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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton tolerant to glyphosate herbicide (*2mepsps*, *Gossypium hirsutum* L.) (GHB614, OECD UI: BCS-GHØØ2-5)
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

a) Composition and origins of component elements

Component elements of the donor nucleic acid that was used for the production of cotton tolerant to glyphosate herbicide (*2mepsps*, *Gossypium hirsutum* L.) (GHB614, OECD UI: BCS-GHØØ2-5) (hereinafter referred to as "GHB614") are shown in Table 1.

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

Component element	Position in vector	Size (bp)	Origin and function
2mepsps gene expression cassette			
Ph4a748At	0026-1036	1011	A sequence including the promoter region of the histone H4 gene from *Arabidopsis thaliana* (Reference 7). It initiates the constitutive transcription of the *2mepsps* gene in plant tissues.
intron1 h3At	1037-1553	517	A sequence including the first intron of the histone H3.3 II gene from *A. thaliana* (Reference 8).
TPotp C	1554-1926	373	A sequence developed based on the coding region of plastid transit peptide derived from RuBisCo small subunit genes of sunflower (*Helianthus annuus*) and corn (*Zea mays*) (Reference 26). It transits the mature 2mEPSPS protein to the plastids.
2mepsps	1927-3264	1338	A gene encoding the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase (2mEPSPS protein) resulted from two point mutations of 5-enol-pyruvylshikimate-3-phosphate synthase gene (*epsps* gene) derived from maize (*Z. mays*) (Reference 27), conferring the tolerance to herbicide glyphosate. The sequence encoding the plastid membrane transit peptide of *epsps* gene has been removed.
3'histonAt	3265-4007	743	A sequence including the 3' untranslated region of the histone H4 gene from *A. thaliana* (Reference 7). It terminates transcription and directs the 3' polyadenylation.
Additional information			
LB	0001-0025	25	Left border repetitive sequence from the T-DNA region of *Rizobium radiobacter* (*Agrobacterium tumefaciens*) (Reference 48).
RB	4008-4032	25	Right border repetitive sequence from the T-DNA region of *R.radiobacter* (*A.tumefaciens*) (Reference 48).
—	4033-4224	192	Plasmid pTiAch5 fragment in the right border repetitive sequence (Reference 50).
*npt*I fragment	4225-4935	711	*npt* I gene fragment derived from transposon Tn903 coding the neomycin phosphotransferase (Reference 32). This sequence does not function due to the fragment.
ORI ColE1	4936-6108	1173	A sequence including the replication origin derived from plasmid pBR322 of *Escherichia coli* (Reference 3).
ORI pVS1	6109-9879	3771	A sequence including the replication origin (Reference 16) of plasmid vector pVS1 of *Pseudomonas* (Reference 22).
aadA	9880-11648	1769	A sequence including the gene resistant to aminoglycoside derivative antibiotics, derived from *E. coli* (Reference 12).
—	11649-11953	305	Plasmid pTiAch5 fragment in the left border repetitive sequence (Reference 50).

(Note: All the rights pertinent to the information in the table above and the responsibility for the

contents rest upon the applicant.)

b) 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 the component elements of the donor nucleic acid are respectively shown 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

The 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) protein (EC 2.5.1.19), one of the enzymes that catalyze the shikimate pathway, a biosynthetic pathway of aromatic amino acids specific to plants and microorganisms, catalyzes the reversible reaction to produce the 5-enol-pyruvylshikimate-3-phosphate (EPSP) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P). The EPSPS protein binds to PEP and S3P to provide an intermediate product of enzyme-substrate complex consisting of three components, though the herbicide glyphosate binds reversibly to PEP binding site and competitively inhibits the activity (Reference 4). As a result, plants fail to synthesize the essential aromatic amino acids for protein synthesis and then die.

The *2mepsps* gene transferred to GHB614 is a gene that has the nucleotide at two sites substituted due to the point mutation in the *epsps* gene, which encode the EPSPS protein cloned from maize (*Z. mays*). The 2mEPSPS protein produced by the *2mepsps* gene has the amino acid sequence in which threonine, 102nd amino acid in the wild-type EPSPS protein, is substituted by isoleucine, and proline, 106th amino acid, substituted by serine, respectively. Consequently, the 2mEPSPS protein offers lower binding affinity for glyphosate, allowing the shikimate synthesis to work without inhibition of activity by glyphosate and then plants to survive even in the presence of glyphosate.

