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 and glufosinate herbicides (*2mepsps*, modified *bar*, *Gossypium hirsutum* L.) (GHB614 × LLCotton25, OECD UI: BCS-GHØØ2-5 × ACS-GHØØ1-3)
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	—

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

1. Information concerning preparation of living modified organisms

This stack line has been produced by crossing GHB614 with LLCotton25, having properties of the two. Thus, information on preparation of GHB614 and LLCotton25, and others are described below.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Component elements of the donor nucleic acids used for the production of GHB614 and LLCotton25 are shown in Table 1 (p. 3) and Table 2 (p. 4), respectively.

Table 1 Component elements of the donor nucleic acid used for the production of GHB614

Component elements	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 derived from *Arabidopsis thaliana* (Reference 7). This sequence constitutively initiates the 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 derived 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 maize (*Zea mays*) (Reference 26). This sequence transits the mature 2mEPSPS protein to the plastids.
2mepsps	1927-3264	1338	A gene encoding the double-mutant 5-enol-pyruvyl-shikimate-3-phosphate synthase (2mEPSPS protein), and resulting from two point mutations in 5-enol-pyruvyl-shikimate-3-phosphate synthase gene (*epsps* gene) derived from maize (*Z. mays*) (Reference 27). This gene confers the tolerance to glyphosate herbicide. The sequence encoding the plastid membrane transit peptide has been removed from the *epsps* gene.
3'histonAt	3265-4007	743	A sequence including the 3' untranslated region of the histone H4 gene derived from *A. thaliana* (Reference 7). This sequence terminates the transcription and causes the 3' polyadenylation.
Additional information			
LB	0001-0025	25	A Left border repetitive sequence of T-DNA derived from *Rizobium radiobacter* (*Agrobacterium tumefaciens*) (Reference 48).
RB	4008-4032	25	A Right border repetitive sequence of T-DNA derived from *R. radiobacter* (*A. tumefaciens*) (Reference 48).
—	4033-4224	192	A fragment derived from Plasmid pTiAch5 in the right border repetitive sequence (Reference 50).
*npt*I fragment	4225-4935	711	A fragment of transposon Tn903-derived *npt I* gene that codes for neomycin phosphotransferase (Reference 33). This sequence does not function, because it is a fragment.
ORI ColE1	4936-6108	1173	A sequence including the replication origin of plasmid pBR322 derived from *Escherichia coli* (Reference 3).
ORI pVS1	6109-9879	3771	A sequence including the replication origin (Reference 18) of plasmid vector pVS1 derived from *Pseudomonas* (Reference 23).

aadA	9880-11648	1769	A sequence including *E. coli*-derived gene that confers the tolerance to aminoglycoside antibiotics (Reference 13).
—	11649-11953	305	A fragment derived from Plasmid pTiAch5 in the left border repetitive sequence (Reference 50).

Table 2 Component elements of the donor nucleic acid used for the production of LLCotton25

Component elements	Position in vector	Size (bp)	Origin and function
Modified *bar* gene expression cassette			
P35S3	250-1634	1385	The promoter region of cauliflower mosaic virus 35S transcript gene. This region initiates the transcription (Reference 30).
Modified *bar*	1635-2186	552	Bialaphos tolerance (*bar*) gene derived from *Streptomyces hygroscopicus*. It confers the tolerance to glufosinate herbicide (Reference 42). Two codons in the N-terminal of the wild *bar* gene, or GTG and AGC, have been replaced with ATG and GAC, respectively. Amino acid has changed from serine to asparagine for the replacement of AGC with GAC, while remaining methionine for the replacement of GTG with ATG.
3'nos	2206-2465	260	The 3' untranslated region of nopaline synthase gene derived from T-DNA of pTiT37. This region terminates the transcription and causes the 3' polyadenylation (Reference 10).
Additional information			
RB	198-222	25	A right border repetitive sequence derived from T-DNA of pTiB6S3 (Reference 15).
LB	2520-2544	25	A left border repetitive sequence derived from T-DNA of pTiB6S3 (Reference 15).
aadA	2544-4618	2075	A sequence including the streptomycin/spectinomycin-tolerant gene derived from the transposon Tn7 (Reference 28).
pVS1ori	4619-8389	3780	The replication origin of plasmid pVS1 derived from *Pseudomonas* (Reference 23).
ColE1	8390-9555	1165	A sequence including ColE1 ori, which is the replication origin of the plasmid pBR322 (Reference 3).

2) Function of component elements

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

Functions of the component elements of the donor nucleic acids used for the production of GHB614 and LLCotton25 are also shown in Table 1 (p. 3) and Table 2 (p. 4), respectively.

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

2mEPSPS protein

The 5-enol-pyruvyl-shikimate-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 reversible reaction that synthesize 5-enol-pyruvyl-shikimate-3-phosphate (EPSP) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P). The EPSPS protein binds to both PEP and S3P to construct a three-component enzyme-substrate complex intermediate. However, the activity of EPSPS is competitively inhibited by glyphosate herbicide binding reversibly to the PEP-binding site (Reference 4). In this case, the plants become unable to synthesize aromatic amino acids, which are essential for protein synthesis, to die after a while.

