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

Name: Bayer CropScience K.K.
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (modified *bar*, *cry2Ae*, *Gossypium hirsutum* L.) (GHB119, OECD UI:BCS-GHØØ5-8)
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 Effects on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Component elements of the donor nucleic acid used for the development of cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (modified *bar*, *cry2Ae*, *Gossypium hirsutum* L.) (GHB119, OECD UI:BCS-GHØØ5-8) (hereinafter referred to as "GHB119") are shown in Table 1.

In addition, the amino acid sequences of the modified PAT protein expressed by the modified *bar* gene and the Cry2Ae protein expressed by the *cry2Ae* gene are shown in Annex 1 (Confidential:Not made available or disclosed to unauthorized persons).

Table 1 Position, size, origin, and function of component elements in vector pTEM12

Component elements	Position in vector	Size (bp)	Origin and function
Modified *bar* gene expression cassette			
3′nos	26 - 335	310	A sequence including the 3′ untranslated region of the nopaline synthase gene derived from pTiT37 of *Agrobacterium tumefaciens* (Depicker *et al*., 1982). It terminates the transcription and causes the 3′ polyadenylation.
Modified *bar*	336 - 887	552	A sequence including the gene encoding phosphinothricin acetyltransferase (PAT protein) derived from *Streptomyces hygroscopicus* (Thompson *et al*., 1987). It confers tolerance to glufosinate herbicide. Two codons at the N-terminal of the wild *bar* gene have been replaced with ATG and GAC, respectively.
PcsvmvXYZ	888 - 1423	536	The promoter region of Cassava Vein Mosaic Virus 35S RNA gene (Verdaguer *et al*., 1996). It initiates constitutive transcription.
cry2Ae gene expression cassette			
P35S2	1424 - 1920	497	A sequence including the promoter region of Cauliflower Mosaic Virus 35S RNA gene (Odell *et al*., 1985). It initiates constitutive transcription.
5′cab22L	1921 – 1990	70	A sequence including the leader sequence of the chlorophyll

			a/b binding protein gene derived from *Petunia hybrida* (Harpster *et al*., 1988). It increases the expression level of the *cry2Ae* gene.
TPssuAt	1991 – 2155	165	The coding region of the RuBisCo small subunit transit peptide gene (*ats1A*) derived from *Arabidopsis thaliana* (De Almeida *et al*., 1989).
cry2Ae	2156 – 4051	1896	The coding region of the insect-resistance gene derived from *Bacillus thuringiensis* subsp. *dakota*. It confers resistance to Lepidoptera. The nucleotide sequence of this gene was modified to enhance its expression in cotton, but the amino acid sequence remains unchanged (Arnaut *et al*., 2005).
3′35S	4052 – 4320	269	A region containing the 3′ untranslated region of the Cauliflower Mosaic Virus 35S RNA gene (Sanfaçon *et al*., 1991). It terminates transcription.
Others			
RB	4321 - 4345	25	A right border repetitive sequence derived from *A. tumefaciens* T-DNA (Zambryski, 1988).
—	4346 - 4537	192	A fragment derived from plasmid pTiAch5 (Zhu *et al*., 2000).
*npt*I-fragment	4538 - 5248	711	A fragment of transposon Tn903-derived *nptI* gene that encodes neomycin phosphotransferase (Oka *et al*., 1981). This sequence does not function because it is a fragment.
ORI ColE1	5249 - 6421	1173	A sequence including the replication origin of plasmid pBR322 derived from *Escherichia coli* (Bolivar *et al*., 1977).
ORI pVS1	6422 - 10192	3771	A sequence including the replication origin (Hajdukiewicz *et al*., 1994) of plasmid pVS1 derived from *Pseudomonas aeruginosa* (Itoh *et al*., 1984).
aadA	10193 - 11961	1769	A sequence including *E. coli*-derived gene that confers the tolerance to aminoglycoside antibiotics (Fling *et al*., 1985).
—	11962 - 12266	305	A fragment derived from plasmid pTiAch5 (Zhu *et al*., 2000).
LB	1 - 25	25	A left border repetitive sequence derived from *A. tumefaciens* T-DNA (Zambryski, 1988).

