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	Soybean tolerant to herbicide glyphosate and acetolactate synthase inhibitors (*gat4601*, *gm-hra*, *Glycine max* (L.) Merr.) (DP-356Ø43-5, OECD UI：DP-356Ø43-5)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid in the soybean tolerant to herbicide glyphosate and acetolactate synthase inhibitors[1] (*gat4601*, *gm-hra*, *Glycine max* (L.) Merr.) (DP-356Ø43-5, OECD UI : DP-356Ø43-5) (hereinafter referred to as "this recombinant soybean") and the origins of component elements are shown in Table 1. In addition, the nucleotide sequences of donor nucleic acid and amino acid sequences are shown in Annex 2.

2) Functions of component elements

(a) Functions of target genes, expression-regulating regions, localization signals, selective markers and other component elements of donor nucleic acid

Functions of component elements of donor nucleic acid are shown in Table 1.

[1] Acetolactate synthase inhibitors include thifensulfuron methyl and chlorimuron ethyl.

Table 1 Composition of donor nucleic acid and the origins and functions of component elements

Component element	Size (bp)	Origin and function
gat4601 gene expression cassette		
SCP1 Promoter	499	Constitutive expression promoter constructed based on a part of 35S promoter region of cauliflower mosaic virus (CaMV) (O'Dell *et al*., 1985) and Rsyn7-SynCoreII promoter (Bowen *et al*., 2000 and 2003) to induce transcription.
TMV omega 5'UTR	67	Enhancer region to promote transcription, derived from tobacco mosaic virus (TMV) omega 5' untranslated region (Gallie and Walbot, 1992).
gat4601 (Modified *gat*)	441	Glyphosate *N*-acetyltransferase gene (modified *gat*: hereinafter referred to as "*gat4601*") produced by modifying nucleotide sequences and amino acid sequences to enhance the *N*-acetylation catalytic activity of herbicide glyphosate based on *N*-acetyltransferase derived from *Bacillus licheniformis* (Castle *et al*., 2004、Keenan *et al*., 2005, GenBank Accession No: AY597417). It encodes the modified GAT protein (hereinafter referred to as "GAT4601") composed of 146 amino acids with a molecular weight of 17kDa.
*pin*II Terminator	316	A terminator region in the protease inhibitor II (*pin*II) gene derived from potato to terminate transcription (Keil *et al*., 1986; An *et al*., 1989).
gm-hra gene expression cassette		
SAMS Promoter	645	Promoter region to induce transcription of S-adenosyl-L-methionine synthetase (SAMS) gene derived from soybean (Falco and Li, 2003).
SAMS Intron	591	Intron region present in the 5' untranslated region of SAMS gene derived from soybean (Falco and Li, 2003).
gm-hra (Modified *als*)	1971	Modified gene (modified *als*: hereinafter referred to as "*gm-hra*") derived from acetolactate synthase gene (*gm-als*) of soybean. It encodes the modified ALS (hereinafter referred to as "GM-HRA") protein composed of 656 amino acids, having a molecular weight of 71kDa. In the N-terminal region of GM-HRA protein, five (5) amino acids (methionine-proline-histidine-asparagine-threonine) have been newly added (Falco and Li, 2003).
gm-als Terminator	652	A terminator region to terminate the transcription possessed by the acetolactate synthase gene (*gm-als*) derived from soybean.

(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

gat4601 gene

The modified *gat* (hereinafter referred to as "*gat4601*") gene transferred to this recombinant soybean is a gene modified to enhance the *N*-acetylation catalytic activity against herbicide glyphosate based on the nucleotide sequences of *N*-acetyltransferase derived from *Bacillus licheniformis*, encoding the modified GAT (hereinafter referred to as "GAT4601") protein (Castle *et al.*, 2004 ; Siehl *et al.*, 2005; Keenan *et al.*, 2005). *B. licheniformis* is a gram-positive bacterium used for production of enzyme for foods (alpha amylase, cyclodextrin-glycosyltransferase, hemicellulase, etc.) in Japan, the US, Canada and Europe. The nucleotide sequences of *gat4601* gene (597th through 1,037th nucleotides in Appendix 1 in Annex 2) and the amino acid sequences of GAT4601 protein are shown in Annex 2.

The herbicide glyphosate inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway for the biosynthesis of aromatic amino acids of tryptophan, tyrosine and phenylalanine in plants. As a result, plants treated with glyphosate cannot synthesize the aromatic amino acids and ultimately die (Figure 1). The GAT4601 protein produced by the *gat4601* gene acetylates the NH group of herbicide glyphosate and transforms the glyphosate to the *N*-acetyl glyphosate which does not inhibit the EPSPS activity, thereby conferring the tolerance to herbicide glyphosate on plants (Figure 1).

Comparison was made for the *N*-acetylation catalytic activity against herbicide glyphosate in terms of k_{cat}/K_M as an indicator and as a result, k_{cat}/K_M was found 4.21 min^{-1} mM^{-1} for the *N*-acetyltransferase protein derived from *B. licheniformis* before modification, while k_{cat}/K_M was found 4,570 min^{-1} mM^{-1} for the GAT4601 protein, showing enhanced activity by about 1,000 times. In fact, in the field tests conducted in the US and Canada, it has been confirmed that this recombinant soybean possesses high tolerance to herbicide glyphosate. The k_{cat} refers to enzyme reaction rate constant, and K_M refers to affinity to substrate.

gm-hra gene

The modified *als* (hereinafter referred to as "*gm-hra*") gene transferred to this recombinant soybean is derived from acetolactate synthase (*gm-als*) gene of soybean, and it encodes the modified ALS (hereinafter referred to as "GM-HRA) protein which is not affected by the herbicide acetolactate synthase inhibitors. The nucleotide sequences (2,697th through 4,667th necleotids in Appendix 1 in Annex 2) of the *gm-hra* gene and the amino acid sequences of GM-HRA protein are shown in Annex 2.

Herbicide acetolactate synthase inhibitors inhibit the acetolactate synthase (ALS) activity for biosynthesis of branched chain amino acids of leucine, valine and isoleucine in plants. As a result, these branched chain amino acids are not

synthesized and plants would die (Figure 2). The GM-HRA protein produced by the *gm-hra* gene is not affected by herbicide acetolactate synthase inhibitors but exhibits the ALS activity even in the presence of the inhibitors and then, the biosynthesis of the branched chain amino acids of leucine, valine and isoleucine becomes possible, conferring the tolerance to herbicide acetolactate synthase inhibitor on plants (Figure 2). Production of recombinant crop using the modified *als* gene has been reported in rice.
(http://www.bch.biodic.go.jp/download/lmo/public_comment/AD41ap.pdf).