In addition, based on the amino acid sequence of the 2mEPSPS protein, overall homology search (Uniprot_Swissprot, Uniprot_TrEMBL, PIR, NRL-3D, DAD and GenPept) and allergen epitope search (Uniprot_Swissprot, Uniprot_TrEMBL, PIR, DAD and GenPept) were conducted. As a result, this protein did not show any homology with known toxins or allergens.

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

For the concentration relating to the affinity (*K*m value: Michaelis constant) for PEP and S3P, comparison was made between the 2mEPSPS protein and the EPSPS protein and as a result, the both proteins were found equivalent to each other with regard to the *K*m value for PEP (Table 2). In addition, for S3P, it was found that the 2mEPSPS protein exhibited slightly lower affinity compared to the EPSPS protein (Table 2). Furthermore, as a result of examination on the enzyme activity, the EPSPS protein exhibited higher maximum velocity (*Vmax*) for both PEP and S3P compared to the 2mEPSPS protein, showing approx. 4.7 times higher *Vmax* value for PEP and approx. 4.0 times higher *Vmax* value for S3P (Table 2). In addition, the glyphosate acts as a competitive inhibitor for PEP and then, a half-maximal inhibitory concentration (IC_{50} value) of glyphosate for PEP was investigated with the PEP concentration set at a value five times the *K*m value. As a result, the 2mEPSPS protein showed approx. 190 times higher IC_{50} value compared to the EPSPS protein (Table 2). Furthermore, the inhibition constant (*K*i value) of glyphosate for PEP was found 2.3 mM for the 2mEPSPS protein and 0.9 μM for the EPSPS protein, showing that the glyphosate offers approx. 2,000 times smaller inhibitory activity for the 2mEPSPS protein compared to the EPSPS protein (Table 2). Consequently, the *K*m values for PEP and S3P were found almost equivalent and thus, it was considered that there is no change in individual binding sites, and other sites were subjected to variations while the 2mEPSPS protein maintained the similar substrate specificity as the EPSPS protein, thereby leading to the induced high tolerance to glyphosate. The EPSPS protein is known to also react with the shikimate, an analogue of S3P, as well as PEP and S3P, though the reactivity of the EPSPS protein with shikimate was low (Reference 14), offering higher substrate specificity.

Table 2 Reaction kinetic constants (*K*m value, IC_{50} value, *K*i value) of the 2mEPSPS protein and the EPSPS protein

Enzyme	*K*m value/PEP[a] (mM)	*K*m value/S3P[a] (mM)	Vmax/PEP[a] (U/mg)	Vmax/ S3P[a] (U/mg)	IC_{50} value /PEP[a] (mM)	*K*i value /PEP (μM)
2mEPSPS	0.07±0.005	0.12±0.01	2.6±0.05	3.0±0.08	18.3±2.7	2300
EPSPS	0.07±0.01	0.09±0.006	12.2±0.42	11.9±0.19	0.098±0.005	0.9

n=2

a: (average ± standard deviation)

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In addition, due to the production of the 2mEPSPS protein, an increase in the EPSPS activity in addition to the existing EPSPS protein is considered to

cause possible effects. However, the EPSPS protein, which is involved in the synthesis reaction in the final stage in the shikimate synthetic pathway, is unlikely to be susceptible to the negative regulation of intermediate metabolite and/or final product; therefore, it is considered that the protein does not take part in the rate-limiting of this pathway (References 6, 18, 19, 20, 21, and 46). Moreover, there is a report that, even in the plant cultured cell producing 40 times more EPSPS protein than usual, there occurs no excessive generation of the final product of aromatic amino acid (Reference 38). Furthermore, based on the results shown in Table 3, it is confirmed that, regarding the aromatic amino acids (tyrosine, tryptophan, and phenylalanine), the final products generated through the shikimate synthetic pathway, the GHB614 line and the recipient cultivar Coker312 are equivalent to each other for the dry weight % of various amino acids in the fuzzy seed (Table 3; Appendix 8, p.10, Table 2).