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

2) Functions 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 individual component elements of the donor nucleic acid are shown in Table

1 (p. 2-3).

(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

Modified PAT protein

In the process of nitrogen metabolism, plants produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. In detoxification of the produced ammonia, glutamine synthase plays a pivotal role, although the enzyme is inhibited in plants sprayed with glufosinate herbicide, resulting in ammonia accumulation and death of the plants.

The modified PAT protein (phosphinothricin acetyltransferase) expressed by the modified *bar* gene can acetylate glufosinate to produce *N*-acetyl-glufosinate, which is not toxic to plants, thereby inactivating the inhibitory action of glufosinate on glutamine synthase (OECD, 1999). This mechanism prevents ammonia accumulation, resulting in survival of the plants, even if they were sprayed with glufosinate herbicide.

The modified PAT protein exhibits a high affinity to glufosinate. Glufosinate is classified into L-amino acid, though it does not cause any acetyl group transfer to other various amino acids. It has little affinity to glutamic acid, which has a particularly high structural similarity to glufosinate, and causes virtually no transfer reaction *in vivo* (Thompson *et al.*, 1987). In addition, even in the presence of excessive amounts of various amino acids, the acetyl group transfer reaction to glufosinate by the modified PAT protein was not inhibited (Wehrmann *et al.*, 1996). Consequently, it is considered that the modified PAT protein possesses a high substrate specificity to glufosinate.

In 2011, amino acid sequence homology of the modified PAT protein was explored in known allergens registered in the database (AllergenOnline). As a result, no homology was found.

The modified *bar* gene is identical to the gene introduced into cotton tolerant to

glufosinate herbicide (LLCotton25) (OECD UI: ACS-GHØØ1-3), which has already been granted an approval for Type 1 Use in Japan in February 2006.

Cry2Ae protein

The Cry2Ae protein encoded by the *cry2Ae* gene introduced into GHB119 is an insecticidal protein (Bt protein) comprising 632 amino acids and having a molecular weight of 71 kDa. The Cry2Ae protein contains the active region (core protein) and 43 amino acids at the N-terminal of the wild-type Cry2Ae protein isolated from *B. thuringiensis* subsp. *dakota*, a gram-positive bacteria ubiquitously existing in soil. The *cry2Ae* gene introduced into GHB119 has a modified codon for enhancing its expression in cotton, but the amino acid sequence expressed by the modified codon remains unchanged (Arnaut *et al.*, 2005).

The Cry2Ae protein exhibits insecticidal activity against tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), old world bollworm (*Helicoverpa armigera*), pink bollworm (*Pectinophora gossypiella*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), and other insect pests of the Lepidoptera (Arnaut *et al.*, 2005; Annex 7: confidential: Not made available or disclosed to unauthorized persons). When ingested by a target insect, the Cry2Ae protein, like any other Bt protein, is digested by a specific protease in the midgut to become an active polypeptide (core protein). The core protein binds to specific receptors on the brush border membrane vesicles (BBMV) on the midgut epithelium, causing formation of ion channels on the midgut columnar cells (Chen *et al.*, 1995), loss of homeostasis, induction of sepsis, and, ultimately, death of the insect (Knowles and Dow, 1993; Broderick *et al.*, 2006).

It has been confirmed that the Cry2Ae protein does not affect the growth or survival of honeybee (*Apis mellifera*) and ladybug (*Coleomegilla maculate*) (Annex 7: Confidential: Not made available or disclosed to unauthorized persons). In addition, in the U.S. studies conducted in 2007 and 2008, it has been confirmed that the Cry2Ae protein has low risk to other non-target insects including green lacewing (*Chrysoperia rufilabris*), springtail (*Folsomia candida*), and water flea (*Daphnia magna*) (Table 2, p. 6). Moreover, the Cry2Ae protein and other Bt proteins are unlikely to affect humans and other mammals, because mammalian digestive organs have proteases and acidic digestive fluids that can digest the Bt proteins or their core proteins and lack the receptors that can bind to these

core proteins.