In order to identify that the GAT4601 protein and the GM-HRA protein do not possess any homology with known allergen proteins, comparison was made for amino acid sequences using the allergen database (FARRP6 database, Release 6, 2006 January version) provided by the Food Allergy Research and Resource Program (FARRP) of University of Nebraska in the US. This database holds the amino acid sequences of a total of 1,541 known allergens and estimated allergens. As a result, there were no known and estimated allergens observed which exhibited significant homology with the both proteins. In addition, for the GAT4601 protein and GM-HRA protein, homology search with known toxic proteins in the database (Genpept "nr" dataset, Release 153.0, 4/15/06), which holds the amino acid sequences of registered proteins without any duplication constructed based on integration of the public databases Genbank, Swiss-Prot, PIR, Protein Research Foundation and Protein Data Bankcid)] was conducted. As a result, for the both proteins, no homology with any known toxic proteins was observed.

The herbicide glyphosate is sprayed on the stems and leaves of weeds at the growing stage, and the herbicide acetolactate synthase inhibitors are sprayed in the periods before growth of weeds to the initial stage of growth to work on the soil. Today, the both herbicides are widespreadly used worldwide including Japan and the US. By cultivation of the soybeans conferred the tolerance to herbicide glyphosate by the transferred *gat4601* gene and the tolerance to herbicide acetolactate synthase inhibitors by the transferred *gm-hra* gene, it is expected that the farmers would be able to effectively utilize the two herbicides which offer different characteristics from each other and they are also given a wider choice of options for weed control.

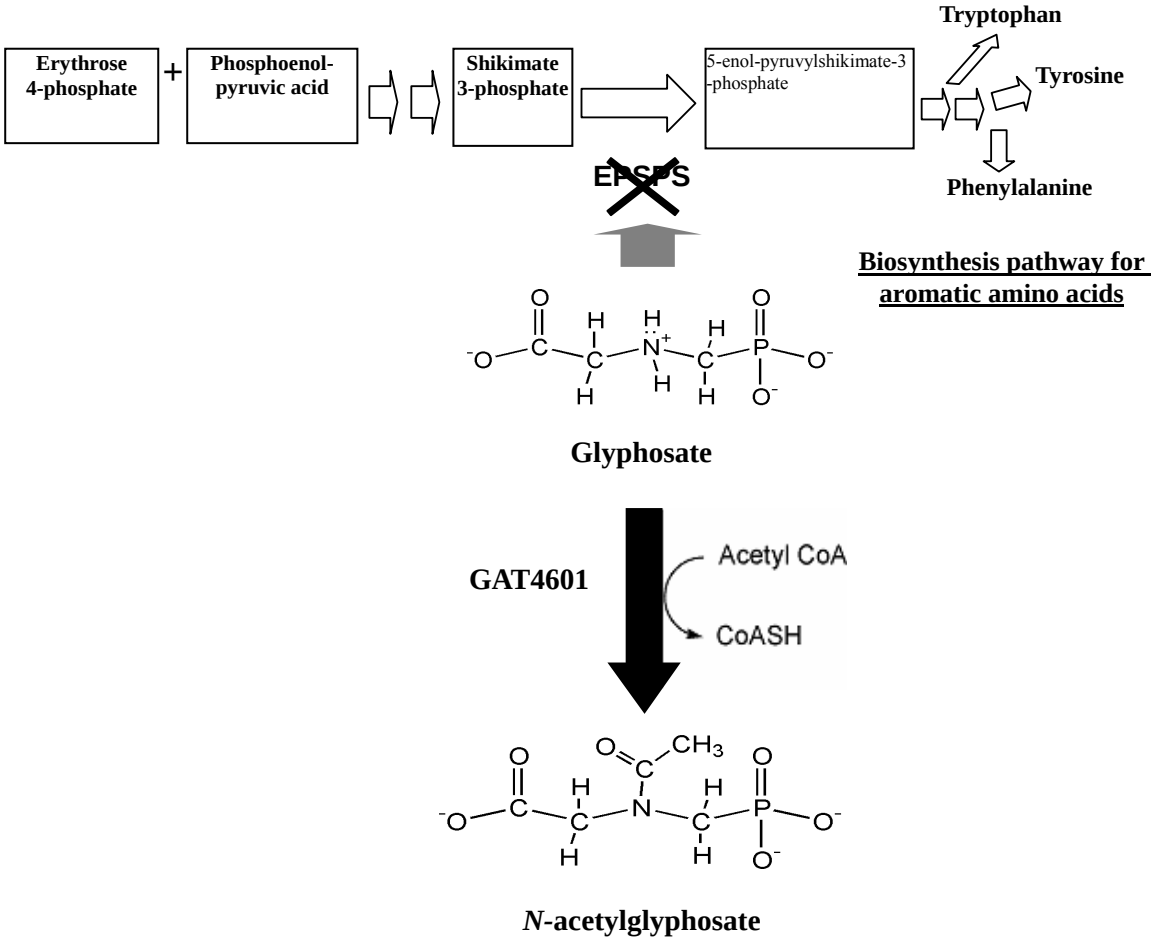

Figure 1 Mechanism of action of GAT4601 protein

The herbicide glyphosate inhibits the activity of EPSPS. As a result, plants treated with herbicide glyphosate cannot synthesize aromatic amino acids and ultimately die. On the other hand, the GAT4601 protein acetylates the NH group of herbicide glyphosate and transforms the glyphosate to the *N*-acetylglyphosate which does not inhibit the activity of EPSPS. Consequently, the biosynthesis of aromatic amino acids becomes possible.