Table 3 Contents of amino acids in the fuzzy seed of GHB614 and Coker312

Amino acid	Dry weight %			Significant difference [b]	
	Coker312 plot[a] (A)	GHB614 plot not treated with glyphosate [a] (B)	GHB614 plot treated with glyphosate [a] (C)	A-B	A-C
Phenylalanine	1.241	1.264	1.256	ns	ns
Tryptophan	0.313	0.321	0.317	ns	ns
Tyrosine	0.593	0.614	0.606	ns	ns

a: Overall average was determined based on 27 measurements at a total of 9 sites (3 measurements each at 9 sites).
b: Treated statistically based on the T-test (level of significance 1%). T-test was conducted in two patterns, (A) and (B), and (A) and (C).
ns: No significant difference
(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

Based on the above understanding, it is considered unlikely that the expression of the *2mepsps* gene would affect the metabolic system of the recipient organism.

(2) Information concerning vectors

a) Name and origin

The vector used for the development of GHB614 is the plasmid pTEM2, which includes the *2mepsps* gene expression cassette transferred into pTYG50 derived from the pGSC1700 constructed based on the plasmid pBR322 derived from *E.*

coli, the plasmid pVS1 derived from *Pseudomonas* and other elements (Reference 9) (Figure 1).

b) Properties

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

The total number of base pairs of the plasmid pTEM2 is 11,953bp. Component elements of this vector are shown in Appendix 1 (p.7, Table 1).

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

The plasmid pTEM2 contains the following sequences outside the T-DNA region, though it has been confirmed as a result of Southern blotting analysis that these sequences are not transferred into GHB614 (Appendix 3, p.14, Figure 10 through p.18, Figure 14).

– The replication origin derived from the plasmid pBR322 of *E. coli* (Reference 3) (ORI ColE1), and the replication origin of the plasmid vector pVS1 of *Pseudomonas* (ORI pVS1) (Reference 16). They function to cause autonomous replication in *E. coli* and *R. radiobacter* (*A. tumefaciens*), respectively.

– Aminoglycoside antibiotics-resistant gene derived from *E. coli* (*aadA*) (Reference 12). This was used as a selective marker in *E. coli* and *R. radiobacter* (*A. tumefaciens*).

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

The range of recipient organisms for the autonomous replication of plasmid pTEM2 is limited to *E. coli* and several gram-negative bacteria, and the plasmid does not possess any infectious characteristics in plants.

