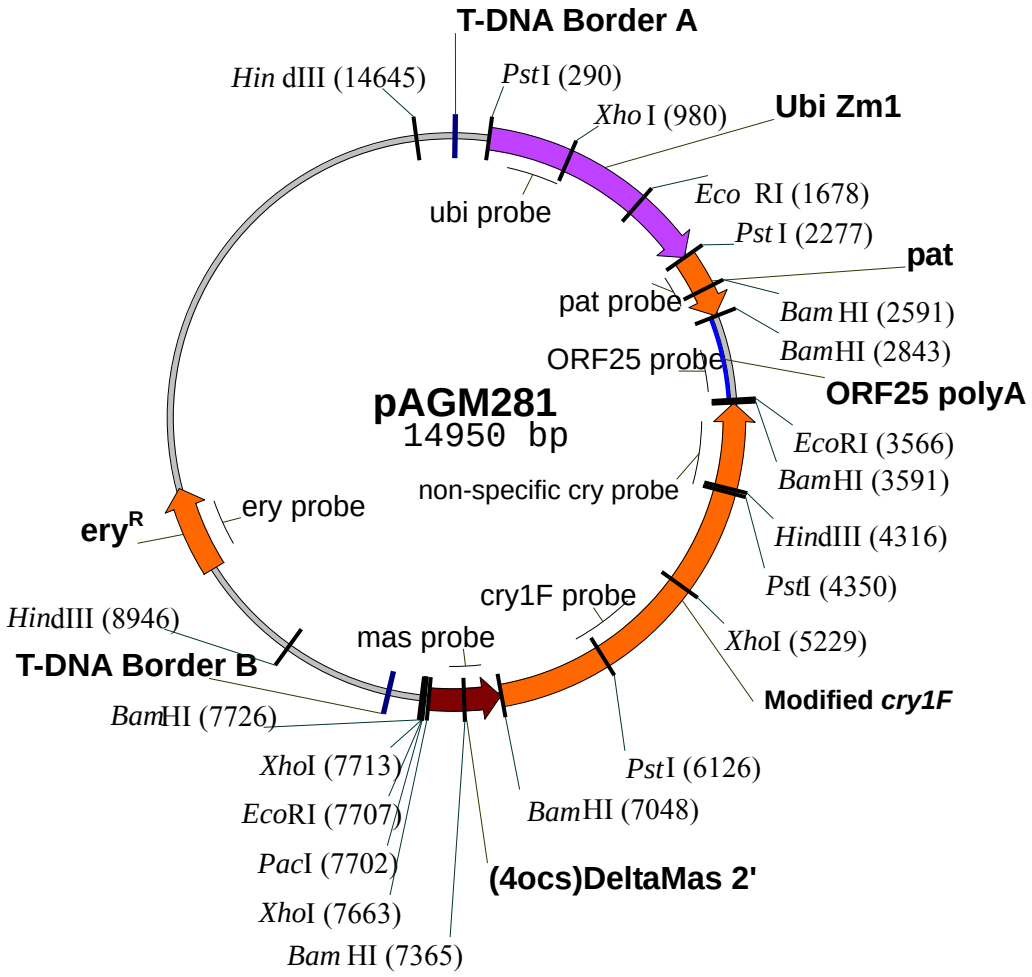
(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

The T-DNA region of the plasmid RK2, the origin of the vectors pAGM281 and pMYC3006, has been replaced by the three types of gene cassettes, modified *cry1F* cassette, modified *cry1Ac* cassette, and *pat* cassette, shown in Table 1 and Table 2. Therefore, the plasmid RK2 does not contain any sequence allowing infection of *Agrobacterium*, and its infectivity is not known.