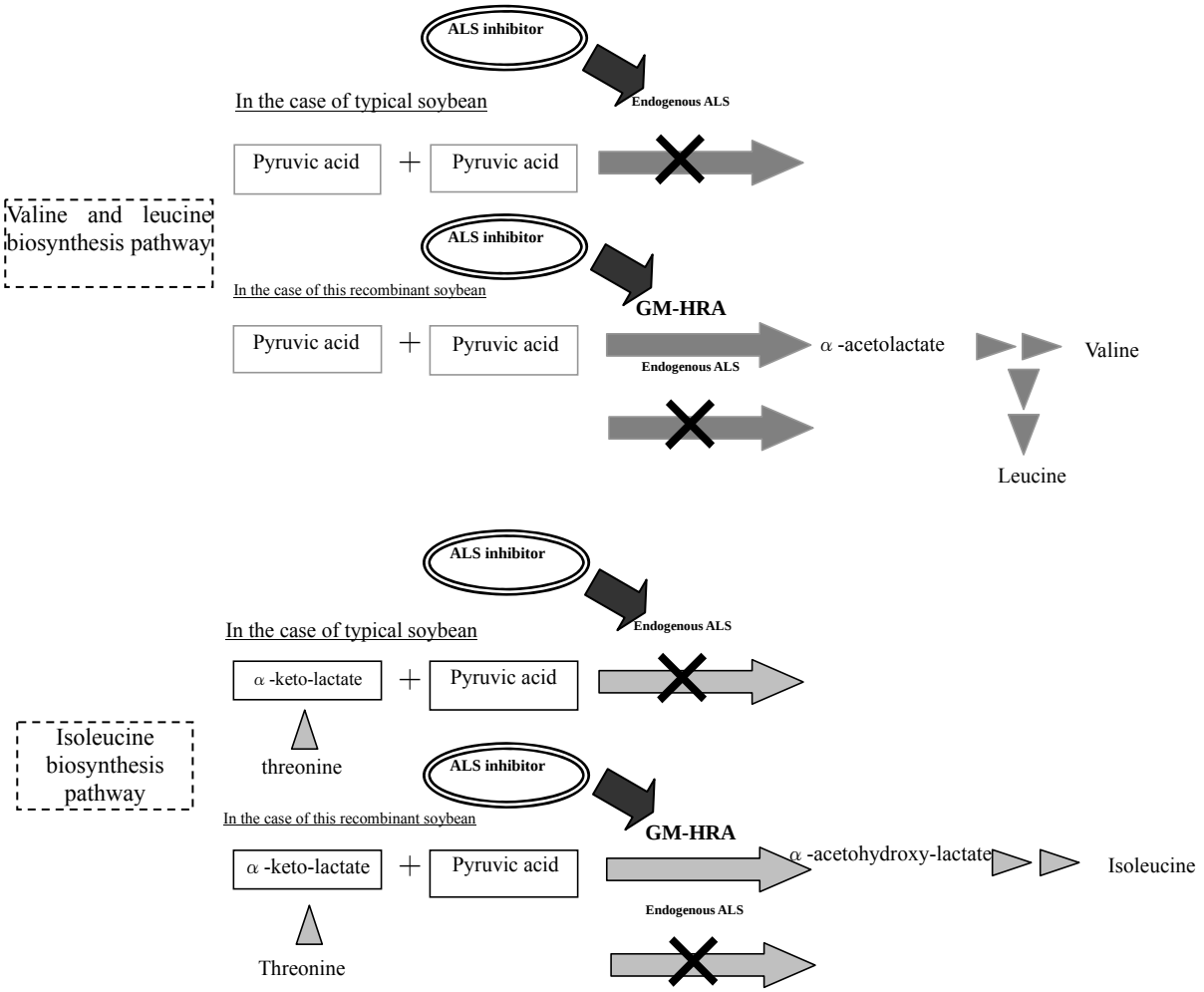

Figure 2 Mechanism of action of GM-HRA protein

Plant-intrinsic acetolactate synthase (ALS) is inhibited by the herbicide acetolactate synthase inhibitors (ALS inhibitors) and then, plants cannot synthesize the branched chain amino acids of valine, leucine and isoleucine and ultimately die. On the other hand, when the GM-HRA protein is produced in plants, plants are not affected by the herbicide ALS inhibitors and thus can synthesize the branched chain amino acids of valine, leucine and isoleucine.

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

gat4601 gene

The GAT4601 protein produced by the *gat4601* gene transferred to this recombinant soybean is a member of *N*-acetyltransferase. It is generally known that *N*-acetyltransferase acetylates the amino acid side chains of *N*-terminal amino acids of proteins and biogenic amine compounds of free amino acid and histone, and antibiotics (Glossary of Biochemistry Terms, 1998). The GAT4601 protein is a protein modified to enhance the *N*-acetylation catalytic activity against herbicide glyphosate and in fact, compared to *N*-acetyltransferase derived from microorganisms, the activity against herbicide glyphosate has been enhanced by about 1,000 times. In order to confirm that the GAT4601 protein does not affect the metabolic system of the recipient organism, evaluation and discussion were conducted as described below.

i) Crystal structure analysis

As a result of three-dimensional structure analysis of GAT4601 protein, it is found that the active center of *N*-acetylation reaction of herbicide glyphosate in GAT4601 protein is located deeply inside the protein and the herbicide glyphosate is taken into the inside, to which the coenzyme acetyl CoA binds to induce the reaction (Figure 3; Keenan *et al*., 2005, Siehl *et al*., 2007). This suggests that only the herbicide glyphosate and other low-molecular weight compounds can reach the active center of the protein without suffering any steric hindrance and become the substrate of this protein, namely that high-molecular weight compounds are unlikely to become the substrate of this protein. In the study using the GAT4602 protein[2] which possesses high homology with the GAT4601 protein, it was reported that the protein did not exhibit any *N*-acetylation catalytic activity against nucleoside, nucleotide, histone, tRNA and other high-molecular weight compounds among the biogenic amines (Siehl *et al*., 2005).

ii) Substrate reactivity test

Reactivity between the low-molecular weight compounds which can become substrate and the GAT4601 protein was examined using 20 types of agricultural chemicals (herbicides, pesticides and disinfectants) (Annex 3, Table 1), 11 types of antibiotics (kanamycin, ampicillin, etc.) (Annex 3, Table 2) and 21 types of amino acids (Annex 3, Table 3). The catalytic activity of *N*-acetylation reaction by the GAT4601 protein was determined in terms of the amount of coenzyme A, a product of reaction of acetyl CoA, the coenzyme of this reaction, accumulated in 30 minutes. As a result, against five (5) amino acids, glycine, serine, threonine, aspartic acid and glutamic acid, the catalytic activity was observed, though no catalytic activity was observed against the other compounds (Annex 3, Tables 1 to 3). Among the five amino acids against which the catalytic activity was observed, aspartic acid showed the

[2] GAT4602 protein possesses 96% identity and 99% similarity of amino acid sequences with GAT4601 protein introduced in this recombinant soybean and the similar N-acetylation catalytic activity against herbicide glyphosate (GAT4601: 4,570 min^{-1} mM^{-1}, GAT4602: 4,160 min^{-1} mM^{-1}).

highest catalytic activity, which was, though, only about 3% of the catalytic activity obtained when the glyphosate was selected as a substrate (Annex 3, Table 3).