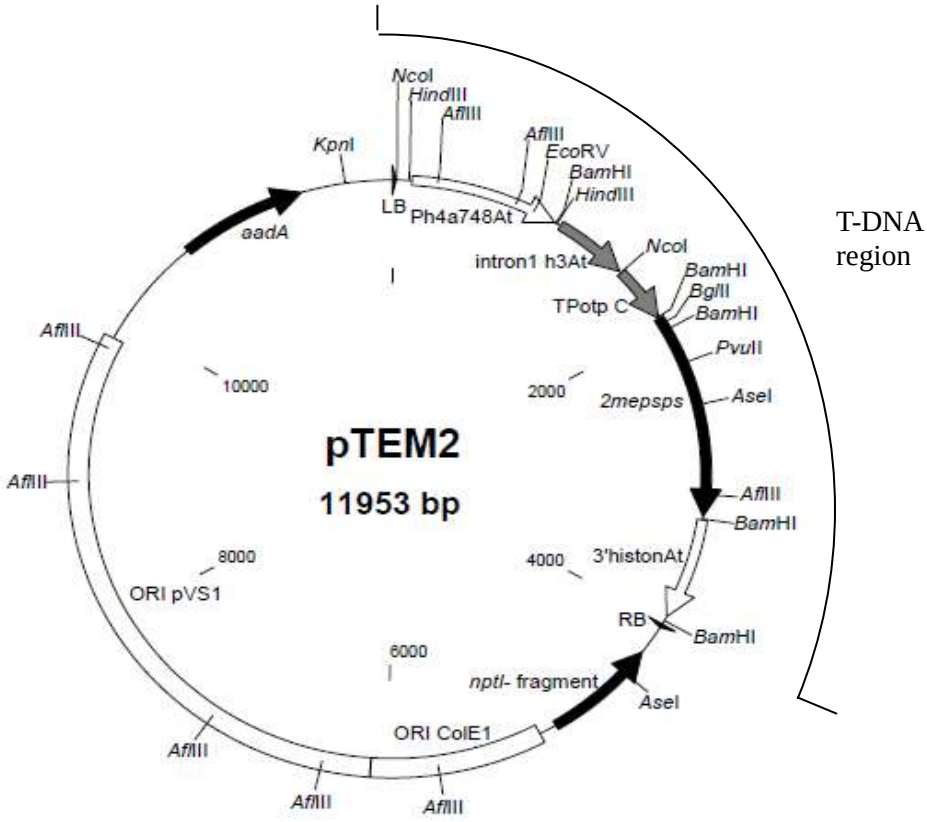

Figure 1 pTEM2 vector map and restriction enzyme cleavage sites

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

(3) Method of preparing living modified organisms

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

In the recipient organism, the *2mepsps* gene expression cassette ([Ph4a748At]-[intron1 h3At]-[TPotp C]-[*2mepsps*]-[3'histonAt]) between LB and RB on the pTEM2 was transferred. Composition of the T-DNA region is shown in Figure 2.

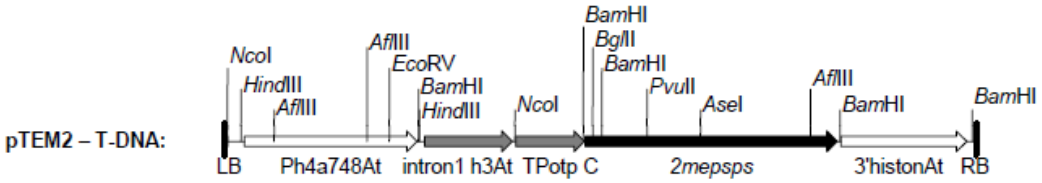

Figure 2 T-DNA region composition and restriction enzyme cleavage sites

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

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

The *Agrobacterium* method was used for transferring the nucleic acid to the recipient cultivar Coker312 (hereinafter referred to as "recipient cultivar"). Transformation was conducted by exposing a piece of the tissue of the recipient cultivar to the culture solution of *R. radiobacter* (*A. tumefaciens*) C58C1^{Rif} strain into which the vector pTEM2 was transferred (Reference 44).

c) Processes of rearing of living modified organisms

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

A piece of the tissue into which the nucleic acid was transferred was selected based on the glyphosate tolerance after removing the *Agrobacterium* on the regeneration medium containing 500 mg/L claforan.

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

Due to the culture on the medium containing 500 mg/L claforan after transferring the nucleic acid, the *Agrobacterium* used for transformation was removed. Furthermore, based on the culture on the medium without containing any claforan, it was confirmed that there exists 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

Grown shoots were transplanted to pots and cultivated in a greenhouse to obtain the original transformants (T_0) of GHB614. Then, based on the comprehensive evaluation of herbicide glyphosate tolerance, agronomic characters and other factors, an elite line was selected. This application includes the strain exhibiting the tolerance to herbicide glyphosate in the original transformant (T_0 generation) and its progeny.

Process of rearing GHB614 is shown in Figure 3.

Figure 3 Process of rearing GHB614

(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 made on a segregation ratio between herbicide glyphosate-tolerant and herbicide glyphosate-sensitive individuals in the generations BC2F2, F1, F2, BC1F1 and BC2F1 of GHB614 and as a result, all the generations examined exhibited the segregation ratio expected in single-gene dominant inheritance with regard to the transferred gene (Table 4). Consequently, it is considered that the replication product of the nucleic acid transferred in GHB614 exists on the genome of cotton at one site.

Table 4 Segregation analysis of GHB614 with regard to tolerance to herbicide glyphosate

Parent	Generation	Ratio	Observed value		Expected value		χ^2 calculated [a]
		R:S	R	S	R	S	
Self-pollinated BC2F1	(1) BC2F2	3:1	28 [b]	8	27	9	0.15
BC2F2 [c] × FM966 [d]	(2) F1	1:1	7	9	8	8	0.25
Self-pollinated heterozygous F1	(3) F2	3:1	113	43	117	39	0.60
Heterozygous F1 × FM966	(4) BC1F1	1:1	9	12	10.5	10.5	0.43
Heterozygous BC1F1 × FM966	(5) BC2F1	1:1	11	6	8.5	8.5	1.47

a. Assumes a one gene locus model. To reject the null hypothesis, the χ^2 value must be greater than 3.84, with one degree of freedom and α=0.05.
b. Tested by homozygosity PCR with the results of 9 homozygous and 19 heterozygous plants.
c. Generated using a heterozygous transgene donor source referred to in the above b.
d. Commercially available non-recombinant cotton cultivar.

