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 (Modified *vip3A*, *Gossypium hirsutum* L.) (COT102, OECD UI: SYN-IR102-7) |
| Content of the Type 1 Use of Living Modified Organism | Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them |
| Method of the Type 1 Use of Living Modified Organism | – |

**Outline of the Biological Diversity Risk Assessment Report**

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

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

### (1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of cotton resistant to Lepidoptera (modified *vip3A*, *Gossypium hirsutum* L.) (COT102, OECD UI: SYN-IR102-7) (hereinafter referred to as "this recombinant cotton") are shown in Table 1.

**Table 1 Composition of the donor nucleic acid pCOT1 used for the development of this recombinant cotton and the origins and functions of component elements**

| Component elements | Size (bp) | Origin and function |
|---|---|---|
| Insect pest-resistant gene cassettes | | |
| Act2 promoter | 1,408 | Promoter region from the *actin* gene (*actin-2* gene) of *Arabidopsis thaliana*, including the first exon and intron (Reference 20). Involved in the constant expression of the target gene (modified *vip3A* gene). |
| Modified *vip3A* gene | 2,370 | A modified version of the native *vip3A* gene found in the *Bacillus thuringiensis* strain AB88, a gram-positive bacteria existing normally in soil (Reference 21), to accommodate the preferred codon usage in plants (Reference 22). The *vip3A* gene encodes the modified Vip3A protein which exhibits insecticidal activity against the insect of order Lepidoptera. In the modified Vip3A protein, the amino acid at position 284 in the amino acid sequence was substituted to glutamine from lysine. |
| NOS terminator | 253 | Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens*. Its function is to terminate transcription of mRNA by polyadenylation (Reference 23). |
| Transformed cell selective marker gene cassettes | | |
| Ubq3 promoter | 1,721 | Promoter region including the first intron from the *polyubiquitin* gene (*ubi3*) of *A. thaliana* (Reference 24). Involved in the constant expression of target gene (*aph4*). |
| *aph4* gene | 1,026 | Phosphotransferase (hygromycin B phosphoferase) gene derived from *Escherichia coli*. Catalyzes the phosphorylation of hygromycin and some related aminoglycosides (Reference 25), thereby conferring resistance to hygromycin. Serves as a selective marker for transformed cells for development of this recombinant cotton. |

| | | |
|---|---|---|
| NOS terminator | 253 | Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Reference 23). Its function is to terminate transcription of mRNA by polyadenylation. |
| Other regions (Plasmid backbone region) | | |
| LB | 25 | Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 26). |
| *spec* | 789 | Streptomycin adenylyltransferase gene (*aadA*) from *E. coli* transposon Tn7 (Reference 27). Used as a vector selectable marker to confer resistance to erythromycin, streptomycin, and spectinomycin. |
| *repA* | 1,074 | Replicon (a part of the minimal function replication unit to control the replication of DNA) region of plasmid pVS1 derived from *Pseudomonas* bacteria. Essential gene for maintenance of vector in *A. tumefaciens* (Reference 28). |
| VS1 ori | 405 | The replication origin consensus sequence derived from the plasmid pVS1 of *Pseudomonas* bacteria. Functions as the replication origin of plasmid in *A. tumefaciens* (Reference 29). |
| ColE1 ori | 807 | Origin of replication of plasmid in *E. coli* (Reference 30). |
| RB | 25 | Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 31). |

2) Function of component elements

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

Functions of component elements of donor nucleic acids used for the development of this recombinant cotton are listed in Table 1.

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

Modified Vip3A protein

The Cry protein derived from *Bacillus thuringiensis*, exhibiting insecticidal activity, is produced during the spore forming period of *B. thuringiensis* and inherent in the cells. In contrast, the Vip protein, to which the modified Vip3A protein expressed in this recombinant cotton belongs, has been discovered as Vegetative Insecticidal Protein produced during vegetative growth of *B. thuringiensis* and secreted outside cells (Reference 21). As the Vip protein, Vip1, Vip2 and Vip3 proteins have been identified and they are classified into 3 ranks and 7 divisions by the *Bacillus Thuringiensis* Nomenclature Committee. The Vip1 and Vip2 proteins exhibit the insecticidal activity against the insects of order Coleoptera, and the Vip3 protein exhibits its insecticidal activity against the insects of order Lepidoptera.