Then, assuming the five types of amino acids to which the catalytic activity was observed and the additional six types of compounds which have the very similar structures to the herbicide glyphosate become the substrate of the GAT4601 protein, the value of k_{cat}/K_m of GAT4601 protein was measured (Annex 3, Table 4). This test was conducted with 100mMKCl added to the reaction solution to attain the *in vivo* conditions as possible. As a result, the k_{cat}/K_m value of GAT4601 protein against herbicide glyphosate was found 267 $min^{-1}mM^{-1}$. In addition, among the five amino acids, aspartic acid and glutamic acid exhibited about 3% of catalytic activity against herbicide glyphosate, though in the test conditions close to the *in vivo* conditions, catalytic activity was not observed in threonine, serine, and glycine (Annex 3, Table 4).

In addition, among the compounds similar to herbicide glyphosate, against only the D-2-amino-3-phosphonopropionate, the catalytic activity was observed, though the catalytic activity was about 5% of the catalytic activity against herbicide glyphosate, and against the L-2-Amino-3-phosphonopropionate, an optical isomer of the herbicide glyphosate, no catalytic activity was observed (Annex 3, Table 4).

Based on the above understanding, it was considered that GAT4601 protein possesses very high substrate specificity to herbicide glyphosate.

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

Figure 3 Structure of GAT4601 protein and the reactive site with herbicide glyphosate

iii) Amino acid analysis

The GAT4601 protein is considered to have high substrate specificity to herbicide glyphosate though as mentioned above, it is confirmed to exhibit about 3% of the catalytic activity against herbicide glyphosate against aspartic acid and glutamic acid. Then, this recombinant soybean was compared with non-recombinant control soybean for amino acid content. The amino acid analysis on the seeds and the forage was conducted in a total of 18 repeats based on sampling of 3 repeats each from 6 fields in the North America. As a result, regarding the amino acid content in the seed and the forage, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Table 2).

Table 2 Amino acid analysis

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In order to examine practical equivalence as food and feed, detail analysis on components has been conducted. In the analysis, GAT4601 protein exhibited weak catalytic activity against aspartic acid and glutamic acid. Then analysis was made for *N*-acetylaspartic acid and *N*-acetylglutamic acid contained in free amino acid and as a result, it was found that the content of *N*-acetylaspartic acid and *N*-acetylglutamic acid is significantly higher in this recombinant soybean compared to conventional soybean (Annex 4, Table 1). Free amino acid is reported to account for less than 1% of the total amino acid content in soybean seed (Takashi *et al*., 2003), though the content of free amino acid in the soybean seeds of both this recombinant soybean and the non-recombinant soybean were found about 0.5% of the total amino acid content, showing no statistically significant difference. In addition, for the composition of free amino acid, a statistically significant difference was observed in proline and valine, though in the other free amino acids, no statistically significant difference was observed between this recombinant soybean and the non-recombinant soybean (Annex 4, Table 2). Also for the proline and valine for which a significant difference was observed, the difference was found falling within the tolerance interval observed in commercial cultivars.

As such, a small amount of free aspartic acid and free glutamic acid were found acetylated in this recombinant soybean, though it was suggested that the content of acetylated amino acid did not affect the total amount and composition of free amino acid. In addition, based on the findings that there was no difference in content and composition of amino acid comprising the protein (Table 2) and that the growth characteristics of this recombinant soybean was found equivalent to that of the non-recombinant soybean from the isolated field test in Japan in FY 2006 and the field test conducted in FY 2005 in the US, it was judged that the productivity of GAT4601 protein by the *gat4601* gene does not affect the metabolic system of the recipient organism.

As can been seen from the fact that even from the non-recombinant soybean, *N*-acetylaspartic acid and *N*-acetylglutamic acid were detected, the acetylated amino acids are produced in many organisms including plants and animals, and the acetylation of *N*-terminal amino acid induced by posttranslational modification of cytoplasmic protein, for example, is the most typical event (Persson *et al*., 1985; Polevoda and Sherman, 2002). In addition, there is no report that *N*-acetylaspartic acid and *N*-acetylglutamic acid serve as harmful substances.

gm-hra gene

The GM-HRA protein produced by the *gm-hra* gene works in the biosynthetic pathway of branched amino acids of leucine, valine and isoleucine, taking the place of endogenous ALS inhibited by the herbicide acetolactate synthase inhibitor (Figure 2). In the biosynthetic pathway of valine and leucine among the synthetic pathways of branched chain amino acids, ALS is subject to feedback control by valine. On the other hand, in the isoleucine biosynthetic pathway, it is generally known that in addition to the ALS feedback control by valine, the threonine dehydratase, a catalytic enzyme at the initial stage, is feedback-controlled by

isoleucine (Glossary of Biochemistry Terms, 1998). Therefore, even if the catalytic activity of ALS is enhanced by the GM-HRA protein and as a result, the amount of branched chain amino acid to be synthesized is increased, it is considered that the content of any specific amino acids is regulated by the action of feedback control. In practice, regarding the branched chain amino acids of the seeds of this recombinant soybean, no statistically significant difference was observed (Table 2). Consequently, it was judged that production of GM-HRA protein in this recombinant soybean does not affect the metabolic system of the recipient organism.

The *gat4601* gene takes part in acetylation of herbicide glyphosate, while the *gm-hra* gene takes part in biosynthesis of branched chain amino acids, therefore, it is considered unlikely that expression of the both genes would affect with each other.

(2) Information concerning vector

1) Name and origin

Name and origin of the vector which was the basis for PHP20163A, the linear DNA fragment composed of the *gat4601* gene expression cassette and *gm-hra* gene expression cassette, is as follows.

Name: PHP20163
Origin: Constructed based on the plasmid pUC19 derived from *Escherichia coli* (*E. coli*).

PHP20163A is a linear DNA fragment composed of *gat4601* gene expression cassette and *gm-hra* gene expression cassette ([SCP1 Promoter]-[TMV omega 5'UTR]-[*gat4601*]-[*pin*II Terminator]-[SAMS Promoter] -[SAMS Intron]- [*gm-hra*] -[*gm-als* Terminator]) extracted by treating the plasmid PHP20163 with restriction enzyme (Figure 4).