In 2011, amino acid sequence homology of the Cry2Ae protein was explored in known allergens registered in the database (AllergenOnline). As a result, no homology was found.

Table 2 Evaluation of effects of the Cry2Ae protein on non-target insects

Species	Growth stage	Evaluated items	Results
Green Lacewing (*Chrysoperia rufilabris*) (Neuroptera)	Larva	Mortality	NOEC[1] 27 μg/g[2]
Springtail (*Folsomia candida*) (Collembola)	Larva	Mortality, reproduction	No mortality at 44 μg/g
Water flea (*Daphnia magna*) (Diplostraca)	Immature	Fatality, development, reproduction	NOEC 330 μg/L

[1]: NOEC: No effect concentration
[2]: Mean value of the amount of Cry2Ae protein in feed determined by ELISA

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

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

Modified PAT protein

The modified PAT exhibits a high substrate specificity to glufosinate and is unlikely to cause any acetyl group transfer to compounds other than glufosinate. Moreover, *N*-acetyl-glufosinate, which is the metabolite of glufosinate, does not inhibit glutamine synthase (OECD, 2002) and is therefore unlikely to affect the metabolic system of the recipient organism.

Cry2Ae protein

There is no report that Bt proteins possess any enzyme activities, and the Cry2Ae protein is likely to work independently from the metabolic system of the recipient organism. Thus, it is considered that these proteins are unlikely to affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

The plasmid pTEM12 used for the development of GHB119 was constructed based on pGSC1700 (Cornelissen and Vandewiele, 1989) (Figure 1, p. 8).

2) Properties

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

The total number of base pairs in the plasmid pTEM12 is 12,266 bp.

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

The plasmid pTEM12 possesses the following sequences outside the T-DNA region; these sequences are located outside the T-DNA region and are not introduced into GHB119 (T3: Figure 2, b, p. 10), which has been confirmed by Southern blotting analysis (Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

- The replication origin derived from the plasmid pBR322 of *E. coli* (ORI ColE1) (Bolivar *et al.*, 1977) and the replication origin of the plasmid vector pVS1 of *P. aeruginosa* (ORI pVS1) (Hajdukiewicz *et al.*, 1994), each of which functions to cause autonomous replication in *E. coli* and *A. tumefaciens*, respectively
- A sequence including a gene conferring tolerance to aminoglycoside antibiotics (*aadA*) derived from *E. coli* (Fling *et al.*, 1985), which was used as a selective marker in *E. coli* and *A. tumefaciens*

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

The plasmid pTEM12 does not have any transmissible element and thus has no infectivity.