(3) Method of preparing living modified organisms

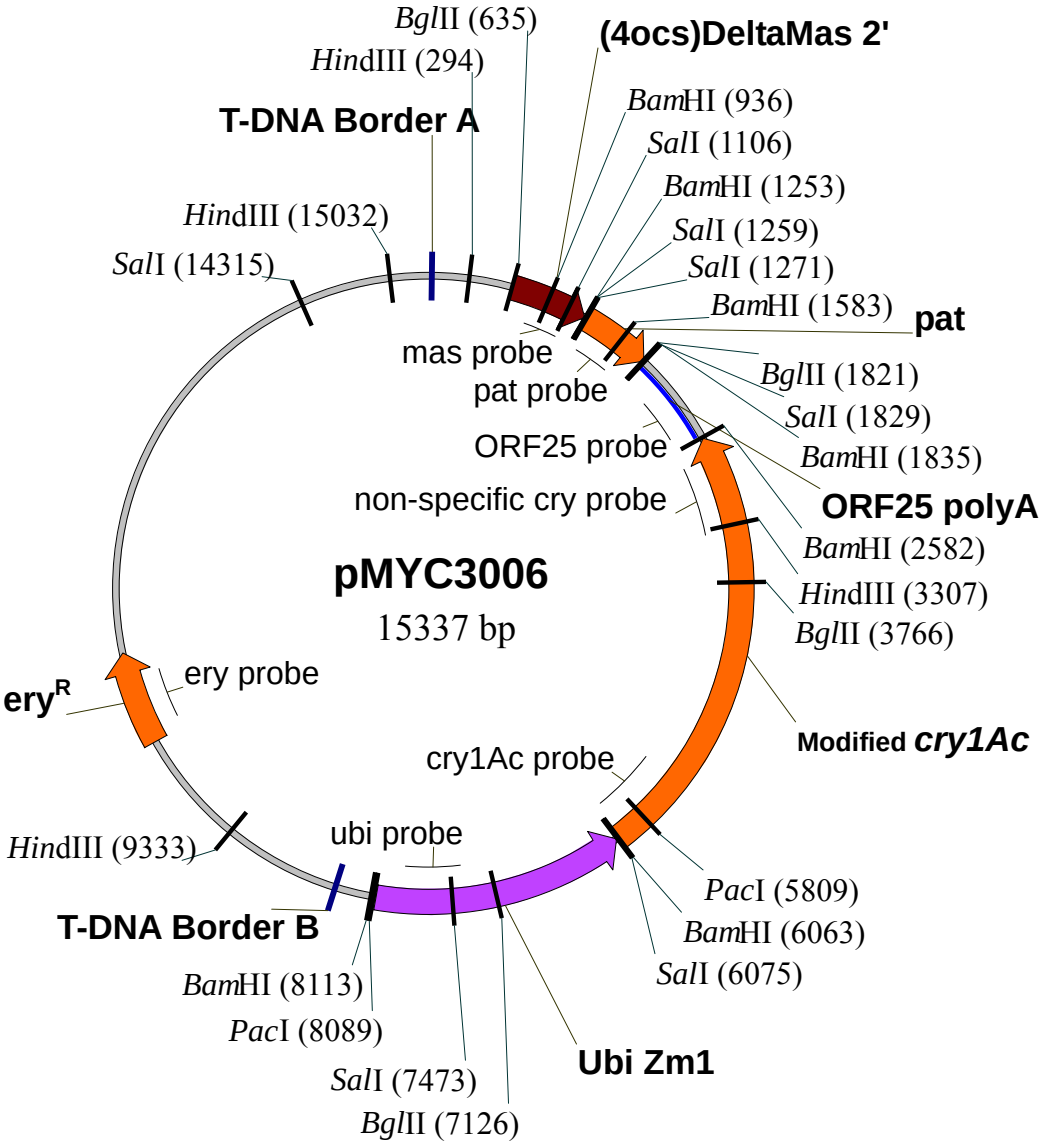
1) Structure of the entire nucleic acid transferred in the recipient organism

The structure of expression vector pAGM281 used for developing Cotton 281 is shown in Figure 1. The structure of expression vector pMYC3006 used for developing Cotton 3006 is shown in Figure 2.