The Vip3A protein has a total length of 88kDa, though the Vip3A protein, when fed by larvae of the target insects of order Lepidoptera, is partially digested in the digestive tracts and becomes a core protein having a length of 62kDa. It is known that the core protein binds to the specific receptors on the intestinal epithelium cells of target insects, causing disturbed ionic balance leading to destructed intestinal epithelium cells and resultantly inhibited digestive process, which contributes to insecticidal activity (Reference 32, Reference 33). This mechanism of action is also attained similarly in the Cry protein. In addition, Lee and his colleagues (Reference 33) have reported that the Vip3A protein and the Cry1Ab protein bind to the brush border membrane vesicles (BBMV) of mid-gut epithelium without any conflict with each other, and they also have revealed that the Vip3A protein does not bind to any aminopeptidase-like and cadherin-like molecules, which are known as the receptors of Cry1Ab protein, in the BBMV of Tobacco Hornworm (*Manduca sexta*), a sensitive species of the insects of order Lepidoptera (Reference 33). Consequently, it is suggested that the Vip3A protein provides the similar mechanism of action as the Cry protein, though the Vip3A protein differs from Cry1Ab protein regarding the receptors involved (Reference 33).

The Vip3A protein has been confirmed to exhibit the insecticidal activity against Cotton bollworm (*Helicoverpa armigera*), Tobacco budworm (*Heliothis virescens*) and other order Lepidopteran insects which are the pest insects of order Lepidoptera for cultivation of cotton in the US. On the other hand, the Vip3A protein has been confirmed not to exhibit any insecticidal activity against honeybees (*Apis mellifera*), which can transmit pollens of cotton, and European corn borer (*Ostrinia nubilalis*) and Monarch butterfly (*Danaus plexippus*), the insects of order Lepidoptera, against which the Cry1Ab protein exhibits its insecticidal activity.

Moreover, it has been also confirmed based on the homology search using the publicly available protein database (SWISS-PROT, FARRP, etc.) that the amino acid sequence in the modified Vip3A protein does not have any homology with known allergens and toxins examined.

APH4 protein

The *aph4* gene, derived from *E. coli*, encodes the APH4 protein which is a hygromycin B phosphoferase. The APH4 protein phosphates and detoxicates the hygromycin and thus the cells containing this protein exhibits resistance to hygromycin (Reference 34). Then the *aph4* gene was used as a selective marker for gene-transferred cells. The APH4 protein has high substrate specificity, and it is known to phosphate the aminoglycoside antibiotics Hygromycin B and Hygromycin B2, and also Destomycin A and Destomycin B which are similar to each other in the structure, though it does not phosphate any other aminocyclitol and aminoglycoside antibiotics (neomycin, streptomycin, gentamicin, kanamycin, spectinomycin, tobramycin, amikacin, etc.) (Reference 34, Reference 35).

For the safety of APH4 protein, the US Environmental Protection Agency (EPA) has approved exemption of this protein from the requirement of establishment of a pesticide residue tolerance (Reference 36).

Moreover, it has been also confirmed based on the homology search using the publicly available protein database (SWISS-PROT, FARRP, etc.) that the amino acid sequence in this protein does not have any homology with known allergens and toxins examined.

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

The modified Vip3A protein expressed due to the modified *vip3A* gene is reported unlikely to possess any enzyme activity and thus, it is considered to function independently from the metabolic system of recipient organism. In addition, the APH4 protein expressed due to the *aph4* gene is an enzyme which phosphates hygromycin B and some relative aminoglycoside antibiotics, though it has very high substrate specificity and there is no substance identified in plant body that could become any substrate (Reference 38); therefore, it is considered extremely low that the proteins could affect the metabolic system of plants.

Based on the above understanding, it is considered unlikely that the transferred genes could affect the metabolic system of recipient organism.