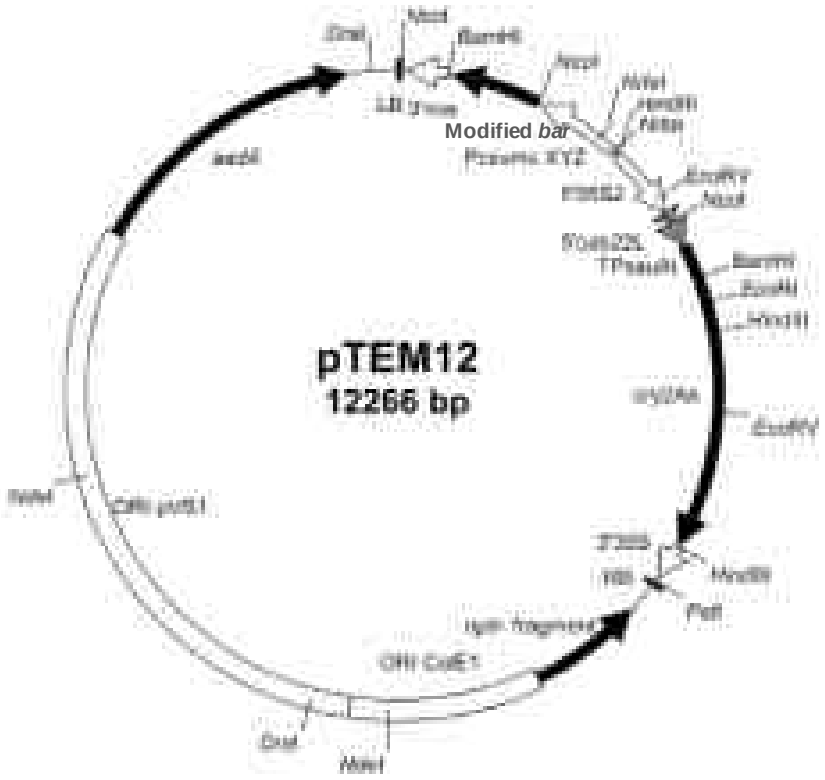

Figure 1 pTEM12 vector map

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

(3) Method of preparing living modified organisms

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

The *cry2Ae* gene expression cassette and the modified *bar* gene expression cassette ([3′nos]-[modified *bar*]-[Pcsvmv XYZ]-[P35S2]-[5′cab22L]-[TPssuAt]-[*cry2Ae*]-[3′35S]) between LB and RB on the plasmid pTEM12 were transferred into the recipient organism (Figure 1, p. 8).

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

The *Agrobacterium* method (Deblaere *et al.*, 1985) was used for transferring the nucleic acid to the recipient organism. Transformation was conducted through co-cultivation, by exposing the embryogenic callus induced from hypocotyls of the recipient cotton cultivar Coker312 to a liquid culture of non-oncogenic *A. tumefaciens* $C58C1^{Rif}$ strain (Van Larebeke *et al.*, 1974) carrying the non-oncogenic helper Ti plasmid pEHA101 and pTEM12.

3) Processes of breeding of living modified organisms

(a) Mode of selection of the cell in which nucleic acid is transferred

Tissue segments into which the nucleic acid was transferred were cultured on the regeneration medium containing 500 mg/L claforan. The regenerated plants were selected based on glufosinate tolerance.

(b) Presence or absence of remaining *Agrobacterium* when the method of transferring nucleic acid is based on *Agrobacterium* method

After transferring the nucleic acid, the transformants were cultured on a medium containing 500 mg/L claforan to remove any residual *Agrobacterium* used for transformation. Then, the regenerated plants were cultured on claforan-free medium to confirm the absence of residual *Agrobacterium*.

(c) Process of breeding 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 Effects on Biological Diversity

The selected plants were transplanted into pots and cultivated in a greenhouse. Then, the plants were selected based on glufosinate tolerance to obtain the original generation (T0) of GHB119. Elite lines were selected based on glufosinate tolerance, agronomic traits, etc. The process of breeding of GHB119 is shown in Figure 2. This application includes the T3 and F1 generations and the progenies of the T3 and F1 generations.

In February 2011, an application for the safety evaluation as food was submitted to the Ministry of Health, Labour and Welfare, and an application for safety evaluation as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries.

[Confidential: Not made available or disclosed to unauthorized persons]

Figure 2 Process of breeding of GHB119

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

(a) Place where the replication product of transferred nucleic acid exists

Examination was done on the segregation ratio between glufosinate herbicide-tolerant and -sensitive individuals in the T1 and BC1F1 generations of GHB119 (Figure 2, a, p. 10). The result fitted the expected segregation ratio for single-locus control with regard to the transferred gene (Table 3). Consequently, it is considered that the inserted DNA transferred into GHB119 resides at a single locus on the cotton genome.