In the *2mepsps* gene transferred to GHB614 two nucleotides are changed by point mutation, compared with the *epsps* gene, which encodes the EPSPS protein cloned from maize (*Z. mays*). Concerning amino acid sequence of the 2mEPSPS protein, which is expressed from the *2mepsps* gene, the 102nd amino acid threonine and the 106th amino acid proline in the wild EPSPS protein have been substituted by isoleucine and serine, respectively. Consequently, the 2mEPSPS protein has lower binding affinity for glyphosate, able to promote the shikimate synthesis without being inactivated by glyphosate. GHB614 can survive even in the presence of glyphosate.

In 2008, amino acid sequence homology of the 2mEPSPS protein was explored inclusively in proteins that had been registered to various databases (Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD, GenPept, and AllergenOnline). As a result, this protein did not show any homology to the known toxins or allergens.

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 produced ammonia, glutamine synthetase plays a pivotal role. If sprayed to plants, glufosinate herbicide inhibits the glutamine synthetase to allow the produced ammonia to accumulate, resulting in death of the plants.

In the N-terminal of the modified *bar* gene, GTG codon has been replaced with ATG, which is a codon suitable for plants, and AGC codon has been replaced with GAC to increase translation efficiency. Serine is changed to asparagine in translation for the replacement of AGC with GAC, while remaining methionine for the replacement of GTG with ATG.

The product expressed from the modified *bar* gene, or the modified PAT protein, can acetylate glufosinate to make N-acetyl-glufosinate, inactivating inhibitory aciton of the glufosinate on glutamine synthase. Thus, even if glufosinate herbicide is sprayed to plants, this mechnism would prevent ammonia from accumulating, resulting in survival of the plants.

In 2009, amino acid sequence homology of the modified PAT protein was explored inclusively in proteins that had been registered to various databases (Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD, GenPept, and AllergenOnline). As a result, this protein did not show any homology to the known toxins or allergens.

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

2mEPSPS protein

Comparison was made between the 2mEPSPS and the EPSPS proteins in the concentration representing the affinity (*K*m value: Michaelis constant) for PEP and S3P. These proteins were found equivalent to each other in *K*m value for PEP, while the 2mEPSPS protein had slightly lower affinity for S3P than the EPSPS protein (Table 3, p. 7). Furthermore, the enzyme activity was evaluated. The EPSPS protein showed higher maximum velocity (Vmax) for both PEP and S3P than the 2mEPSPS protein; Vmax value of the EPSPS protein was approximately 4.7 times higher for PEP and approximately 4 times higher for S3P (Table 3, p. 7) than 2mEPSPS protein. Because glyphosate acts as an inhibitor competing with PEP, the half-maximal inhibitory concentration (IC_{50} value) of glyphosate against PEP was evaluated, with PEP concentration set at five times of the *K*m value.

The 2mEPSPS protein showed approximately 190 times higher IC_{50} value than the EPSPS protein (Table 3, p. 7). Moreover, the inhibition constant (*K*i value) of glyphosate against PEP was evaluated. The 2mEPSPS and the EPSPS proteins showed a *K*i value of 2.3 mM and 0.9 μM, respectively; inhibitory activity of glyphosate on the 2mEPSPS protein was approximately 2,000 times lower than that on the EPSPS protein (Table 3). Since the 2mEPSPS and the EPSPS proteins showed similar *K*m values for PEP and S3P, the substrate specificity appears to have almost been conserved, with PEP- and S3P-binding sites not changed, between the 2mEPSPS and the EPSPS proteins. Thus, mutation in other sites appears to have induced the high glyphosate tolerance. The EPSPS protein is known to react also with shikimate, which is an analogue of S3P, as well as PEP and S3P. Nevertheless, the EPSPS protein is considered to have high substrate specificity, because of low reactivity between the EPSPS protein and shikimate (Reference 16).

Table 3 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:(mean value ± standard deviation)

Addition of the produced 2mEPSPS protein to the existing EPSPS protein might have influence by increasing EPSPS activity. However, the EPSPS protein, which participates in the synthesis reaction at the final stage of the shikimate synthetic pathway, is considered not to be involved in the rate-limiting step of this pathway, because unlikely to be negatively regulated by the intermediate metabolites and/or final products (References 6, 19, 20, 21, 22, and 46). Moreover, it has been reported that according to a report, cultured plant cells producing a 40-time or more amont of the EPSPS protein cannot excessively produce aromatic amino acids as a final product (Reference 38). Regardless of spray of glyphosate herbicide, there were no statistically significant differences in the content of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) between GHB614 seeds produced the EPSPS protein as well as the 2mEPSPS protein and seeds of the recipient cultivar (Table 4, p. 8).

Table 4 Content of various 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 mean was determined from 27 measurements obtained in a total of 9 test plots (3 measurements per test site × 9 test sites).
b: Treated statistically by t-test (level of significance: 1%). The t-test was conducted in two patterns: (A) vs. (B), and (A) vs. (C).
ns: No statistically significant differences
(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, expression of the *2mepsps* gene is considered unlikely to affect the metabolic system of the recipient organism.