R = Resistant to glyphosate, S = Susceptible to glyphosate

(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 replication products of transferred nucleic acid and stability of its inheritance through multiple generations

As a result of Southern blotting analysis done for the DNA extracted from GHB614 (BC2F4) with cleavage made by some restriction enzymes, using the Ph4a748At, intron1h3At+TPotp C, *2mepsps*, 3' histonAt and the intact T-DNA region as probes, it was confirmed that one copy of T-DNA region was transferred (Appendix 3, p.4, Figure 2 through p.8, Figure 6). In addition, as a result of sequence analysis for the T-DNA region transferred in GHB614, it was confirmed that the sequence of the transferred T-DNA region was identical to the

T-DNA region on pTEM2 (Appendix 2, pp.15-22).

Moreover, as a result of Southern blotting analysis for the DNA extracted from the two generations of GHB614 (T7 and BC2F3), the identical band patterns were identified in the both generations and thus, it was confirmed that the transferred T-DNA region was stably inherited across the multiple generations (Appendix 3, p.10, Figure 8).

Furthermore, as a result of Southern blotting analysis for the DNA extracted from GHB614, using the five DNA fragments covering the entire sequences outside the T-DNA region on the plasmid pTEM2 as probes, no band was detected in any probes and thus, it was confirmed that no sequences outside the T-DNA region were transferred in GHB614 (Appendix 3, p.14, Figure 10 through p.18, Figure 14).

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

—

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

For GHB614 (BC2F4, measurement was made with the ELISA method on the content of the 2mEPSPS protein in the leaf, stem, root, square, apex and pollen at the first growth stage (2- to 3-leaf stage), second growth stage (4- to 6-leaf stage), third growth stage (immediately before flower opening) and fourth growth stage (flowering time). Measurement was made at all the growth stages for leaf, the second and fourth growth stages for stem and root, and the fourth growth stage for square, apex and pollen. In addition, the level of total extractable protein (TEP) was measured to determine the proportion of the 2mEPSPS protein in the TEP. As a result, an average 2mEPSPS protein content in the leaf was found ranging from 11.16 ± 3.73 μg/g in the first growth stage to 0.45 ± 0.22 μg/g in the fourth growth stage, showing decreasing protein contents over time with the progress of growth. In addition, the 2mEPSPS protein proportion in the TEP in the leaf was also found decreasing with the progress of growth, though it exhibited a temporary increase in the third growth stage. The 2mEPSPS protein content in the stem was found 1.94 ± 0.61 μg/g in the second growth stage and 1.58 ± 0.96 μg/g in the fourth growth stage, showing a decreasing tendency over time, and the 2mEPSPS protein proportion in the TEP also exhibited decreasing tendency over time. The 2mEPSPS protein content in the root was found 0.99 ± 1.00 μg/g in the second growth stage and 4.04 ± 1.71 μg/g in the fourth growth stage, showing an increasing tendency over time, and the 2mEPSPS protein proportion in the TEP was also found increasing over time. Furthermore, the 2mEPSPS protein content in the square, apex and pollen in the

fourth growth stage was found 5.35 ± 0.25 μg/g, 5.47 ± 0.22 μg/g, and 0.16 ± 0.01 μg/g, respectively. Consequently, the 2mEPSPS protein was detected in all the samples examined, though the expression level varied according to tissue or growth stage (Table 5; Appendix 4, Table 7 and Table 10).

Table 5 Average 2mEPSPS protein content in different plant tissues of GHB614 (μg/g fresh weight ± standard deviation [a]) [%TEP]

Tissue type	LOD ($\times 10^{-3}$μg/g fresh weight)	1st growth stage	2nd growth stage	3rd growth stage	4th growth stage
Leaf	4.47	11.16±3.73 [0.121]	7.94±2.87 [0.090]	6.52±7.20 [0.385]	0.45±0.22 [0.028]
Stem	8.34	ND	1.94±0.61 [0.062]	ND	1.58±0.96 [0.039]
Root	27.33	ND	0.99±1.00 [0.074]	ND	4.04±1.71 [0.176]
Square	27.33	NA	NA	NA	5.35±0.25 [0.175]
Apex	8.10	ND	ND	ND	5.47±0.22 [0.338]
Pollen	16.08	NA	NA	NA	0.16±0.01 [0.001]

a: Standard deviation was calculated based on 20 measurements for leaf, root, and stem (5 samples × 2 sample extracts from 2 replicate plots × 2 measurements on each extract from 2 replicate plots), and 4 measurements for not yet opened square, growing point and pollen (one sample × 2 sample extracts from 2 replicate plots × 2 measurements on each extract from 2 replicate plots).