Table 3 Confirmation of segregation ratios

Generation	Number of tested plants	Expected segregation ratio	Observed values		χ^2 Value[1]
			Tolerant	Sensitive	
T1	16	3 : 1	11	5	0.33
BC1F1	19	1 : 1	10	9	0.05

[1]: Assumes a single-locus model. To reject the null hypothesis, the χ^2 value must be equal to or greater than 3.84, with one degree of freedom and p = 0.05.

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

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

As a result of Southern blotting analysis using GHB119 (T3: Figure 2, b, p. 10), it was confirmed that one copy of the T-DNA region had been transferred (Annex 1: Confidential: Not made available or disclosed to unauthorized persons). In addition, the result of the sequence analysis demonstrated that the nucleotide sequence of the transferred DNA into GHB119 was identical to that of the T-DNA region on the vector pTEM12 (Annex 2: Confidential: Not made available or disclosed to unauthorized persons). The map of the transferred DNA is shown in Figure 3(p.12).

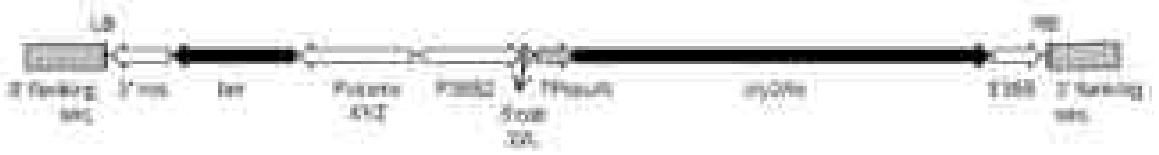

Figure 3 Map of the transferred DNA into GHB119

In the figure, "*bar*" indicates the "modified *bar*."

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

In addition, genomic DNAs extracted from leaves of three GHB119 generations (F1, BC1F1, and BC2F1: Figure 2, c, p. 10) were treated with the restriction enzyme *Eco*RV, which cleavage two sites within the T-DNA region, and then subjected to Southern blotting analysis using the T-DNA region as a probe. As a result, all three generations exhibited the same band pattern of expected size, demonstrating that the transferred nucleic acid is stably inherited across multiple generations (Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

(c) Nearby or separate location of multiple copies, if present, on chromosome

This item is not applicable because there is only one copy of the T-DNA region in GHB119.

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

In 2007, five GHB119 plants (BC2F4: Figure 2, d, p. 10) cultivated in a greenhouse in the U.S. were subjected to ELISA for measuring the expression levels of the modified PAT protein and the Cry2Ae protein in the root, stem, leaf, flower bud, apex, boll, whole aerial parts, pollen, nectar, flower, and seed at various growth stages (vegetative period, just prior to flowering period, flowering period, and harvest period). As a result, the modified PAT protein was detected from all tissues and nectar. Meanwhile, the Cry2Ae protein was below the detection limit (10.8 ng/g sample) in the nectar but was detected from all other tissues. Consequently, it has been confirmed that the modified PAT protein and the Cry2Ae protein are stably expressed across GHB119 individual plants (Annex 3: Confidential: Not made available or disclosed to unauthorized persons).

In the isolated field test conducted in Japan in 2011, the expression of the modified PAT protein and the Cry2Ae protein was studied using the same generations used in the isolated field test (T6: Figure 2, f, p. 10) and the subsequent generation derived from the harvested seeds. The expression of the modified PAT protein and the Cry2Ae protein was checked by the glufosinate herbicide-spraying test and the immunochromatography method, respectively. As a result, all GHB119 individuals in both tested generations exhibited

glufosinate tolerance and expressed the Cry2Ae protein. Consequently, it has been confirmed that both proteins are stably expressed across GHB119 generations (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

(e) 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

GHB119 does not contain any DNA sequence related to transmission, and therefore, it is considered that the DNA would be unlikely transmitted to wild animals and wild plants in a natural environment.