Modified PAT protein

The product expressed from the modified *bar* gene, or the modified PAT protein, has high affinity for glufosinate. While glufosinate is classified into L-amino acid, the modified PAT protein cannot transfer the acetyl group to various amino acids. Although glutamic acid is similar to glufosinate especially in structure, the modified PAT protein cannot cause substantial transfer reaction with glutamic acid in vivo, because of its little affinity for glutamic acid (Reference 42). It has been reported that the modified PAT protein is not prevented from transferring the acetyl group to glufosinate even in the excessive presence of various amino acids (Reference 45). Thus, the modified PAT protein has high substrate specificity for glufosinate, considered unlikely to affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

GHB614: pTEM2 derived from the pGSC1700 (Reference 9), which was constructed from the plasmid pBR322 of *E. coli*, the plasmid pVS1 of *Pseudomonas*, and other elements (Figure 1,

p. 10).

LLCotton25: pGSV71 (Reference 23), which was constructed from the plasmid pBR322 of *E. coli* and the plasmid pVS1 of *Pseudomonas* (Figure 2, p. 11).

2) Properties

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

GHB614: pTEM2; 11,953 bp

LLCotton25: pGSV71; 9,555 bp

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

In the non-T-DNA region of either pTEM2 or pGSV71, there is a sequence of gene that confers tolerance to aminoglycoside antibiotics including streptomycin and spectinomycin (*aadA)*. Southern blotting analysis has confirmed that these sequences were not transferred into the recipient organism.

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

Neither pTEM2 nor pGSV71 has transmissible elements, conferring infectivity.

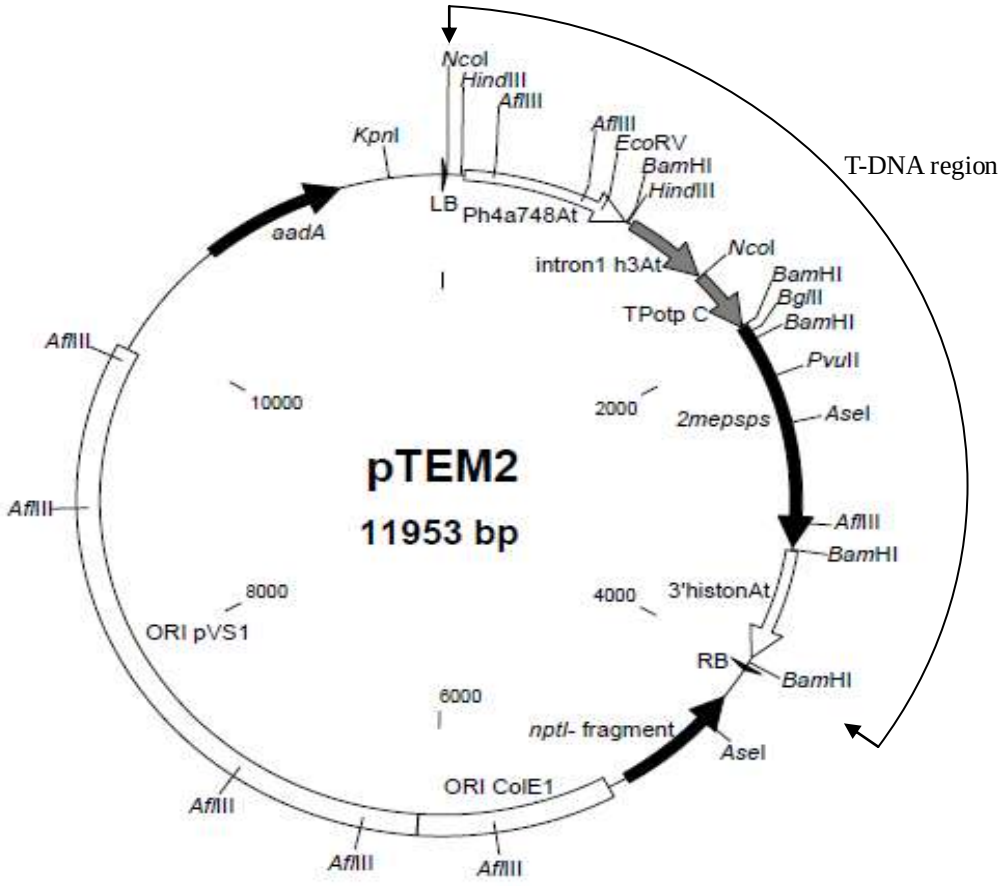

Figure 1 pTEM2 vector map and restriction enzyme cleavage sites
(All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon the applicant.)