TEP: Total extractable protein

ND: Not determined

NA: Not applicable

Fuzzy seed (kernel＋lint coat) samples of GHB614 (BC2F2) were collected from a total of 9 test sites in the U. S. At each test site, there were GHB614 line plots treated with glyphosate herbicide {sprayed three times with glyphosate at 0.75 lb active ingredient /acre (0.084 g/m^2)} and GHB614 plots not treated with glyphosate herbicide. Fuzzy seed was separated into two fractions, kernel and lint coat, and the 2mEPSPS protein content in each fraction was determined based on the ELISA method. In addition, from the 2mEPSPS protein content and the weight in each fraction, the 2mEPSPS protein content in fuzzy seed was calculated and the total crude protein (TCP) level in fuzzy seed was determined to obtain the 2mEPSPS protein content (%) in the TCP of fuzzy seed. As a

result, independently from whether or not the glyphosate herbicide was sprayed during the growth, the 2mEPSPS protein was detected in the both fractions kernel and lint coat, and the average 2mEPSPS protein content in the fuzzy seed of GHB614 was found 19.2 ± 3.1 μg/g for the samples not treated with glyphosate, and 21.2 ± 4.0 μg/g for the samples treated with glyphosate. In addition, the 2mEPSPS protein content in the TCP in fuzzy seed was found 0.0093 ± 0.0018% for the samples not treated with glyphosate, and 0.0100 ± 0.0019% for the samples treated with glyphosate (Table 6, Appendix 5, Table 2).

Table 6 2mEPSPS protein contents in fuzzy seed (kernel + lint coat) harvested from GHB614 treated and not treated with herbicide glyphosate (as detected by ELISA method)

Sample	2mEPSPS protein content (μg/g ± standard deviation [a])		2mEPSPS protein content in total crude protein (TCP) (%[b])	
	Not treated with glyphosate	Treated with glyphosate	Not treated with glyphosate	Treated with glyphosate
Kernel	36.3±7.2	40.2±9.0	NA	NA
Lint coat	0.08±0.06	0.14±0.15	NA	NA
Fuzzy seed	19.2±3.1	21.2±4.0	0.0093±0.0018	0.0100±0.0019

a: Overall average and standard deviation were determined based on 108 actual measurements at a total of 9 test sites (12 measurements each at 9 sites). For fuzzy seed (kernel＋lint coat), average value was determined for three plots at each test site, and from the average values at a total of 9 test sites, overall average and standard deviation were determined.

b: 2mEPSPS as % of crude protein for kernel and lint coat is not applicable (NA) because protein cotent determinations were not made on these samples. For fuzzy seed, from the average value of TCP protein level at each test site and the average value of the 2mEPSPS protein content at each test site of three plots, the 2mEPSPS protein content in the TCP (%) was determined and then, overall average value and standard deviation across a total of 9 test sites were determined.

TCP: Total crude protein

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

Moreover, glyphosate herbicide (active ingredient content 0.41%) of 300 mL/m^2 was sprayed to 20 seedling individuals 2 weeks after sowing of the seeds germinated from the seeds of GHB614 (BC2F5) used in the isolated field tests conducted in 2008 and 40 seedling individuals 2 weeks after sowing of the seeds germinated from the seeds of the next generation of BC2F5. As a result, in all the generations tested, GHB614 grew normally even after 2 weeks from glyphosate spraying and showed tolerance to the glyphosate (Appendix 7, Figure 9 and Table 5, Table 10).

Based on the above understanding, it was confirmed that the gene transferred in GHB614 is stably expressed across individuals and generations.

(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
The nucleic acid transferred in GHB614 contains no sequence that possesses transferring factor and therefore, it is considered unlikely that the transferred nucleic acid would be transmitted to wild animals and wild plants under a natural environment.

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

Specific identification of GHB614 is available by PCR method using the flanking sequences of DNA transferred in GHB614 (Appendix 9). This PCR method is utilized practically and confirmed to be effective for cultivation management.