2) Properties

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

The total number of base pairs of linear DNA fragment PHP20163A is 5,362 bp. The entire nucleotide sequences of the plasmid are shown in Annex 2.

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

PHP20163A is a linear DNA fragment composed of only the target gene cassette region, without containing any sequences which possess the other specific functions.

(c) Presence or absence of infectious characteristics of vector

For PHP20163A used for transferring, the entire nucleotide sequences have

been clarified, and no sequence is contained that allows transfer to other microorganisms; therefore, there is no infectivity of vector.

(3) Method of preparing living modified organisms

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

Locations and orientations of component elements of the nucleic acid in the PHP20163A used for the transferring and the restriction enzyme cleavage sites are shown in Figure 4.

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

Figure 4 Composition of nucleic acid in the linear DNA fragment PHP20163A and the restriction enzyme cleavage sites

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

The particle gun bombardment method was used to transfer the nucleic acid into the recipient organism (Klein *et al*., 1987).

3) Processes of rearing of living modified organisms

This recombinant soybean is genetically modified soybean developed by the US Pioneer Hi-Bred International, Inc., and the process of selection and rearing is as described below.

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

Process of selection of transformant is shown in Figure 5.

(b) Presence or absence of remaining Agrobacterium

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(c) Processes of rearing and pedigree trees

Process of rearing of this recombinant soybean is shown in Figure 6. The scope of application for approval of this recombinant soybean includes the progeny lines of T1 and later generations shown in the process of rearing.

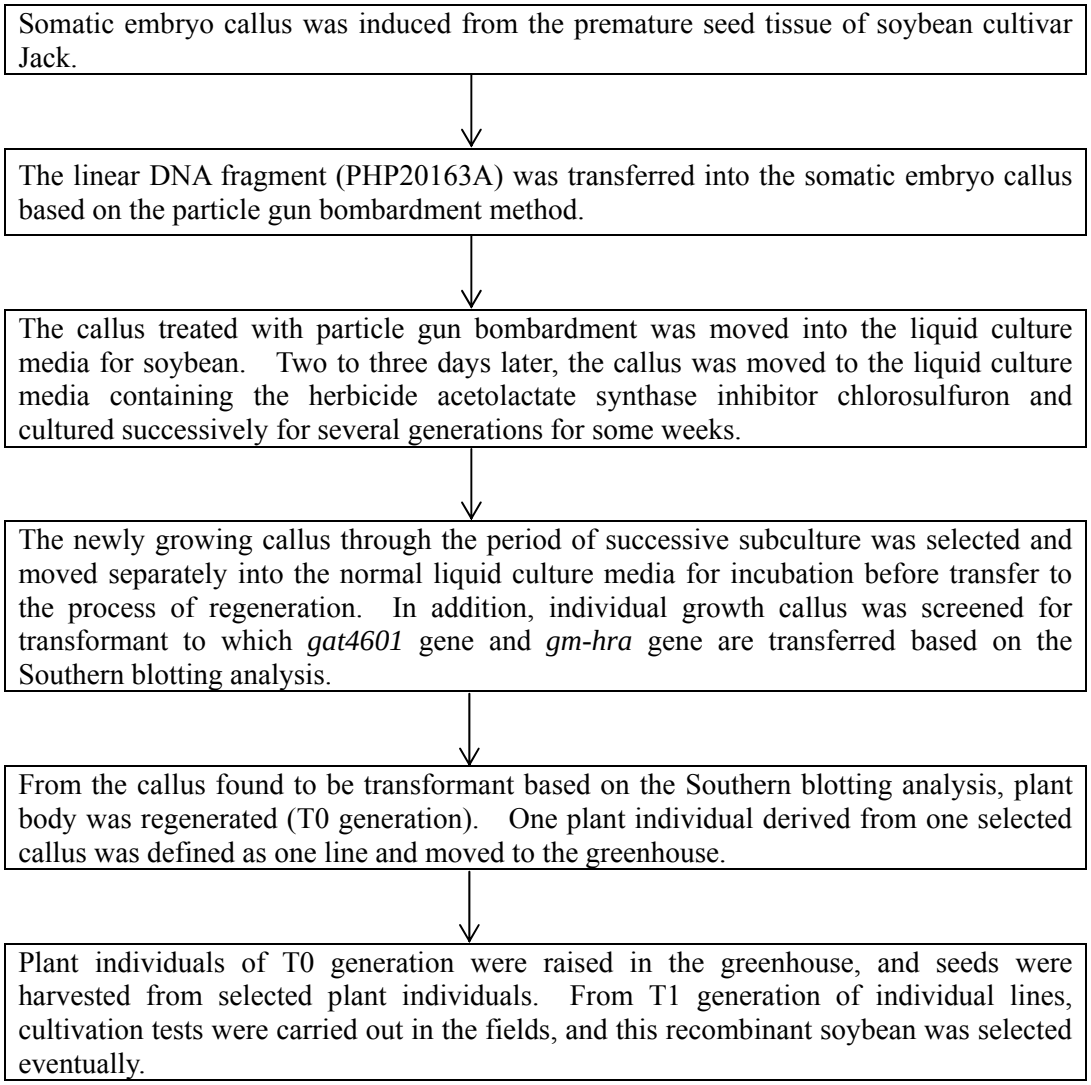

Figure 5 Processes of transferring the nucleic acid into the recipient organism and of selecting this recombinant soybean

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

Figure 6 Process of rearing of this recombinant soybean

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

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

This recombinant soybean of T1 and F3 generations was examined for presence of *gat4601* gene based on the PCR method and Western blotting analysis. As a result, the mode of inheritance of the transferred nucleic acid exhibited a segregation ratio corresponding to 3:1 (Table 3). In addition, in the F3 generation, the same plant body used in the Western blotting analysis was examined for segregation ratio of *gm-hra* gene based on the Southern blotting analysis and as a result, it similarly exhibited a segregation ratio corresponding to 3:1. As such the transferred nucleic acid is found stably inherited in accordance with the Mendel's laws, therefore, it is confirmed that the transferred nucleic acid exists on the genome of soybean.