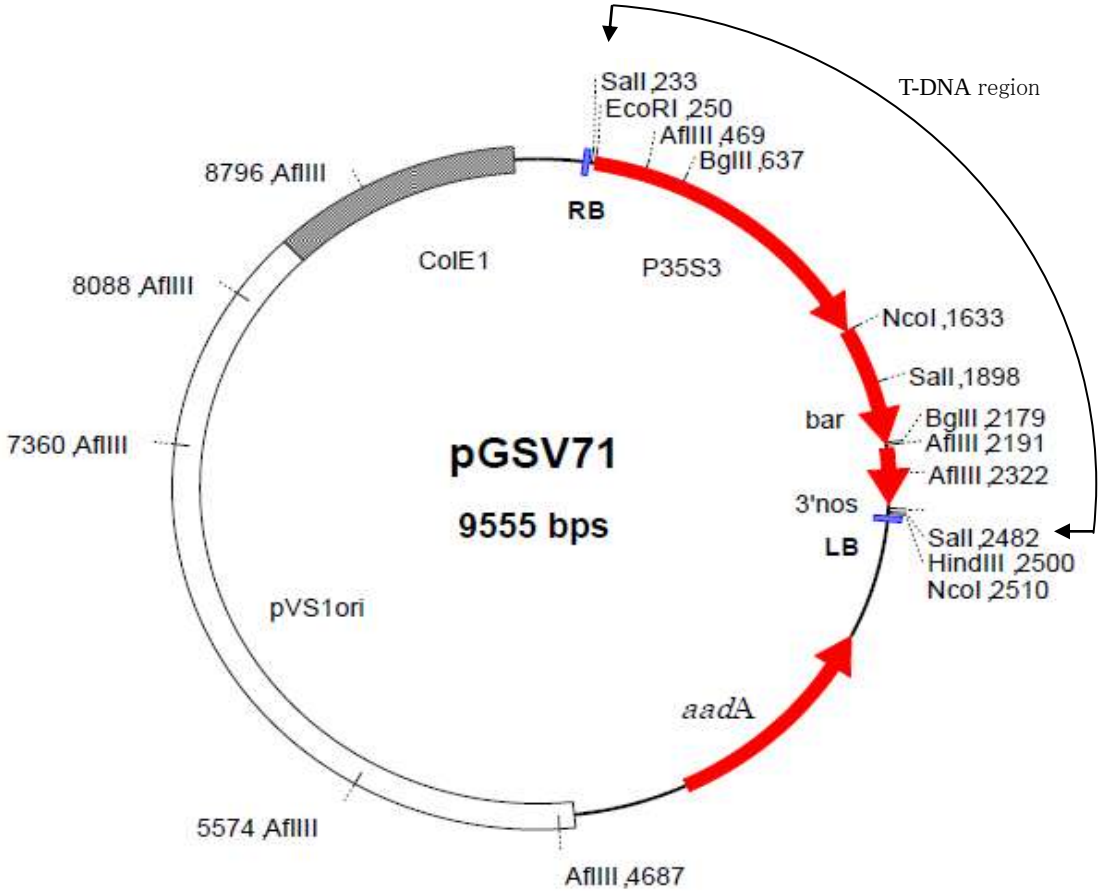

Figure 2 pGSV71 vector map and restriction enzyme cleavage sites

The "bar" refers to the modified *bar* gene.

(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

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

When GHB614 and LLCotton25 were produced, the LB-RB region of pTEM2 and pGSV71 were transferred into the recipient organism, respectively. Constituents of the transferred nucleic acids are shown in Figure 1 (p. 10) and Figure 2 (p. 11).

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

For either GHB614 or LLCotton25, *Agrobacterium* method was used to transfer the nucleic acid into the recipient organism.

3) Processes of rearing of living modified organisms

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

Transformed cells of GHB614 and LLCotton25 were selected using media supplemented with glyphosate and glufosinate, respectively.

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

Either GHB614 or LLCotton25 cells, into which each nucleic acid was transferred, was cultuted in media that contained Claforan (a brand name of a cephem antibiotic) at 500 mg/L. *Agrobacterium* bacteria used for the transformation were removed from the media. Moreover, these cells were cultured in media that did not contain Claforan. It was confirmed that no *Agrobacterium* bacteria used remained in this media.

(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 investigated, 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

For either GHB614 or LLCotton25, regenerated individuals were transplanted into pots, and grown under room temperature to obtain the original transformants (T0 generation). Elite lines of GHB614 and LLCotton25 were selected, considering the desired traits, or tolerance of GHB614 to glyphosate herbicide and tolerance of LLCotton25 to glufosinate herbicide, the agronomic traits, and others. GHB614 and LLCotton25 were

backcrossed with the same commercially available cultivar, and then crossed with each other to create this stack line. Process of rearing this stack line is shown in Figure 3.

This application includes the cross generation (F1 generation) that was produced by crossing between the backcross progeny of GHB614 and that of LLCotton25, and the progeny of this generation.

Information on approval of GHB614 and LLCotton25 in Japan is summarized in Table 5.

Table 5 Information on approval of GHB614 and LLCotton25 in Japan

	Environmental safety	Safety as food	Safety as feed
GHB614	July, 2009: Pending application	January, 2009: Pending application	January, 2009: Pending application
LLCotton25	February, 2006: Approved for Type I Use Regulation	June, 2004: Approved safety of use as food	February, 2006: Approved safety of use as feed
This tack line	November, 2009: Pending application	2009 Scheduled for application	2010 Scheduled for notification

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

Figure 3 Process of rearing this stack line cotton

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

1) Place where the replication product of transferred nucleic acid exists

It has been confirmed in both GHB614 and LLCotton25 that the transferred nucleic acid existed on the cotton genome.