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

GHB119 can be identified by the PCR method employing primers corresponding to the neighboring sequences of the transferred DNA. The quantification limit of this detection method is 0.08%, and the reliability of the method has been verified as a result of tests performed within the company and at two other laboratories (Annex 4: Confidential: Not made available or disclosed to unauthorized persons).

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

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

GHB119 shows tolerance to glufosinate herbicide due to the modified PAT protein encoded by the modified *bar* gene. The modified PAT protein metabolizes glufosinate to produce *N*-acetyl-glufosinate, which does not inhibit glutamine synthase (OECD, 2002), and it is therefore considered that the metabolite is unlikely to affect the metabolic system of the recipient organism. Moreover, this metabolite is designated as a compound subject to the maximum residue limit for glufosinate in cottonseed (4 ppm) (The Japan Food Chemical Research Foundation), and toxicity studies were conducted with mammals

including rats, mice, and dogs demonstrated that its toxicity is lower than that of glufosinate, which is classified as an ordinary substance* (Bayer CropScience K.K., 2009).

In addition, GHB119 shows resistance to tobacco budworm (*H. virescens*), pink bollworm (*P. gossypiella*), cotton bollworm (*H. zea*), and other Lepidopteran insect pests in cotton cultivation due to the Cry2Ae protein encoded by the *cry2Ae* gene.

(b) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified crops and taxonomical species to which the recipient organism belongs, and the degree of difference, if present

In 2010, an isolated field tests was conducted in an open field at the Frontier Science Research Center, University of Miyazaki (hereinafter referred to as "isolated field"); however, sufficient amount of data for assessing the Adverse Effects on Biological Diversity could not be obtained due to delayed start of cultivation and insect damage by Lepidopteran. Therefore, another isolated field test was conducted in 2011 to compare GHB119 (T6: Figure 2, f, p. 10) with the recipient cultivar Coker312 (hereinafter referred to as "non-recombinant cotton") (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). In addition, a study was conducted in a P1P laboratory at Bayer CropScience K.K. Yuki Research Center in 2008 to compare the tolerance to low temperature at the early stage of growth of GHB119 (BC2F5: Figure 2, e, p. 10) and the non-recombinant cotton (Annex 6: Confidential: Not made available or disclosed to unauthorized persons).

a. Morphological and growth characteristics

In the isolated field test, we compared GHB119 with the non-recombinant cotton of the following items: the uniformity of germination, flowering time, boll opening time, leaf shape, plant type, flower color, boll shape, lint color, seed shape, seed color, leaf length, leaf width, plant height, number of flower buds, number of nodes, total number of branches,

* Substances that are not regarded as poisonous or not designated as poisonous or deleterious substances specified under the Poisonous and Deleterious Substances Control Law. The LD50 value for oral exposure is 300 mg/kg or higher.

number of harvested bolls per plant, weight of aerial parts, weight of under-ground parts, boll length, boll width, boll weight, number of segments of a boll, number of seeds per boll, and 100-seed weight.

As a result, a difference was observed in the germination rate 14 days after sowing between GHB119 (61%) and the non-recombinant cotton (98%) (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). The flowering time and the boll opening time were three days later and two days earlier, respectively, in GHB119 than in the non-recombinant cotton (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). The plant height and the number of flower buds were smaller in GHB119 compared to the non-recombinant cotton, while the boll weight was larger in GHB119 than in the non-recombinant cotton; the differences observed for these items were statistically significant (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). In other items, no difference or statistically significant difference was observed between the two cotton lines (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

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

In the P1P laboratory in Japan, young plants of GHB119 and the non-recombinant cotton were cultivated at 5°C under 12-h day length for 1 month, and degree of withering was evaluated every week. As a result, no statistically significant difference was observed between the two lines at any of the evaluation time points, and all individuals of both lines were dead four weeks after they had been transferred to the low-temperature condition (Annex 6: Confidential: Not made available or disclosed to unauthorized persons).