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

**Figure 1 Structure of expression vector pAGM281**




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

**Figure 2　Structure of expression vector pMYC3006**

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

The expression vectors pAGM281 and pMYC3006 were introduced into the experimental cotton GC510 line (*G. hirsutum*), the recipient organism of Cotton 281 and Cotton 3006, by the *Agrobacterium* method. Slices of cotyledon of the GC510 line cotton were co-cultivated with *Agrobacterium* that holds either expression vector pAGM281 or pMYC3006, and the T-DNA region on the expression vector was integrated into the genome of cotton.

3) Processes of rearing of living modified organisms

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

In the media containing glufosinate and antibiotics carbenicillin, callus was produced and then regenerated to plant body 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 section disc.

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

Any remaining *Agrobacterium* was killed by adding the antibiotics carbenicillin in the process of introducing the callus, and it was confirmed that there was no remaining *Agrobacterium*.

(c) Process of rearing and genealogical tree

The Cotton 281 and Cotton 3006 were crossed individually with the truebreed cotton (PSC355) for selective breeding. Then the BC3F1 generations of Cotton 281 and Cotton 3006 were crossed with each other to produce this recombinant cotton which expresses the modified Cry1F, modified Cry1Ac and PAT proteins. This recombinant cotton was bred based on the commonly used line breeding method by self-pollination. First, in the F2 generation, the strains, which contain the both genes, modified *cry1F* and modified *cry1Ac*, were selected by the PCR method, and the strains, which do not contain the both genes, were eliminated before the time of flower initiation. For the selected strains which came into flower, self-pollination was applied to obtain the F3 generation. Repeating this process could increase the rate of dominant homo individuals and in fact, in the F7 generation which is to be commercialized, all of the 368 seeds examined have expressed the both proteins, modified Cry1F and modified Cry1Ac.

The safety of Cotton 281 and Cotton 3006 was certified in September 2005 and the safety of this recombinant cotton was certified in October 2005, in accordance with “Food Sanitation Law” by the Ministry of Health, Labour and Welfare. In addition, the application for the safety evaluation of Cotton 281 and Cotton 3006 in accordance with “Feed Safety Law” were filed with the Ministry of Agriculture, Forestry and Fisheries in July 2005.

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

1) Location of the copy of transferred nucleic acid

The transferred DNA follows the Mendel's Law of Genetics once it has been introduced on the chromosome of plants. Analysis was conducted to identify what ratio of segregation the traits introduced in the Cotton 281 and Cotton 3006 exhibit in the population of F2 generation. As a result of self-pollination among the BC3F1 generation individuals, which possess either gene, modified *cry1F*/*pat* or modified *cry1Ac*/*pat* gene, in the heterogeneous condition, the BC3F2 generations exhibited the segregation ratio as expected based on the Mendel's Law on the nuclear genes. Therefore, based on the agreement of test results with the segregation ratio to be expected on the assumption that the nucleic acid transferred to the Cotton 281 and Cotton 3006 is located on the chromosomes, it was confirmed that the transferred nucleic acid is present on the chromosome. In addition, segregation of traits in the F1 and F2 generations of this recombinant cotton was examined. As a result, the segregation ratio was found as expected based on the Mendel's Law on the nuclear gene, and it was confirmed that the transferred nucleic acid is present on the chromosome of this recombinant cotton.

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

The number of copies of the transferred modified *cry1F* gene, modified *cry1Ac* gene and *pat* gene and stability of the inheritance through multiple generations were examined by Southern blotting analsyis.