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

By Southern blotting analysis, it has been confirmed in both GHB614 and LLCotton25 that one copy of the T-DNA region was transferred into the genome DNA, and that inheritance of the transferred gene was stable over multiple generations.

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

——

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

The stability of protein expression in each parent line has been confirmed by the following methods.

GHB614: ELISA analysis and glyphosate herbicide spraying test

LLCotton25: ELISA analysis and glufosinate herbicide spraying test

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

Neither the nucleic acid transferred into GHB614 nor LLCotton25 has transmission-associated DNA sequence, considered likely to be transmitted in natural environments to wild animals and plants, and others.

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

GHB614 and LLCotton25 can be identified by PCR method that employs primers corresponding to its surrounding region of their transferred DNA sequence.

To detect and identify this stack line requires to conduct analysis with both the methods for each cotton seed or plant body; if both the results are positive, the subject can be regarded as this stack line.

(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 stack line has the following traits for each parent line:

GHB614: Glyphosate-herbicide tolerance conferred by the *2mepsps* gene

LLCotton25: Glufosinate-herbicide tolerance conferred by the modified *bar* gene

The 2mEPSPS protein, which is expressed in GHB614, is an enzyme that binds to both phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) to catalize reaction synthesizing 5-enol-pyruvyl-shikimate-3-phosphate (EPSP) in the shikimate pathway in the same way as EPSPS. It has been reported that, since EPSPS is not a rate-limiting enzyme in the shikimate pathway, increase in EPSPS activity leads to no excess production of the aromatic amino acid, which is the final product of this pathway (Reference 38). The 2mEPSPS and the EPSPS proteins show a similar *Km* value for their substrates, or PEP and S3P (Table 3, p. 7), considered to be equivalent in substrate specificity. Although known to have reactivity with shikimic acid, which is an analog of S3P, EPSPS is considered to have high substrate specificity, because this reactivity is low (Reference 16). Thus, the 2mEPSPS protein has also high substrate specificity, considered unlikely to affect the metabolic system of the recipient organism.

The modified PAT protein, which is expressed in LLCotton25, is an enzyme that can inactivate glufosinate by transferring the acetyl group to it. While glufosinate is classified into L-amino acid, the modified PAT protein cannot transfer the acetyl group to various amino acids. Although glutamic acid is similar to glufosinate especially in structure, the modified PAT protein cannot cause substantial transfer reaction with glutamic acid in vivo, because having little affinity for glutamic acid (Reference 42). It has been reported that the modified PAT protein is not prevented from transferring the acetyl group to glufosinate even in the excessive presence of various amino acids (Reference 45). Thus, the modified PAT protein has high substrate specificity for glufosinate, considered unlikely to affect the metabolic system of the recipient organism.

For this stack line, therefore, it is considered unlikely that these proteins interact

with each other to affect the metabolic system of the recipient organism.

Actually, in order to confirm that there are no interactions between the 2mEPSPS and the modified PAT proteins in this stack line, biological assays by herbicide spraying were performed in the U. S. in 2008 (Table 6, p. 17).

Biological assay with glyphosate herbicide

After grown to the 2nd to 3rd true-leaf stage under room temperature, seedlings of this stack line, GHB614, and the non-recombinant cotton were sprayed with glyphosate herbicide at the standard concentration [active ingredient: 0.75 lb (340 g)/acre], 8-time, 16-time, and 32-time concentrations. Seven and 14 days later, extent of phyto-toxicity was rated. In all the test plots, neither different point nor statistically significant difference in the extent of phyto-toxicity was observed between this stack line and GHB614 (Table 6, p. 17).

Biological assay with glufosinate herbicide

After grown to the 2nd to 3rd true-leaf stage under room temperature, seedlings of this stack line, LLCotton25, and the non-recombinant cotton were sprayed with glufosinate herbicide at the standard concentration [active ingredient: 0.52 lb (236 g)/acre], 8-time, 16-time, and 32-time concentrations. Seven and 14 days later, extent of phyto-toxicity was rated. Fourteen days compared with 7 days after the spray, a little progress in herbicide injury was observed in the non-recombinant cotton of the standard-concentration test plot. In all the test plots, from 7 days after the spray, changes in the extent of phyto-toxicity were not observed in this stack line or LLCotton25 (Table 6, p. 17). In the standard-, 16-time-, and 32-time-concentration plots, no different points were observed between this stack line and LLCotton25. In the 8-time concentration [active ingredient: 4.16 lb (1887 g)/acre] plot, statistically significant difference was observed between this stack line and LLCotton25; however, it seems difficult to attribute this difference to the interaction between the 2mEPSPS and the modified PAT proteins.

These resutlts suggest that no interaction may occur between the 2mEPSPS and the modified PAT proteins in this stack line, and that the two traits that this stack line obtained may have not changed through the crossing.

Thus, difference in physiological or ecological properties between this stack line and cotton, which is a taxonomical species to which the recipient organism belongs, will be estimated, based on the results of the individual examination of the parent lines, or GHB614 and LLCotton25.