c. Overwintering ability and summer survival of matured plant

In the isolated field, GHB119 and the non-recombinant cotton were left cultivated after the harvest time in winter; as a result, all plants weredead in February 2012 (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

d. Fertility and size of pollen

No statistically significant difference was observed between the size of pollens collected from GHB119 and the non-recombinant cotton cultivated in the isolated field. In addition, the pollens of both lines showed high fertility (99% or higher) without any statistically significant difference between the two lines (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

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

Regarding seed production, GHB119 was compared with the non-recombinant cotton in the number of harvested bolls per plant, the number of seeds per boll, and 100-seed weight. As a result, no statistically significant difference was observed between GHB119 and the non-recombinant cotton in any of the items examined (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Regarding the shedding habit, shedding was not observed in GHB119 and the non-recombinant cotton (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Regarding dormancy and germination rate, seeds of GHB119 and the non-recombinant cotton harvested in the isolated field were sown either immediately after harvest or after 3-month air drying at room temperature to evaluate the germination rate. As a result, the germination rates of the just-harvested seeds of GHB119 and the non-recombinant cotton were 70% and 66%, respectively, while the germination rates of the 3-month air-dried seeds of GHB119 and the non-recombinant cotton were 94% and 86%, respectively. Under either condition, no statistically significant difference was observed between the two cotton lines (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

f. Crossability

Since related species that can cross with cotton are not voluntarily grown in Japan, crossability was not examined.

g. Productivity of harmful substances

In the isolated fields, a succeeding crop test was carried out to evaluate the effect of substances secreted from the roots on other plants, a plow-in test was carried out to evaluate the effect of substances in the plant body on other plants after dying, and a soil microflora test was carried out to evaluate the effect of substances secreted from the roots on microorganisms in soil.

Succeeding crop test

After cultivating GHB119 and the non-recombinant cotton in the isolated field, radish seeds were sown in the remaining soil, and comparison was made between the two plots for germination rate, plant height, fresh weight, and dry weight. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Plow-in test

Plants of GHB119 or the non-recombinant cotton harvested from the isolated field were mixed into soil, and radish seeds were sown in the soil to compare the germination rate, plant height, fresh weight, and dry weigh between the two plots. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Soil microflora test

Soil was sampled after cultivating GHB119 or the non-recombinant cotton in the isolated field, and comparison was made for the number of bacteria, actinomycetes, and fungi with the dilution plate method. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

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

A review was made by persons with specialized knowledge and experience concerning Adverse 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 Effects on Biological Diversity

This recombinant cotton was developed by introducing the expression vector pTEM12 constructed from the plasmid pGSC1700 into the recipient organism by the *Agrobacterium* method.

Based on the segregation ratio of the transferred gene and the Southern blot analysis, it has been confirmed that one copy of the T-DNA, which contains the modified *bar* gene encoding the modified PAT protein (phosphinothricin acetyltransferase) derived from *S. hygroscopicus* and the *cry2Ae* gene encoding the Cry2Ae protein derived from *B. thuringiensis*, is present on the chromosome of this recombinant cotton and is stably inherited across multiple generations. In addition, it has been confirmed by ELISA that the target genes are stably expressed across multiple generations.

1) Competitiveness

Cotton, the taxonomical species to which the recipient organism belongs, has been used for a long time in Japan, though there is no report that it has grown voluntarily in the natural environment.

As a result of studies on various characteristics in competitiveness of this recombinant cotton, which were carried out in 2008 in the P1P laboratory in Japan and in 2011 in the isolated field in Japan, differences or statistically significant differences were observed between this recombinant cotton and the non-recombinant control cotton in the flowering time, boll opening time, plant height, number of flower buds, and boll weight.