In order to examine the number of copies of transferred nucleic acid, Southern blotting analysis was conducted on the F3 generation of this recombinant cotton. As a result, it was confirmed that one copy of the modified *cry1F* gene, one copy of the modified *cry1Ac* gene, and two copies of the *pat* gene and one fragment of *pat* gene (partial *pat* gene) were transferred in this recombinant cotton. The partial *pat* gene is found present in the Cotton 281, and the partial *pat* gene in this recombinant cotton is derived from the Cotton 281. The schematics of genes transferred in the Cotton 281 and Cotton 3006 are provided in Figure 3 and Figure 4, respectively. The partial *pat* gene is about 1/16 in length compared to the full-length *pat* gene. In the Western blotting analysis on this recombinant cotton to examine the productivity of PAT protein in the *pat* gene fragment, no partial PAT protein was detected. In addition, it was confirmed that this recombinant cotton contains no gene transferred, which encodes the Erythromycin resistance ($ery^R$) derived from bacteria present in the transformation vector. Furthermore, in order to examine the stability of inheritance of the transferred nucleic acid through multiple generations, Southern blotting analysis was conducted on the F7 generation of this recombinant cotton. As a result, it was found that the transferred genes are stably inherited in posterity.

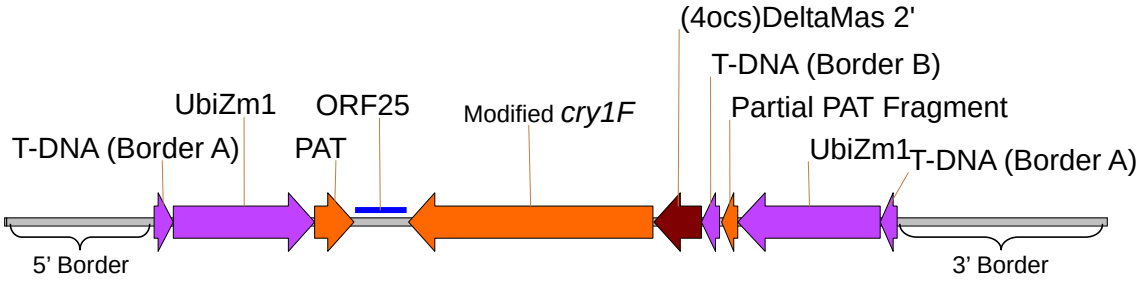



**Figure 3 Schematic representation of the entire inserted region of T-DNA derived from Cotton 281 and the neighboring borders**

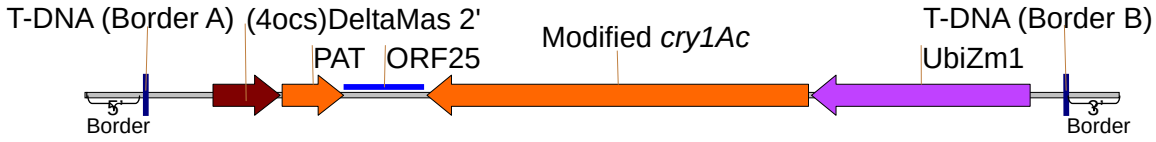



**Figure 4 Schematic representation of the entire inserted region of T-DNA derived from Cotton 3006 and the neighboring borders**

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

In this recombinant cotton, 2 copies of *pat* gene are present, one copy derived from Cotton 281 and the other one derived from Cotton 3006. The *pat* gene present in Cotton 281 has been introduced as T-DNA region of the expression vector pAGM281 along with the modified *cry1F* gene. In addition, the *pat* gene present in Cotton 3006 has been introduced as T-DNA region of the expression vector pMYC3006 along with the modified *cry1Ac* gene. The modified *cry1F*/*pat* gene introduced into Cotton 281 and modified *cry1Ac*/*pat* gene introduced into Cotton 3006 are located on the genomes specific to Cotton 281 and Cotton 3006, respectively. This recombinant cotton has been produced by crossing Cotton 281 and Cotton 3006, and two independent nuclear genes have exhibited the segregation ratios in accordance with the Mendel's Law in the traits segregation tests on F1 and F2 generations. In addition, also as a result of detection of this recombinant cotton 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 this recombinant cotton.