**(2) Information concerning vectors**

1) Name and origin

For the development of this recombinant cotton, the vectors pCOT1 were used. This vector was constructed based on the pBluescript II SK(+) derived from *E. coli*.

2) Properties

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

The total number of base pairs of the vector pCOT1 is 11,801 bp, and the nucleotide sequences have been disclosed.

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

The vector pCOT1 contains the *spec* gene which expresses the resistance to streptomycin, erythromycin, and spectinomycin as a selective marker for growth of vector in microorganisms, though the gene is not transferred in this recombinant cotton.

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

The vectors contain no sequence that exhibits the infectivity.

**(3) Method of preparing living modified organisms**

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

Two gene expression cassettes (modified *vip3A* gene cassette and *aph4* gene cassette) between RB and LB of T-DNA region of the vector pCOT1 used for the production of this recombinant cotton are transferred in the recipient organism.

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

*Agrobacterium* method was used to transfer the T-DNA region of the vector pCOT1 to the petiole tissues of cotton.

3) Processes of rearing of living modified organisms

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

Transferred genes were cultured on the medium containing the antibiotic hygromycin to select the cells which express the APH4 protein.

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

The antibiotic Cefotaxime was added to the medium for selective culture of transformed cells to remove any residual *Agrobacterium* used for transformation. Then, transformed cells were cultured on the medium containing no Cefotaxime and it was confirmed that there is no remaining *Agrobacterium*.

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

From the cells selected after transferring of genes, plant body was regenerated and conditioned then cultivated in a greenhouse. Then, plant body was analyzed based on the TaqMan PCR to select the individuals that contain the modified *vip3A* gene and the *aph4* gene. Through the self-pollination or backcrossing of the selected individuals with an elite species of cotton, the progeny was raised. The progeny was used for collection of necessary information for assessment of Adverse Effect on Biological Diversity.

Regarding this recombinant cotton, Type I Use (cultivation, storage, transportation, disposal and acts incidental to them in isolated fields) in accordance with the "Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms" was approved in May 2007 by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment. In addition, application for approval of safety of use for food and application for approval of safety of use for feed will be made sequentially to the Ministry of Health, Labour, and Welfare and the Ministry of Agriculture, Forestry and Fisheries, respectively.

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

1) Place where the replication product of transferred nucleic acid exists (on the chromosome, in the cell organelle, or in the protoplasm)

Based on the findings that the transferred genes in this recombinant cotton are stably inherited across multiple generations in accordance with the law of Mendelian inheritance, the transferred nucleic acid is considered to exist on the chromosome.

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

Southern blotting analysis was conducted using the T-DNA region and the backbone region of the vector pCOT1 as probes by breaking the genome DNA extracted from multiple generations of this recombinant cotton through the restriction enzyme treatment. As a result, when the T-DNA region of the vector pCOT1 was used as a probe, the identical band was detected, suggesting that one copy of T-DNA is transferred in multiple generations. Consequently, it was indicated that one copy of the modified *vip3A* gene cassette and the *aph4* gene cassette is stably inherited across multiple generations, and it was confirmed that the transferred genes in individual generations are identical to each other.

When the backbone region of the vector pCOT1 was used as a probe, no band was detected in all generations examined.

Based on the above results, it was confirmed that one copy of T-DNA region of the vector pCOT1 is transferred in the genome in this recombinant cotton at one site and it is stably inherited in offspring.

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

In 2001, in the fields in the US at 3 sites, this recombinant cotton was cultivated, and individual tissue samples were taken at individual growth stages to determine the level of expression of the modified Vip3A protein and the APH4 protein based on the ELISA method. Pollen and nectar samples were collected from the plant body cultivated in greenhouses. As a result, the modified Vip3A protein was detected in leaves, roots, flower buds, bolls, pollens, and seeds, though not detected in lint and nectar. In addition, in 2006, the average expression level of the modified Vip3A protein was examined based on the ELISA method using the leaf samples taken from three generations of this recombinant cotton cultivated in a greenhouse at Syngenta Corporation in the US and as a result, it was confirmed that the modified Vip3A protein was stably expressed across the generations. For the APH4 protein, the level of expression was found below the limit of quantification, though the expression was observed in all the generations examined.