Table 3 Analysis for the segregation ratio of transferred gene

Generation tested	Test method	*gat4601* gene-positive individual	*gat4601* gene-negative individual	*gat4601* gene-positive individual expected value	*gat4601* gene-negative individual expected value	P-value
T1	PCR method	59	23	61.5	20.5	0.61
F3	Western blotting analysis	75	15	67.5	22.5	0.09

The parent individuals of the generations tested were seeds derived from the individuals which are heterozygous for the target gene and screened for the presence of *gat4601* gene based on the PCR method for the T1 generation and Western blotting analysis for the F3 generation. Based on the assumption that when the transferred gene exists on the genome at one site, an expected value of segregation ratio becomes 3:1, chi-square (χ^2) test was conducted.

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

In order to identify the number of copies of the transferred nucleic acid to this recombinant soybean and the stability of its inheritance, the Southern blotting analysis was conducted for the inbred posterity T4 and T5 generations (see Figure 6). The analysis was conducted in 2005 using the plants cultivated by the US Pioneer Hi-Bred International, Inc. DNA samples were extracted from the leaves of 30 and 24 individuals of the inbred posterity T4 and T5 generations respectively and cleaved by the restriction enzyme *Hin*d III. The transferred PHP20163A contains the *Hin*d III site at two locations, the border between *pin*II Terminator and SAMS Promoter (1,378th) and the intermediate segment of *gm-hra* gene (3,796th) (see Figure 4). As probes, the *gat4601* gene region and the *gm-hra* gene region

were used. The analytical results of the Southern blotting analysis are summarized in Table 4 and detailed in Annex 5.

When the *gat4601* gene region is used as a probe, the site of the restriction enzyme *Hin*d III is found located outside the *gat4601* gene in the transferred linear DNA fragment PHP20163A at one site (1,378th). Then, it was expected that cleavage at another one site in the plant genome side would result in detection of a band of the fragment of 1,378 bp or more when one copy is transferred. In actuality, in this recombinant soybean, a band of about 6,000 bp was detected (Annex 5, Figure 3 and Figure 4).

When the *gm-hra* gene region is used as a probe, it was expected that the 2,419 bp *Hin*d III fragment (1,378 - 3,796) located at the center of the linear DNA fragment PHP20163A and the fragment of 1,567 bp or more located at the site closer to the plant genome side from the *Hin*d III site (3,796th) would be detected as a band respectively. In addition, the *gm-hra* gene has high homology with the soybean-intrinsic *als* gene and then, it was expected that in addition to the bands derived from the transferred gene, several DNA fragments derived from the *als* gene would be detected. In fact, multiple bands derived from the intrinsic *als* gene were observed, and as the specific bands to this recombinant soybean, the band of 2,419 bp and the band of about 8,000 bp were observed (Annex 5, Figure 5 and Figure 6).

Based on the above results, it was found that one copy of the PHP20163A used for the transfer is transferred to the soybean genome of this recombinant soybean. In addition, in 30 plant individuals of the inbred posterity T4 generation tested and 24 plant individuals of the inbred posterity T5 generation, the detected band patterns are all found identical (seeTable 4 and Annex 5), therefore it was confirmed that the transferred gene to this recombinant soybean is stably inherited in the posterity.

The map of the transferred gene to this recombinant soybean based on the analytical results of the Southern blotting analysis is shown in Figure 7.

Table 4 Summary of results of Southern blotting analysis

Generation analyzed	Probe used for detection	Restriction enzyme	Expected fragment length (bp) [a]	Detected fragment length (bp) [b]
T4	*gat4601*	*Hin*d III	> 1,378	Approx. 6,000
T5	*gat4601*	*Hin*d III	> 1,378	Approx. 6,000
T4	*gm-hra*	*Hin*d III	> 1,567 2,419	Approx. 8,000 * 2,419
T5	*gm-hra*	*Hin*d III	> 1,567 2,419	Approx. 8,000 * 2,419

*: When the *gm-hra* gene was used as a probe, several DNA fragments derived from the intrinsic *als* gene were detected since the *gm-hra* gene has high homology with the soybean-intrinsic *als* gene. The DNA fragment of approx. 8,000 bp shown above was found almost identical in size to the DNA fragment derived from the intrinsic *als* gene, though it was apparently darker than the band in the non-recombinant soybean and then it was judged as the DNA fragment derived from the transferred gene (see Annex 5, Figures 5 and Figure 6).

a: The size of DNA fragment expected to be detected by treatment with the restriction enzyme *Hin*d III when one copy of the linear DNA fragment PHP20163A is transferred (see Figure 4 in this Assessment Report).

b: Apparent size of detected DNA fragment based on the molecular weight marker.

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Figure 7 Map of the transferred gene to this recombinant soybean based on the Southern blotting analysis

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

This item does not apply since the number of copies is found one based on the Southern blotting analysis.

4) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid

In order to identify that the GAT4601 protein produced by the *gat4601* gene transferred to this recombinant soybean and the GM-HRA protein produced by the transferred *gm-hra* gene are stably produced even in the progeny of this recombinant soybean, the herbicide spraying test and the quantitative analysis based on the ELISA method were conducted using the two generations of the inbred posterity T5 and T6 generations (see Figure 6).

i) Herbicide spraying test

Herbicide spraying test was carried out in 2005 at the US Pioneer Hi-Bred International, Inc. in four (4) repeats with two individuals of each of this recombinant soybean and non-recombinant soybean sprayed. On the 20th

day after sowing, a mixture of herbicide glyphosate (1.752 kg ae/ha) and acetolactate synthase inhibitors [chlorimuron ethyl (33.4 g ai/ha) + thifensulfuron methyl (10.7 g ai/ha)] was sprayed and two weeks later, evaluation was made on the impact of herbicide spraying. The formulation of herbicides used in the test is one of the herbicide spraying methods applicable to this recombinant soybean after its commercialization. The severity of herbicide injury was visually evaluated based on the scale from 0% (no impact) through 100% (complete death).

As a result, the both generations of the inbred posterity T5 and T6 generations exhibited no herbicide injury from the spraying of tank-mixed herbicides of glyphosate and acetolactate synthase inhibitors (Table 5). In addition, based on the fact that no herbicide injury was observed in all the recombinant soybean cultivars sprayed with a mixture of herbicide glyphosate and acetolactate synthase inhibitors, it was confirmed that the transferred genes are stably expressed across the individuals.

Table 5 Comparison of herbicide injury due to spraying of tank-mixed herbicides[1]

	This recombinant soybean (T5 generation)	This recombinant soybean (T6 generation)	Non-recombinant soybean
Sprayed with herbicides	0±0.0[2]	0±0.0	89 ± 2.1
Not sprayed with herbicides	0±0.0	0±0.0	0±0.0

1: Severity of herbicide injury in individuals was evaluated based on the scale from 0 (intact) to 100% (complete death). The values listed above refer to average of 8 individuals.
2: The figures following ± refer to standard errors.