Based on the above understanding, it is considered that the 2 copies of *pat* gene in this recombinant cotton are located separately 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

This recombinant cotton exhibited satisfactory pest control activity in the field tests conducted in 2001 (F3 generation) and 2002 (F5 generation) against the order

Lepidoptera including Tobacco budworm (*Heliothis virescens*), Beet Armyworm (*Spodoptera exigua*), and Cotton bollworm (*Heliocoverpa zea*) which damage cotton. Thus, it was considered that the modified Cry1F protein and the modified Cry1Ac protein are stably produced in this recombinant cotton.

The PAT protein has been used as a selectable marker for Cotton 281 and Cotton 3006, and it was confirmed to be tolerant to glufosinate in the T0 generation. In this recombinant cotton produced by crossing Cotton 281 and Cotton 3006, the glufosinate spraying test (on the F6 generation) in the isolated field tests in Japan confirmed that the PAT protein is stably produced.

In addition, expression of the modified Cry1F protein, modified Cry1Ac protein and PAT protein in various sites of this recombinant cotton was examined based on the ELISA analysis. In the analysis, samples of F3 generation raised in 2001 at 6 fields in the US (Arizona, California, Mississippi, North Carolina, and 2 sites in Texas) were tested. As a result, in the terminal leaf, flower bud, cottonseed boll, the whole plant and seed, the modified Cry1F protein, modified Cry1Ac protein and PAT protein were found expressed. The expression level of the modified Cry1F protein ranged from 0.91 ng/mg in the cottonseed boll to 40.21 ng/mg in the whole plant, and the expression level of the modified Cry1Ac protein was from less than the detection limit of cottonseed boll (0.025 ng/mg) to 3.04 ng/mg in the flower bud. In addition, the expression level of the PAT protein ranged from less than detection limit of terminal leaf and flower bud (0.056 ng/mg) to 0.85 ng/mg in the seed. In the pollen, the modified Cry1F protein and PAT protein were little expressed, but only the modified Cry1Ac protein was found expressed (1.02 to 2.46 ng/mg). Then, in order to examine the stability of expression of the modified Cry1F protein, modified Cry1Ac protein and PAT protein among generations, expression of the proteins in the seeds of F6 generation raised in 2003 at 6 fields in the US (Arizona, California, Mississippi, North Carolina, and 2 sites in Texas) was determined based on the ELISA analysis. As a result, it was confirmed that also in the F6 generation, the modified Cry1F protein (1.50 to 4.15 ng/mg), modified Cry1Ac protein (0.16 to 0.89 ng/mg) and PAT protein (0.22 to 1.02 ng/mg) were stably expressed. The fields examined are all typical cotton cultivation areas, which have such suitable climate conditions for cotton growth as at least 500 mm of rainfall per season and 160 days or more at temperatures exceeding 15℃, though the topographical conditions vary.

Based on the above understanding, it was considered that the modified Cry1F protein, modified Cry1Ac protein and PAT protein are stably expressed even in various environmental conditions.

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 recombinant cotton does not contain any sequence allowing transmission; therefore there is no possibility that the genes transferred to this recombinant 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 this recombinant cotton, 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 this recombinant cotton. The detection limit (detectable lower limit) of this method is 20 copies of PCR-amplified product, and this method has been confirmed reliable enough by the reproducibility among measurements, dilution test, and test for checking crossability with other recombinant cotton and non-recombinant cotton.

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

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)

It was confirmed that the modified Cry1F protein and modified Cry1Ac protein, which are encoded by the modified *cry1F* gene and modified *cry1Ac* gene, are expressed in various tissues of this recombinant cotton and thus the resistance to order Lepidoptera is conferred to this recombinant cotton.

In addition, it was also confirmed that the PAT protein, which is encoded by the *pat* gene used as a selectable marker, is also expressed in various tissues of this recombinant cotton and thus the tolerance to glufosinate herbicide is conferred to this recombinant cotton.