Based on the above understanding, it was confirmed that the modified Vip3A protein and the APH4 protein in this recombinant cotton are stably expressed across individuals and through generations.

4) 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

The transferred nucleic acid does not contain any sequence allowing transmission. Therefore, it is considered unlikely that the nucleic acid transferred to this recombinant

cotton could be transmitted to any other wild animals and wild plants.

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

Existence of the target genes in this recombinant cotton can be confirmed based on the results of Southern blotting analysis using the modified *vip3A* gene as a probe after breaking the genome DNA by the restriction enzyme. In addition, a method for specific detection of this recombinant cotton was developed based on the nucleotide sequence of transferred genes and the nucleotide sequence of the neighboring genome.

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

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

This recombinant cotton is given the trait to be resistant to Lepidoptera due to the modified Vip3A protein that is expressed by the modified *vip3A* gene, and the trait to be a selective marker due to the APH4 protein that is expressed by the *aph4* gene. This recombinant cotton, which expresses the modified Vip3A protein, exhibits the Lepidoptera resistance to Cotton bollworm (*Helicoverpa armigera*), Tobacco budworm (*Heliothis virescens*) and other pest insects of the order Lepidoptera, which are the pest insects for cultivation of cotton in the US.

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

In 2007, isolated field tests were carried out at the Kanza Site of Central Research Station, R&D Division, Syngenta Japan K.K. using this recombinant cotton and the non-recombinant control cotton.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, examination was conducted regarding the progress of germination, germination rate, flowering date, flower shape and petal color, leaf length and width, boll length and width, boll opening time (boll opening date of the first boll), the color of fiber, harvest time (the day on which the plant lost the vigor and leaf drop was observed), plant height, the number of nodes, the number of flower buds, the total number of branches, the number of bolls harvested per plant, the total number of bolls per plant, the number of segments of a boll, the number of seeds per boll, seed color and shape, fresh weight of a boll, and the weights of above- and under-ground parts at the harvest time. For the germination rate, leaf length and leaf width, boll length and width, plant height, the number of nodes, the number of flower buds, the total number of branches, the number of bolls harvested per plant, the total number of bolls per plant, the number of seeds per boll, fresh weight of a boll, and the weights of above- and under-ground parts at the harvest time, statistical treatment was conducted. As a

result, in all the items examined, no significant difference or difference was observed between this recombinant cotton and the non-recombinant control cotton.

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

The first leaf stage seedlings of this recombinant cotton and the non-recombinant control cotton were cultivated in the low-temperature conditions representing the winter season to compare the severity of damage due to the low temperature stress. As a result, this recombinant cotton and the non-recombinant control cotton both died completely, and there was no difference observed in the rate of death between the both plants.

(c) Wintering ability of the matured plant

The plant bodies of this recombinant cotton and the non-recombinant control cotton cultivated in the isolated field tests shed the leaves and died due to the low temperatures and frosting in the winter season. Based on the above findings, it was judged that there would be no difference between this recombinant cotton and the non-recombinant control cotton regarding the wintering ability of the matured plant.

(d) Fertility and size of the pollen

Pollens were collected from this recombinant cotton and the non-recombinant control cotton, and observed under a microscope for comparison of fertility, shape and size of pollens. As a result, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton regarding the fertility of pollen stained with Acetocarmine solution. In addition, the shape of pollen was found circular for the both plants, and no significant difference was observed in the diameter of pollen between the both plants.

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

Regarding seed production, comparison was made for the total number of bolls per plant and the number of seeds per boll and as a result, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

Regarding shedding habit of the seed, examination was made for the presence or absence of seeds shed naturally from open bolls at the harvest time and the number of seeds shed. As a result, no seed shed was identified in both this recombinant cotton and the non-recombinant control cotton, showing no difference in the shedding habit between the both plants.