Table 6 **Rating of the extent of phyto-toxicity caused by harbicide spray [1] (mean value ± standard deviation)**

Glyphosate herbicide				
	Standard concentration[2]	8-time concentration	16-time concentration	32-time concentration
7 days after the spray				
This stack line	0±0 (10)[5]	2.33±0.50 (9)	3.44±0.53 (9)	4.30±0.48 (10)
GHB614	0±0 (9)	2.00±0.71 (9)	3.00±0.54 (8)	4.13±0.35 (8)
Significant difference[4]	—	ns	ns	ns
Non-recombinant cotton	0.63±1.19 (8)	3.00±0 (7)	4.67±0.50 (9)	5.00±0 (10)
14 days after the spray				
This stack line	0±0 (10)	2.33±0.50 (9)	3.44±0.53 (9)	4.30±0.48 (10)
GHB614	0±0 (9)	2.00±0.71 (9)	3.00±0.54 (8)	4.13±0.35 (8)
Significant difference[4]	—	ns	ns	ns
Non-recombinant cotton	1.88±0.64 (8)	3.00±0 (7)	5.00±0 (9)	5.00±0 (10)

Glufosinate herbicide				
	Standard concentration[3]	8-time concentration	16-time concentration	32-time concentration
7 days after the spray				
This stack line	0±0 (10)	1.50±0.53 (10)	2.00±0 (10)	3.00±0 (10)
LLCotton25	0±0 (10)	1.00±0 (10)	2.00±0 (10)	3.00±0 (10)
Significant difference[4]	-	s	—	—
Non-recombinant cotton	4.00±0 (7)	5.00±0 (4)	5.00±0 (9)	5.00±0 (9)
14 days after the spray				
This stack line	0±0 (10)	1.50±0.53 (10)	2.00±0 (10)	3.00±0 (10)
LLCotton25	0±0 (10)	1.00±0 (10)	2.00±0 (10)	3.00±0 (10)
Significant difference[4]	-	s	—	—
Non-recombinant cotton	4.14±0.38 (7)	5.00±0 (4)	5.00±0 (9)	5.00±0 (9)

[1]: Rating of the extent of phyto-toxicity (visual evaluation)

0 = <10% phyto-toxicity may show trace amount of bronzing to the cuticle of cotyledons

1 = 10-20% phyto-toxicity, moderate bronzing of cotyledons, trace etching of the true leaves

2 = 21-40% phyto-toxicity, moderate bronzing of cotyledons, minor etching and curling of the true leaves.

3 = 41-60% phyto-toxicity, moderate to severe bronzing and necrosis of the cotyledons, moderate etching and curling of true leaves.,

4 = 61-80% phyto-toxicity, moderate to severe etching, curling and necrosis to cotyledons and true leaves.

5 = 81-100% phyto-toxicity, severe chlorosis, necrosis and leaf drops to cotyledons and true leaves

[2]: Active ingredient 0.75 lb (340 g)/acre

[3]: Active ingredient 0.52 lb (236 g)/acre

[4]: Mann-Whitney U test (significance level of 5%) ns: There were no statistically significant differences between this stack line and its parent lines. s: There were statistically significant differences between this stack line and its parent lines. —: In this rating, as variance is not recognized, the statistical analysis was impossible in this test.

[5]: The figure in parenthesis shows the number of seedling individuals that were used for the spraying test after the growth following germination; ten seeds were sowed.

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

GHB614 and LLCotton25 were compared with the non-recombinant control cotton in physiological or ecological properties.

GHB614: In 2008, isolated field testing was conducted at the National Institute for Agro-Environmental Sciences (Appendix 1). In 2007, cold-tolerance at the early stage of growth was examined at a special screened greenhouse in Japan (Appendix 2).

LLCotton25: In the fiscal year 2003, isolated field testing was conducted at the National Agricultural Research Center for Kyushu Okinawa Region, the National Agricultural Research Organization (today, the National Agricultural Research Center for Kyushu Okinawa Region, the National Agriculture and Food Research Organization) (Appendix 3). In 2002, fertility and size of the pollen were evaluated in France (Appendix 4).

(a) Morphological and growth characteristics

Evaluation items associated with morphological and growth characteristics are shown in Table 7 (p. 20).

For GHB614, statistically significant difference from the non-recombinant control cotton was observed in the germination rate of the seeds used for the isolated field testing (hereinafter referred to as "seeds for growing experiment") (Appendix 1, p. 11, Table 4). However, this difference is considered to be attributed to different sampling locations of the two lines of seeds, and the unseasonable broken before the harvesting of the non-recombinant cotton.

For LLCotton25, statistically significant difference from the non-recombinant control cotton was observed in the plant height after 60 days of the seeding and in the number of nodes after 60 and 120 days of the seeding. However, no statistically significant differences was not observed at the other evaluation days in the plant height or the number of nodes (Appendix 3, p. 8, Table 3).