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

Isolated field tests were conducted at National Agricultural Research Center for Kyushu Okinawa Region (KONARC) in FY 2003, and the difference between this recombinant cotton and the non-recombinant control cotton was examined.

(a) Morphological and growth characteristics

Uniformity of germination was attained 6 days after sowing for both this recombinant cotton and the non-recombinant control cotton.

Regarding the plant height at individual stages of growth, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

Regarding the flowering characteristics, the number of flower buds varied widely between individuals, though no significant difference was observed between this recombinant cotton and the non-recombinant control cotton. The calyx consisted of three (3) large, heart-shaped sepals with the serrate edge. The number of petals was 5 with the lapped base. The number of pistils was one (1), and many stamens

grew from lower ovary, though they did not yet reach the stigma. The base of the ovary had 6 small nectary ducts, though giving off little fragrance. The flowers were in white color on the first day of flower opening for both this recombinant cotton and the non-recombinant control cotton, turned light pink on the second to third days, and withered on the third to fourth days and petals fell down. In the shape of flower organ, no difference was observed between this recombinant cotton and the non-recombinant control cotton.

Regarding the boll opening characteristics, the bolls became largest in size about one and a half month after flower opening, and the bolls opened for both this recombinant cotton and the non-recombinant control cotton. No difference was observed in the harvest time.

The shape of boll was found oval for both this recombinant cotton and the non-recombinant control cotton, and the bolls started to swell one week after flower opening. Regarding the number of harvested bolls per plant and the number of non-harvested bolls, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton, though the number of bolls harvested was larger in this recombinant cotton (19.2 bolls against 11.7 bolls). This was ascribable to the possible fact that the growth of the non-recombinant control cotton and the maturity of bolls were delayed due to the damage by cotton leafroller (*Notarcha derogata*) and Asian corn borer (*Ostrinia furnacalis*). Regarding the weight of boll, no difference was observed. The number of segments of a boll was 3 to 5 for both this recombinant cotton and the non-recombinant control cotton, and 21 to 33 seeds per boll were produced.

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

In order to evaluate the cold-tolerance at the early stage of growth of this recombinant cotton, 20 individuals at the 2-foliage leaf stage were put in a climate chamber set at 4℃ and 12-hour day length and examined for response over time. On the next day after putting in the climate chamber, cotyledons started to wither and discolor and then, 6 days after putting in the climate chamber, all the individuals died. Consequently, it was confirmed that seedlings of this recombinant cotton could not survive under the low-temperature conditions below 4℃ similarly as typical cotton.

(c) Wintering ability of the matured plant

The plants of this recombinant cotton and the non-recombinant control cotton cultivated in the open ground field at National Agricultural Research Center for Kyushu Okinawa Region (KONARC) died completely due to the exposure to low temperatures and frosting before the end of December, and the matured plants were hard to overwinter and no difference was observed between this recombinant cotton and the non-recombinant control cotton in the wintering ability.

(d) Fertility and size of the pollen

In Japan, there are no plans for seed of this recombinant cotton to be commercialized, and the cotton is not cultivated for commercial use. Therefore, if

this recombinant 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 recombinant 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. Cotton bolls become open normally 40 to 60 days after flower opening ("Encyclopedia of Agriculture" Yokendo Co., Ltd.). In the isolated field tests, comparison was made between this recombinant cotton and the non-recombinant control cotton for boll opening characteristics. As a result, it was found that this recombinant cotton and non-recombinant control are comparable to typical cotton in the time of cotton boll opening and the progress of cotton boll opening. In addition, it was also observed that after boll opening, the down of bolls of this recombinant cotton is similar to that of the non-recombinant control cotton as the fiber twines around seeds to cause the bolls to be difficult to separate. Therefore, it was considered that this recombinant cotton is equivalent to the non-recombinant control cotton in the shedding habit.