(6) Difference from the recipient organism or the 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

GHB614 shows tolerance to glyphosate herbicide due to the expression of the *2mepsps* gene.

b) 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 2008, the isolated field tests were conducted at the National Institute for Agro-Environmental Sciences to make comparison between GHB614 (BC2F5) and the commercially available cultivar FM966 (hereinafter referred to as "control cultivar"), the recurrent parent of backcross in the rearing process of GHB614 used for the isolated field tests with the similar genetic background, regarding the morphological and growth characteristics, wintering ability of the matured plant, productivity, shedding habit, dormancy and germination rate of the seed, and productivity of harmful substances (Appendix 7). In addition, in 2007 in the special screened greenhouse in Japan, comparison was made between GHB614 (T6) and Coker312, the recipient cultivar having the same genetic background as GHB614 used for the special screened greenhouse, regarding the cold-tolerance at the early stage of growth (Appendix 6).

(a) Morphological and growth characteristics

Comparison was made for the items regarding morphological and growth characteristics between GHB614 and the control cultivar; the uniformity of

germination, flowering time, boll opening time, harvesting time, leaf shape, plant type, flower color, boll shape, lint color, seed shape, seed color, germination rate, leaf size (leaf length and leaf width), plant height, number of appearing flower buds, number of nodes, number of vegetative branch, number of fruiting branch, total number of branches, number of bolls per plant, number of non-harvested bolls per plant, total number of bolls per plant, weights of aerial- and under-ground parts at the harvest time, fertility of the pollen, pollen length, boll size (length and width), boll weight, number of segments of a boll, number of seeds per boll, and 100-kernel weight.

As a result, in the individual growth stages of GHB614 and the control cultivar or at the time of uniformity of germination, flowering time, boll opening time, and harvesting time, no difference was observed between the both plants. In addition, also regarding leaf shape, plant type, flower color, boll shape, lint color, seed shape and color, no difference was observed between the both plants. Furthermore, regarding leaf size, plant height, number of appearing flower buds, number of nodes, number of vegetative branch, number of fruiting branch, total number of branches, boll size, boll weight, number of bolls per plant, number of non-harvested bolls per plant, total number of bolls per plant, weights of aerial- and under-ground parts at the harvest time, number of segments of a boll, number of seeds per boll, and 100-kernel weight, no statistically significant difference was observed between GHB614 and the control cultivar (Appendix 7, Table 2 through Table 4). Regarding the germination rate of the seeds exposed to the cultivation experiments in the isolated field (hereinafter referred to as "seeds for cultivation experiment"), a statistically significant difference was observed between the both plants, though the seeds for cultivation experiment were collected at different sites, and the control cultivar was considered not to produce the seeds that offered as high germination rate as those of GHB614 due to the unseasonable weather before harvesting (Appendix 7).

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

In the special screened greenhouse tests conducted to evaluate the cold-tolerance of young plant body of GHB614 and the recipient cultivar, changes in the degree of withering over time was visually evaluated under the conditions (5°C and 10-hour day length). As a result, at all the points of time of evaluation, no statistically significant difference was observed between the both lines, and it was confirmed that all the individuals of the both lines died on the 24th day after exposure to the low-temperature condition (Appendix 6, Table 13).

c) Wintering ability and summer survival of the mature plant

GHB614 and the control cultivar sown in June 2008 in the isolated field were left cultivated even after the harvest time in November and as a result, all the plants were found withered and dead in February in the following year due to frost (Appendix 7, Figure 8).

d) Fertility and size of the pollen

Pollens were collected from GHB614 and the control cultivar cultivated in the isolated field, and stained with acetocarmine solution and observed under a microscope. As a result, 99.7% of the pollens from GHB614 and 99.8% of the pollens from the control cultivar were found stained, showing a high fertility of the pollens without any statistically significant difference between the both lines (Appendix 7, Table 4 and Figure 7). In addition, regarding the size of pollen, no statistically significant difference was observed between the both lines (Appendix 7, Table 4 and Figure 7).

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

As a result of comparison for the number of bolls per plant, number of non-harvested bolls per plant, total number of bolls per plant, and number of seeds per boll between GHB614 and the control cultivar harvested in the isolated field, no statistically significant difference was observed between the lines tested (Appendix 7, Table 4).

In addition, as a result of observation in the same isolated field test for shedding of the seeds of GHB614 and the control cultivar from the opened bolls, no shedding was observed between the both lines (Appendix 7, Table 3).