(All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Du Pont Kabushiki Kaisha)

ii) Quantitative ELISA analysis

Quantitative ELISA analysis was conducted for the leaf tissue collected, 10 mg per individual, from three (3) individuals of each of this recombinant soybean 2 weeks after herbicide spraying and the non-recombinant soybean not sprayed with herbicides. The amount of GAT4601 protein produced was found 4.6 ± 1.1 ng/mg dry weight in the T5 generation and 3.4 ± 0.33 ng/mg dry weight in the T6 generation. In addition, the amount of GM-HRA protein produced was found 18 ± 6.6 ng/mg dry weight in the T5 generation and 13 ± 3.0 ng/mg dry weight in the T6 generation. As a result, no statistically significant difference was observed between the both generations and it was confirmed that the both proteins are stably produced through generations (Table 6).

Table 6 Amount of GAT4601 protein and GM-HRA protein produced in this recombinant soybean

	T5 generation[1]	T6 generation[1]	P-value	Non-recombinant soybean[2] (Reference)
Production of GAT4601 protein (ng/mg dry weight)	4.6 ± 1.1 [3] (3.3 - 6.7)	3.4 ± 0.3 (3.0 - 4.0)	0.65	Below the limit of determination[4]
Production of GM-HRA protein (ng/mg dry weight)	18.0 ± 6.6 (7.0 - 30)	13.0 ± 3.0 (7.7 - 18)	0.43	Below the limit of determination[5]

1: n=3
2: n=6
3: The figures following ± refer to standard errors. Values in parentheses refer to (minimum value minus maximum value measured by analysis).
4: The limit of determination for GAT4601 protein: 0.11 ng/mg dry weight.
5: The limit of determination for GM-HRA protein: 0.27 ng/mg dry weight.
(All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Du Pont Kabushiki Kaisha)

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

There is no sequence contained in the nucleic acid transferred that can be transmitted to any other wild animals and wild plants, therefore, there is no risk of transmission of nucleic acid transferred.

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

1) Methods

DNA extracted from leaves was used as a template. Primers were set in the SCP1 promoter region and GAT coding region, and the PCR was conducted at an annealing temperature of 55°C and the number of cycles of 35. The agarose gel electrophoresis was conducted to detect the approx. 700 bp band specific to this recombinant soybean.

2) Sensitivity

DNA samples of 40 ng each were diluted and analyzed based on the PCR method and as a result, the limit of detection (LOD) was found 40 pg.

3) Reliability

Five (5) individuals of each of this recombinant soybean and the non-recombinant soybean were tested at two different locations and analyzed in six (6) repeats to identify the reproducibility.

(6) Difference from the recipient organism or the taxonomic 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

With the production of GAT4601 protein due to the expression of the transferred *gat4601* gene and the acetolactate synthase (GM-HRA), which is not affected by herbicide acetolactate synthase inhibitors, due to the expression of the transferred *gm-hra* gene, the tolerance to herbicide glyphosate and acetolactate synthase inhibitors is conferred to this recombinant soybean.

The GAT4601 protein produced by the expression of the *gat4601* gene provides the *N*-acetylation of herbicide glyphosate to transform the glyphosate to the *N*-acetyl glyphosate which does not possess any herbicidal activity and as a result, it confers tolerance to herbicide glyphosate on plants (see Figure 1). On the other hand, the GM-HRA protein produced by the expression of the *gm-hra* gene works in the pathway for biosynthesis of branched chain amino acids of leucine, valine and isoleucine, taking place of the plant-intrinsic acetolactate synthase, the activity of which is inhibited by the herbicide acetolactate synthase inhibitors and as a result, it confers tolerance to herbicide acetolactate synthase inhibitors on plants (see Figure 2).

In fact, in the greenhouse tests and field tests in US, it was confirmed that this recombinant soybean suffered no herbicide injury when sprayed with herbicide glyphosate and acetolactate synthase inhibitors and exhibited practically sufficient tolerance (see (4)-4)). In addition, as mentioned later, also in the isolated field test in Japan, as a result of spraying test of herbicide glyphosate and acetolactate synthase inhibitors, it was confirmed that this recombinant soybean exhibits tolerance to the both herbicides (Annex 6, Table 1 and Figures 2 to 5).

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

In order to identify any differences between this recombinant soybean and the taxonomic species to which the recipient organism belongs, isolated field test was conducted in 2006 in Japan using the inbred posterity T6 generation of this recombinant soybean (see Figure 6) and the non-recombinant soybean cultivar Jack of the recipient organism as a control plant to examine the items described below (Annex 6).

(a) Morphological and growth characteristics

Differences in morphological and growing characteristics were examined between this recombinant soybean and the non-recombinant control soybean in three (3) repeats with reference to the evaluation criteria for soybean characteristics and the list of soybean characteristics issued by the Ministry of Agriculture, Forestry and Fisheries with respect to a total of 25 items: uniformity of germination, germination rate, time of flower initiation, shape of leaflet, the number of leaflets, trichome color, trichome quantity, color of flowers, maturation period, plant shape, main stem length, the number of main stem nodes, height of the lowest node of pod formation, the number of branches, difficulty in pod bursting, the total number of pods per plant, the total weight of pods per plant, the number of seeds per pod, the total number of seeds per plant, the total weight of seeds per plant, the number of ripe seeds per plant, the weight of ripe seeds per plant, weight of 100 seeds, shape of seed and hilum color.

As a result, except the main stem length, in all other items examined, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 3). In the main stem length, a statistically significant difference was observed (47.5 cm on average in this recombinant soybean compared to 55.2 cm on average in the non-recombinant control soybean).

Then, regarding the main stem length, examination was conducted in the other cultivation plots for confirmation (plot for confirmation of gene expression and backup plot) and as a result, in the plot for confirmation of gene expression and backup plot, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 4).

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

Wagner pots (1/5000a) were filled with field soil, to which the seeds of this recombinant soybean and the non-recombinant control soybean were sown, 15 seeds per pot, and the pots were assigned five (5) plants each and placed in a thermo-hygrostat greenhouse (25°C and 16-hours day length and 8-hours night length). When the soybean plants reached the V1 stage, they were moved to a growth chamber and exposed to the low-temperature conditions (12-hours day length at 12°C and 12-hours night length at 2°C). One month later, the plants were visually examined for any damage due to low temperatures. The test was conducted in three (3) repeats, and the t-test was carried out between this recombinant soybean and the non-recombinant control soybean.