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℃ or more) in soil (OGTR 2002). In addition, in the field tests conducted at 20 sites in the US, no difference was observed between this recombinant cotton and the non-recombinant control cotton in dormancy of the seeds. Therefore, 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.

The germination rate was found 92% for this recombinant cotton and 98% for the non-recombinant control cotton, and no significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

(f) Crossability

Crossability of this recombinant cotton with the non-recombinant control cotton due to dispersion of pollens was examined. In the isolated field at National Agricultural Research Center for Kyushu Okinawa Region (KONARC), 180 grains

of seeds from the non-recombinant control cotton cultivated adjacent to this recombinant cotton, and 20 grains of seeds from this recombinant cotton were sown in a vinyl house. At the 2-foliage leaf stage, glufosinate solution of a 200-fold dilution factor (normally used concentration) was sprayed at 1.5cc/$m^2$, and 5 days after spraying, the number of surviving individuals was examined. As a result, 20 plants of this recombinant cotton examined could all survive, while 3 of 180 plants of the non-recombinant control cotton examined survived. In addition, as a result of glufosinate spraying test in which glufosinate solution of a 200-fold dilution factor was sprayed to the non-recombinant control cotton at 1.5 cc/$m^2$ in the isolated field test, all of five plants examined died. Therefore, it was considered that the 3 plants surviving in the tests were conferred with tolerance to glufosinate by crossing. Based on the above results, it was suggested that 1.7% crossing were produced due to wind- or insect-pollination, though there are reports that the occurrence of natural crossability of cotton is 2.0% (an average for 11 sites; range between 0 and 5.9%) (Umbeck *et al.* 1991); therefore the measured crossability was equivalent to that for typical cotton.

(g) Productivity of harmful substances

To compare the productivity of harmful substances of this recombinant cotton to the non-recombinant control cotton, succeeding crop test, plow-in test and soil microflora test were conducted.

**[Succeeding crop test]**

As a result of succeeding crop test of radish (test plant) using the soil cultivated with this recombinant cotton and the non-recombinant control cotton, regarding the number of germinated plants, plant height, root length and the live weight of above-ground part of radish (test plant), no statistically significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

**[Plow-in test]**

As a result of plow-in test by using the plant body of this recombinant cotton and the non-recombinant control cotton, regarding the number of germinated plants, plant height, root length, and the live weight of above-ground part of radish (test plant), no statistically significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

**[Soil microflora test]**

Soil at sowing time, in the growing period, and at harvest time was collected, and ATP biomass, the count of viable cells of microorganisms in soil, the number of actinomyces, the number of filamentous fungi, the number of *Pseudomonus* were determined. In addition, at harvest time, representative crop body was pulled out and microflora of lateral root was examined. As a result, in the soil at sowing time, regarding the count of viable cells (diluted bouillon media), statistically significant difference was observed between the recombinant zone and the control zone (recombinant zone: $1.21\times10^7$ CFU/g dry soil vs. control zone: $8.38\times10^6$ CFU/g dry soil), though the difference was found within normal variation and thus,

it was judged that the soil was appropriate for tests. In the soil during the growing period, in all items examined, no statistically significant difference was observed between the recombinant zone and the control zone. Also in the soil at harvest time, in the microflora nearby lateral roots, no difference was detected between the recombinant zone and the control zone.

Based on the above results, regarding the productivity of harmful substances, it is considered that this recombinant cotton could not produce any unexpected harmful substances.

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

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

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

(1) Competitiveness

The plant body of cotton species (*Gossypium hirsutum* L.), to which the recipient organism belongs, dies during winter seasons in Japan. Moreover, the level of seed dormancy is extremely low. Therefore, it is considered unlikely that cotton becomes self-seeding in Japan. Cotton has long been distributing as seed cotton in Japan, however, there has been no report that cotton becomes self-seeding in Japan.