Table 7 Evaluation items associated with morphological and growth characteristics of GHB614 and LLCotton25

Item	GHB614	LLCotton25
Uniformity of germination	○	○
Flowering time	○	○
Boll opening time	○	○
Harvesting time	○	○
Leaf shape	○	○
Plant shape	○	○
Flower shape and color	○	○
Boll shape	○	○
Lint color	○	○
Seed shape	○	○
Seed color	○	○
Germination rate	○*	○
Leaf length	○	○
Leaf width	○	○
Plant height	○	○*
Number of appearing flower buds / Effective number of flower buds	○	○
Number of nodes	○	○*
Number of vegetative branches	○	-
Number of fruiting branches	○	○
Total number of branches	○	○
Number of harvested bolls per plant	○	○
Number of non-harvested bolls per plant	○	○
Total number of bolls per plant	○	○
Weights of aerial-ground parts	○	○
Weights of under-ground parts	○	○
Boll length	○	○
Boll width	○	○
Boll weight	○	○
Number of segments of a boll	○	○
Number of seeds per boll segment	-	○
Number of seeds per boll	○	○
100-kernel weight	○	-

○: Evaluation was conducted.

—: No evaluation was conducted.

*:Some test plots showed statistically significant differences (significance level of 5%). For the detailed information, see I-6-2)-(a) (p. 28) and II-1-(1) (pp. 33-34).

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

Both the young plant bodies of GHB614 and LLCotton25 died under the low-temperature conditions (4 to 5°C) like the non-recombinant control cotton (Appendix 2, p. 9, Table 13, and Appendix 3, p. 18, Table 12).

(c) Over wintering ability and summer survival of the mature plant

Both GHB614 and LLCotton25 continued to be grown also after the harvest time at the isolated field in Japan, and were found dead when they were observed in February of the following year (Appendix 1, p. 13, and Appendix 3, p. 18).

(d) Fertility and size of the pollen

GHB614 and LLCotton25 were compared with the non-recombinant control cotton in the fertility and size of the pollen; neither GHB614 nor LLCotton25 showed statistically significant differences or different points (Appendix 1, p. 11, Table 4, and p. 12, Figure 7; Appendix 4, p. 9, Tables 1-2, and p. 14, Figures 9-11).

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

Concerning seed production, GHB614 and LLCotton25 were compared with the non-recombinant control cotton in the number of harvested bolls per plant, the total number of bolls per plant, and the number of seeds per boll; neither GHB614 nor LLCotton25 showed statistically significant differences (Appendix 1, p. 11, Table 4, and Appendix 3, p. 14, Table 9).

Concerning the shedding habit, cotton seeds are considered to have a small chance of shedding from the open bolls, because having difficulty of separating from them owing to the entwined lint (Reference 55). In the isolated field testing, GHB614 showed no shedding of the seeds like the non-recombinant control cotton (Appendix 1, p. 18, Table 3). LLCotton25 is confirmed not to be different from the non-recombinant control cotton in the morphology and opening properties of bolls, although LLCotton25 was not examined for the shedding habit (Appendix 3, pp. 11-13).

GHB614 and LLCotton25 seeds harvested in the isolated field were used to evaluate their dormancy and germination rate. For GHB614, the just-harvested seeds and those stored under room temperature for 3 months after the harvesting were compared in the germination rate with the corresponding seeds of the non-recombinant control cotton. Between these lines, there were no statistically significant differences; the 3-month-stored

seeds of both the lines showed a germination rate of 96% or more (Appendix 1, p. 19, Table 9). For LLCotton25, the seeds stored under room temperature for approximately 1 month after the harvesting were compared in the germination rate with those of the non-recombinant control cotton. The seeds of both the lines showed a germination rate of 100% (Appendix 3, p. 17, Table 11, and p. 18, Table 12).

(f) Crossability

Since the related species able to cross with cotton have not grown voluntarily in Japan, crossability of GHB614 was not examined. For reference, however, the crossability between LLCotton25 and the non-recombinant cotton cultivated 1 m apart was evaluated in the isolated field testing to confirm no possibility of their crossing (Appendix 3, p. 17, Table 11).

(g) Productivity of harmful substances

Both GHB614 and LLCotton25 underwent the succeeding crop test, plow-in test, and soil microorganism test. Neither GHB614 nor LLCotton25 showed statistically significant differences from the non-recombinant control cotton in various evaluation items concerning germination and growth of radish, which was used as a test plant in the succeeding crop test and plow-in test, and in the number of living soil microorganisms (Appendix 1, pp. 16-18, Tables 6-8, and Appendix 3, pp. 22-28, Tables 17-30).

3. Information concerning the use of living modified organisms

(1) Content of the use

Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them

(2) Method of the use

———

(3) Method by which the applicant for approval collects information after starting the Type 1 Use

———

(4) Action to avoid Adverse Effect on Biological Diversity when it may occur

See the Emergency Action Plan.

(5) Results of the use in environments similar to those in which the use in laboratories or the Type 1 Use is planned

—

(6) Information on the use in foreign countries

The state of application and approval of GHB614 and LLCotton25 in foreign countries is summarized in Table 8; the state of application and approval of GHB614, LLCotton25, and this stack line in Japan is summarized in Table 5 (p. 13).

Table 8 The state of approval of GHB614 and LLCotton25 in foreign countries

Country	Regulatory agency	Year and month of approval	
		GHB614	LLCotton25
U. S.	United States Department of Agriculture (USDA)	May, 2009: Confirming safety	March, 2002: Confirming safety
	United States Food and Drug Administration (FDA)	April, 2008: Confirming safety	April, 2003: Confirming safety
Canada	Health Canada	April, 2008: Confirming safety	August, 2004: Confirming safety
	Canadian Food Inspection Agency (CFIA)	April, 2008: Confirming safety	September, 2004: Confirming safety

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

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.