Furthermore, seeds obtained from GHB614 and the control cultivar harvested in the isolated field were sown immediately after harvesting (hereinafter referred to as "harvested seed") and after 3-month air drying at room temperature (hereinafter referred to as "air-dried seed") to evaluate the germination rate. As a result, the germination rate of the harvested seed was found 60.0% for GHB614 and 58.0% for the control cultivar. In addition, the germination rate of the air-dried seed was found 96.0% for GHB614 and 98.0% for the control cultivar. Consequently, in the both conditions, no statistically significant difference was observed in germination rate between the lines tested, and the 3-month air-dried seed showed higher germination rates for the both lines (Appendix 7, Table 9).

f) Crossability

In Japan, no wild relatives exist which can cross with cotton. Thus, crossability of this recombinant cotton was not assessed.

g) Productivity of harmful substances

In order to check in the isolated field whether the substances are excreted from the roots which can affect other plants, exists in the plant body which can affect other plants after dying, and are excreted from the roots which can affect microorganisms in soil, the succeeding crop test, plow-in test, and soil microflora test were carried out respectively.

Succeeding crop test:
In the remaining soil after cultivating GHB614 and the control cultivar in the isolated field up to the harvest time for approx. 5 months, radishes were sown as test plant, and the comparison was made for germination rate, plant height, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed between the lines tested (Appendix 7, Table 6).

Plow-in test:
Aerial-parts were harvested from GHB614 and the control cultivar cultivated in the isolated field up to the harvest time for approx. 5 months, dried and reduced to powder to prepare samples. In the soil mixed with 1% each of the samples, radishes were sown as test plant, and the comparison was made for germination rate, plant height, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed between the lines tested (Appendix 7, Table 7).

Soil microflora test:
The soil was obtained after cultivating GHB614 and the control cultivar in the isolated field up to the harvest time for approx. 5 months, and was diluted as appropriate. Bacteria and actinomycete were incubated on the PTYG medium, and filamentous fungi were incubated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, in all the items examined, no statistically significant difference was observed between the lines tested (Appendix 7, Table 8).

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

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

This recombinant cotton is given a trait to be tolerant to glyphosate herbicide by the transferred *2mepsps* gene, though it is considered unlikely that the glyphosate would exert pressure for selection under a natural environment. Consequently, it is considered unlikely that this trait would increase the competitiveness of this recombinant cotton.

In 2008, in the isolated fields in Japan, with regard to various traits relating to the competitiveness, the morphological and growth characteristics, wintering ability of the matured plant, the productivity, shedding habit and dormancy of seeds, and germination rate were examined. As a result, regarding the germination rate of seeds for cultivation experiment, a statistically significant difference was observed between the cotton event lines examined, though the seeds for cultivation experiment were collected at different sites, and the control cultivar was considered not to produce the seeds that offered as high germination rate as those of GHB614 due to the unseasonable weather before harvesting. In addition, regarding the germination rate of harvested seeds and their air-dried seeds, no statistically significant difference was observed between GHB614 and the control cultivar; therefore, the difference observed in the seeds for cultivation experiment was considered not resulting from the effects of gene modification. In addition, as a result of investigation conducted in the special screened greenhouse in Japan in 2007 for comparison of cold-tolerance at the early stage of growth between this recombinant cotton and the recipient organism, no statistically significant difference was observed between the both plants.

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

It is generally known that the seeds of the cotton plant contain gossypol, which exhibits toxicity to nonruminant species of animals, and cyclopropene fatty acid, which inhibits the desaturation of saturated fatty acids leading to changed color in hen eggs or decreased hatchability. However, there is no case report that wild animals prey on the seeds of the cotton plant. In addition, regarding the cotton, there is no report that it possesses productivity of any harmful substances that could affect the inhabitation or growth of wild animals and wild plants.

This recombinant cotton produces the 2mEPSPS protein that confers the tolerance to glyphosate, though it has been confirmed that this protein does not have any homology of amino acid sequence with known allergens or toxins. In addition, the 2mEPSPS protein is considered to possess high substrate specificity similarly to the EPSPS protein and then, it is considered that this protein does not affect the metabolic system of the recipient organism nor produce any harmful substances.

Furthermore, as a result of succeeding crop test, soil microflora test, and plow-in test conducted in the isolated field tests in Japan to examine the ability of this recombinant cotton to produce any harmful substances (the substances secreted from the roots with a threat to affect other plants and microorganisms, and the substances contained in plant bodies with a threat to affect other plants after dying), no statistically significant difference from the control plots was observed.

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 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.