As a result of confined field test in Japan, with regard to various traits relating to competitiveness, in all items examined, no significant difference was observed between this recombinant cotton and a non-recombinant control cotton line.

This recombinant cotton is given the traits to be resistant to order Lepidoptera due to the transferred modified *cry1F* gene and modified *cry1Ac* gene, and to be tolerant to herbicide glufosinate due to the *pat* gene. However, the damage by Lepidoptera is not the main factor to inhibit the cotton growth under natural environment in Japan. In addition, it is highly unlikely that glufosinate will function as a selective pressure under the natural environment; therefore it is not likely to consider that this recombinant cotton becomes competitiveness and could grow and become self-seeding in Japan.

Based on the above understanding, no wild animals and wild plants that can be affected by this recombinant cotton is specified, 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

There has been no report that cotton, to which the recipient organism belongs, produces any harmful substances that could affect wild animals and wild plants.

This recombinant cotton produces PAT protein that possesses the tolerance to glufosinate herbicide; however, it has not been reported that this protein is harmful. In addition, PAT protein is a member of enzymes that specifically acetylate the glufosinate herbicide and transform it into acetyl glufosinate and it has high substrate specificity and thus it is unlikely to affect any other metabolic systems.

In addition, this recombinant cotton produces the modified Cry1F and Cry1Ac protein,

which may arise a concern that the pollen grains of this recombinant cotton affect the order of non-target Lepidoptera insects; however, as discussed in the section of "Competitiveness," it is considered unlikely that this recombinant cotton could grow and become self-seeding in Japan. Even if this recombinant cotton (seeds) grew after spilled and produced flowers, the pollen grainss of cotton are relatively heavy and sticky and thus, they are unlikely to disperse. Therefore, it is considered unlikely that non-target insects that do not eat cotton are exposed to the pollens of this recombinant cotton.

In addition, in the confined field tests in Japan, the productivity of harmful substances (including secretion from roots to affect the other plants, secretion from roots to affect microorganisms in soil, and substances in the plant body to affect the other plants after dying out) has been investigated, but no significant difference has been observed between this recombinant cotton and the non-recombinant control cotton.

Therefore, it is not likely to consider that this recombinant cotton becomes dominant over the recipient organism cotton and affect wild animals and wild plants.

Based on the above understanding, no wild animals and wild plants that can be affected by this recombinant cotton is specified , the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances 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 above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant cotton in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above is valid.

**Annex List**

Annex 1: Insecticidal spectrum and potential effects on non-target organisms of the modified Cry1F protein and modified Cry1Ac protein
(Not made available or disclosed to unauthorized person)

Annex 2: Nucleotide sequence and flanking region sequence of transferred genes to Cotton 281
(Not made available or disclosed to unauthorized person)

Annex 3: Nucleotide sequence and flanking region sequence of transferred genes to Cotton 3006
(Not made available or disclosed to unauthorized person)

Annex 4: Separation of traits in F2 generation
(Not made available or disclosed to unauthorized person)

Annex 5: Identification of the number of copies of transferred nucleic acid and stability of its inheritance through multiple generations
(Not made available or disclosed to unauthorized person)

Annex 6: Methods for detection of this recombinant cotton
(Not made available or disclosed to unauthorized person)

Isolated field test report:

Safety Assessment Test Report on Cotton resistant to Lepidoptera and tolerant to glufosinate herbicide in isolated field
(Not made available or disclosed to unauthorized person)

1. Objectives
2. Test method
    1) Test sample
    2) Description of tilling and sowing
    3) Test design
3. Description of progress of test
    1) Description of weather
    2) Description of growth
4. Test data
    1) Morphological and growth characteristics
    2) Identification of expression of introduced genes
    3) Natural crossability
    4) Weediness
    5) Difference in sensitivity to pests
    6) Visiting insect fauna (Flowering period)
    7) Productivity of harmful substances
5. Conclusion