This stack line cotton has been produced by crosssing GHB614 with LLCotton25 in accordance with the crossbreeding method. The Biological Diversity Risk Assessment Meeting judged that neither of these parent lines might exert Adverse Effect on Biological Diversity, if used in accordance with the Type 1 Use Regulation, which is to be applied also to this stack line cotton.

Both the 2mEPSPS and the modified PAT proteins have high substrate specificity, considered unlikely to affect the metabolic system of the recipient organism. Moreover, thoses proteins are known to function independently by their own action mechanism. Therefore, it was considered unlikely that the proteins expressed in this stack line cotton from individual parent lines would additionally affect the metabolic pathway of plants.

Actually, comparison was made in glyphosate-herbicide tolerance between this stack line cotton and one parent line GHB614, as well as in glufosinate-herbicide tolerance between this stack line cotton and the other parent line LLCotton25. In the test plots sprayed with the standard-, 16-time-, and 32-time-concentration glufosinate herbicide, there were no different points between this stack line and LLCotton25. In the test plot sprayed with the 8-time-concentration glufosinate herbicide, statistically significant difference was observed between this stack line and LLCotton25; however, it seems difficult to attribute this difference to the interaction between these proteins. In all the test plots sprayed with the glyphosate herbicide, neither different points nor statistically significant differences were observed between this stack line and GHB614. Thus, the 2mEPSPS and the modified PAT proteins are considered unlikely to affect each other in the plant body of this stack line cotton.

Based on the above understanding, it is considered unlikely that notable changes in traits have occurred in this stack line cotton, except for the traits it received from both the parent lines.

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 imported to Japan and used for processing for a long time. However, there is no report that cotton has grown voluntarily in natural environments.

As traits related to the competitiveness of GHB614 and LLCotton25, which are a parent line of this stack line, the following were evaluated: the morphological and growth characteristics, cold-tolerance at the early stage of growth, overwintering ability of the matured plant, fertility and size of the pollen, production, shedding habit, dormancy, and germination rate of the seeds. For GHB614, a statistically significant difference from the non-recombinant control cotton was observed in the germination rate of the seeds for cultivation experiment. However, this difference is considered to be attributed to different harvesting locations of the two lines of seeds, and the broken weather before the harvesting of the non-recombinant cotton. No statistically significant differences were observed in the germination rate of the seeds harvested in the isolated field between GHB614 and the non-recombinant control cotton. This suggests that the difference in the germination rate of the seeds for cultivation experiment may not be attributed to the genetic transformation. For LLCotton25, plant height and the number of nodes were evaluated on four different days. The first and second evaluation days saw a statistically significant difference from the non-recombinant control cotton in the plant height and the number of nodes, respectively. However, such differences are repeated as inconstant, because the other evaluation days saw no statistically significant differences in these evaluation items between the lines. In the other traits, moreover, neither GHB614 nor LLCotton25 showed different points or statistically significant differences from the non-recombinant control cotton. Thus, it seems impossible that only the statistically significant differences in the above-mentioned traits increase the competitiveness of this stack line cotton.

While having tolerance to both the glyphosate and glufosinate herbicides, this stack line cotton is unlikely to increase its competitiveness in natural environments where the glyphosate/glufosinate herbicide cannot be expected to be sprayed.

Eventually, the applicant's conclusion is considered reasonable that this stack line cotton might not exert Adverse Effect on Biological Diversity because of the competitiveness.

(2) Productivity of harmful substances

Cotton seeds contain gossypol, which exhibits toxicity in nonruminant animals, and

cyclopropene fatty acid, which causes discoloration and decreases hatchability in hen eggs by suppressing desaturation of the saturated fatty acids. There is no report that cotton seeds are eaten by wild animals. However, cotton seeds are not known to produce allelpathic substances that could affect the inhabitation or growth of wild animals and plants.

It has been confirmed that both the 2mEPSPS and the modified PAT proteins, which are expressed in this stack line cotton, have no sequence homology to the known allergens structurally.

Moreover, both the 2mEPSPS and the modified PAT proteins have high substrate specificity; it is considered impossible that these proteins act on the metabolic system of the recipient organism to produce harmful subatances.

Actually, the succeeding crop test, soil microflora test, and plow-in test were performed to examine GHB614 and LLCotton25 for the ability to produce harmful substances (substances that are secreted from roots to affect other plants and soil microorganisms, substances contained in plant bodies that affect other plants after their death, etc.),and compare these lines with the control in this ability. In all these tests, neither of these lines showed statistically significant differences from the control, considered likely to have newly obtained the ability to produce harmful substances.

Eventually, the applicant's conclusion is considered reasonable that this stack line cotton might not exert Adverse Effect on Biological Diversity because of productivity of harmful substances.

(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

On the basis of this discussion, the conclusion of the Biological Diversity Risk Assessment Report is considered reasonable that, in Japan, this stack line cotton might not exert Adverse Effect on Biological Diversity, if used in accordance with the Type 1 Use Regulation.