The flowering time of this recombinant cotton was three days later than that of the

non-recombinant control cotton. This could be attributed to a potential decrease in germination ability of this recombinant cotton seed, which is reflected in their low germination rate (61%) 14 days after sowing. The initial growth of this recombinant cotton was delayed compared to the non-recombinant control cotton, which seems to have contributed to the delay in flowering time, but this recombinant cotton showed steady growth thereafter and opened their bolls two days earlier than the non-recombinant control cotton. Nevertheless, these differences were merely slight differences, and it is unlikely that they would enhance the competitiveness of this recombinant cotton. A difference was observed for seed germination rate used in the cultivation experiment, but no statistically significant difference was observed for seeds harvested from the isolated field. Therefore, the observed difference may not be attributed to some genetic factors.

The plant height and the number of flower buds were smaller for this recombinant cotton compared to the non-recombinant control cotton, which could be attributed to the delay in the initial growth. The boll weight was larger in this recombinant cotton than in the non-recombinant control cotton, and the fewer number of flower buds was considered to contribut to the larger boll size. The boll weight of this recombinant cotton did not exceed the values of the commercial cultivar cultivated in the same field in the same year, so the observed difference was considered to be within the range of varietal difference.

This recombinant cotton is given the traits to be tolerant to glufosinate herbicide by the modified PAT protein and resistant to Lepidopteraby the Cry2Ae protein. However, since it is difficult for the cultivated cotton to grow voluntarily in the natural environment in Japan, it is unlikely that these traits could increase the competitiveness of this recombinant cotton in the Japanese natural environment.

Based on the above understanding, it was judged that the conclusion made 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 Effects on Biological Diversity attributable to competitiveness is reasonable.

2) Production of harmful substances

Cotton, the taxonomical species to which the recipient organism belongs, contains gossypol, which exhibits toxicity to nonruminant animals, and cyclopropene fatty acid,

which inhibits desaturation of saturated fatty acids leading to discoloration or decreased hatchability of hen eggs. However, there is no report that cotton seeds are eaten by wild animals. In addition, it is not known that cotton produces any allelopathic substances that could affect the inhabitation or growth of wild animals and plants.

It has been confirmed that the modified PAT protein and the Cry2Ae protein, which are expressed in this recombinant cotton by the transferred genes, have no sequence homology with any known allergens.

As a result of the soil microflora test, the plow-in test and the succeeding crop test carried out in the isolated fields in Japan to examine the production of harmful substances by this recombinant cotton (the substances secreted from the roots, which can affect other plants and microorganisms in soil; the substances existing in the plant body, which can affect other plants after dying), no difference was observed between this recombinant cotton and the non-recombinant control cotton.

The modified PAT protein has a high substrate specificity, and it is considered that this protein unlikely affect the metabolic pathway of the recipient organism and produce any harmful substances. When this recombinant cotton is exposed to glufosinate, *N*-acetyl-glufosinate is produced by the modified PAT protein. *N*-acetyl-glufosinate is designated as a compound subject to the maximum residue limit for glufosinate in cottonseed, and it has been confirmed that its toxicity is lower than that of glufosinate, which is classified as an ordinary substance. Since this recombinant cotton is given resistance to Lepidoptera by the Cry2Ae protein, there was a concern that this recombinant cotton would affect the survival of Lepidoptera living in Japan, which eat the plant body or pollen of this recombinant cotton. However, cotton is not reported to grow voluntarily in Japan, so it is extremely unlikely that imported cotton seeds that were spilled during transportation grow or become self-seeding in the natural environment. Even if they grew, the pollens of cotton are relatively heavy and sticky, so they have little chance to be widely dispersed by wind. Therefore, it is considered extremely unlikely that Lepidoptera species living in Japan would eat this recombinant cotton or be exposed to its pollens.

Based on the above understanding, it was judged that the conclusion made 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 Effects on Biological Diversity attributable to productivity of harmful substances is reasonable.

3) Crossability

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

(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 the Type 1 Use Regulation, causes Adverse Effects on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.

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