The germination rate was found 97% or more for the seeds from both this recombinant cotton and the non-recombinant control cotton, showing no significant difference between the both plants.

It is generally known that the level of seed dormancy of cotton is low (Reference 12) and then, the test concerning dormancy was not conducted.

(f) Crossability

In Japan, no wild relatives exist which can cross with tetraploid cotton cultivar (*Gossypium hirsutum*) to which this recombinant cotton belongs. Thus, crossability was not assessed.

(g) Productivity of harmful substances

Regarding the productivity of harmful substances of this recombinant cotton, the following evaluation tests were carried out in the isolated fields.

Plow-in test:
From each plant body, above-ground parts (leaves and stems) were harvested, and dried and powdered then mixed with soils, to which the seeds of radish were sown as test plant. As a result of determination of germination rate and dry weight, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton regarding the germination rate and dry weight of radish.

Succeeding crop test:
To each soil after cultivating the plant body, the seeds of radish were sown and the seedlings were cultivated in a greenhouse. As a result of determination of germination rate and dry weight, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton regarding the germination rate and dry weight of radish.

Soil microflora test:
At the time of harvesting of this recombinant cotton and the non-recombinant control cotton, soil was sampled from the cultivation field to measure the number of colonies of filamentous fungi, bacteria and Actinomyces for the microorganisms in soil based on the dilution plate technique. As a result, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

## 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 "Act on 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

Cotton (*Gossypium hirsutum* L), the biological species to which the recipient organism belongs, has been long used in Japan, though there is no report that it has become self-seeding in Japan.

This recombinant cotton is given the trait to be resistant to insects of the order Lepidoptera due to the transferred modified *vip3A* gene. However, it is considered that the insect damage by Lepidoptera is not the major factor making the cotton difficult to grow in the natural environment in Japan and also that the given Lepidoptera resistance would not affect the competitiveness. Consequently, it is considered unlikely that the given traits would allow the cotton, a cultivated crop, to grow wildly in the natural conditions and also enhance the competitiveness.

As a result of isolated field tests in Japan, regarding the characteristics relating to competitiveness, no significant difference was observed between this recombinant cotton and the non-recombinant control cotton.

Moreover, with the expression of APH4 protein due to the transferred *aph4* gene, this recombinant cotton is given the tolerance to some aminoglycoside antibiotics, though the trait is considered unlikely to enhance the competitiveness.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant cotton poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

#### (2) Productivity of harmful substances

For the cotton, the biological species to which the recipient organism belongs, there is no report that it produces any harmful substances such as allelochemicals that could affect wild animals and wild plants.

This recombinant cotton produces the modified Vip3A protein which possesses insecticidal activity against pest insects of the order Lepidoptera and the APH4 protein which confers the tolerance to some aminoglycoside antibiotics, though neither of the proteins has been confirmed to have amino acid sequence homology with any known

allergen and toxin.

The modified Vip3A protein is considered unlikely to have any enzyme activity, and the APH4 protein has very high substrate specificity and there is reportedly no substance that can become any substrate in plant body.

Consequently, it is considered unlikely that these proteins would affect the metabolic system of recipient organism and produce any harmful substances. There is a concern about possible impacts of pollens of this recombinant cotton on the non-target species of insects of order Lepidoptera, though the pollens of cotton are relatively heavy and viscous and then, they are considered unlikely to disperse. Even if the pollens of cotton disperse, the extent of dispersing is considered extremely limited. In addition, there is no report that imported cotton seeds were spilled and then cotton grew or became self-seeding in the natural conditions in Japan.

In addition, as a result of succeeding crop tests, plow-in tests and soil microflora tests carried out in the isolated field to examine the production of harmful substances of this recombinant cotton (the substances excreted from the roots which can affect other plants, the substances existing in the plant body which affect other plants after dying, and the substances excreted from the roots which can affect microorganisms in soil), no statistically significant difference was observed between this recombinant cotton and the non-recombinant control cotton in the productivity of all possible harmful substances.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected, if cannot be specified and that the use of this recombinant cotton poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

**(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 recombinant cotton in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Bibliography