As a result, regarding the degree of low-temperature injury, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 5). Consequently, it was concluded that this recombinant soybean is equivalent to the non-recombinant control soybean in the cold-tolerance at the early stage of growth.

(c) Wintering ability or summer survival of the matured plant

The matured plants were left to stand without harvesting and after the plants died down due to low temperatures, a total of 30 individuals of this recombinant soybean and the non-recombinant control soybean were selected, five (5) plants each from each repeat, and a vinyl tunnel was installed to cover the selected plants. For three (3) weeks from December 8, the inside of vinyl tunnel was heated with a hot-air blowing heater (SHINX Co. LTD., HURRYHEAT SH1) (thermostat setting temperature: 25℃) to identify whether the plants could regrow. During the 3-weeks heating period, the tunnel inside temperature was kept at around 20℃ day and night. In order to check the environment in the tunnel, 30 seeds of soybean were sown adjacent to the individual plants.

As a result, the plants of this recombinant soybean and the non-recombinant control soybean were found all died completely after harvesting time, and no difference was observed between the both plants. In addition, even after 3-weeks heating, with regard to both this recombinant soybean and non-recombinant soybean, no new germination or regeneration of plant tissue was observed, and the plants were all found dead completely.

For the soybean seeds sown in the tunnel, the 30 seeds were all germinated during the heating period and then, it was confirmed that soybean could grow in the conditions. Based on the above understanding, it was concluded that this recombinant soybean is equivalent to the non-recombinant control soybean with respect to the wintering ability.

(d) Fertility and size of the pollen

Flowers of this recombinant soybean and non-recombinant control soybean were collected immediately before flower initiation, five (5) flower from each repeat (a total of 3 repeats), and anther was removed under a microscope. In the acetocarmine solution for staining, pollens were taken out from the anther by crushing the anther with the tip of tweezers, the pollens were left for 20 minutes or more and photographed in appropriate fields of view. Later, for the photo prints, 5 fields of view were selected for each repeat and about 100 pollens per field of view were examined for stained pollens. The pollens found stained with the solution were evaluated to possess pollen fertility, and t-test was conducted based on the comparison between this recombinant soybean and non-recombinant control soybean. In addition, with respect to size, one appropriate field of view per flower was selected, measurement of diameter was made for 10 pollens, and then t-test was conducted based on the comparison between this recombinant soybean and the non-recombinant control soybean.

As a result, 98% or more of pollens of this recombinant soybean and non-recombinant soybean were found stained with acetocarmine solution, and no statistically significant difference was observed between the both plants regarding the vital reaction of pollens. Consequently, it was suggested that there is no difference in the fertility of pollen between the both plants. In

addition, also for the size (diameter) of pollen, no statistically significant difference was observed between the both plants. Based on the above results, it was concluded that this recombinant soybean is equivalent to the non-recombinant control soybean in fertility and size of the pollen.

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

Regarding the characteristics referring to the production of seeds, the total number of pods per plant, the total weight of pods per plant, the number of seeds per pod, the total number of seeds per plant, the total weight of seeds per plant, the number of ripe seeds per plant, the weight of ripe seeds per plant and weight of 100 seeds were examined and as a results, in all the items examined, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 3). Therefore, it was confirmed that this recombinant soybean is equivalent to the non-recombinant control soybean regarding the production of seeds. In addition, the both plants were found difficult to burst the pods and it was confirmed that the both plants are also equivalent to each other regarding the shedding habit of the seed (Annex 6).

In addition, with respect to the dormancy and germination rate, examination was made as follows. Three (3) seeds collected from 12 plants at the center of each plot (36 seeds in total/plot) were left to stand on the water-dampened filter paper on a petri dish (on the 63rd day after seed sampling) under the conditions (25°C and 16-hours day length and 8-hours night length). The test was conducted in 3 repeats and 4 days later, examination was made for germination and t-test was conducted between this recombinant soybean and the non-recombinant control soybean. The seeds not germinated were examined for vital reaction by staining with tetrazolium chloride. The seeds used for the examination had been allowed to air-dry and then stored at room temperatures.

As a result, for the harvested seeds of both of this recombinant soybean and the non-recombinant control soybean, the germination rates were 97% or more, and no statistically significant difference was observed between the both plants (Annex 6, Table 7). Among 108 seeds of this recombinant soybean, 3 seeds were not germinated. These seeds were subjected to vital staining with tetrazolium chloride, though the non-germinated seeds were all found dead. Based on the facts that there is no report so far that cultivated species of soybean possess any dormancy of the seeds and that no difference was observed from the above examination between this recombinant soybean and the non-recombinant control soybean in germination rate and the seeds not germinated were all found dead, it was considered that this recombinant soybean is equivalent to the non-recombinant control soybean with respect to the dormancy of the seed. Based on the above results, it was concluded that this recombinant soybean and the non-recombinant control soybean are equivalent to each other with respect to the production, shedding habit, dormancy and germination rate of the seed.

(f) Crossability

In order to evaluate whether the crossability of this recombinant soybean is similar to that of conventional soybeans, the following experiments were conducted in 2006 in the field of the US Pioneer Hi-Bred International, Inc. (Johnston, Iowa, the US) using the T5 generation (Figure 6).

In the field, 68 seeds of this recombinant soybean and 30 seeds of low linolenic acid soybean variety 93M01 were sown as shown in Figure 8 (A) to make uniform the opportunity of crossing between this recombinant soybean and the pollination strain 93M01 (planting distance of 30 cm and ridge spacing of 80 cm) (hereinafter referred to as "recombinant test plot"). With a buffer zone provided 30 m or more distant from the recombinant test plot, 68 seeds of non-recombinant soybean (cultivar Jack) and 30 seeds of soybean variety 93M01 were similarly sown to make uniform the opportunity of crossing between the non-recombinant soybean and the pollination strain 93M01 [(hereinafter referred to as "non-recombinant test plot", Figure 8 (B)] In order to identify the crossing with the cultivar Jack by DNA marker, the 93M01, which has different genetic backgrounds, was selected as pollination strain.

In the maturation period, the seeds were collected from 30 individuals of soybean variety 93M01 in the recombinant test plot, and 300 seed of them were sown in the field. From the primary leaf (V1 stage), leaf tissue was collected to extract the DNA. Using the DNA as template, the real-time PCR analysis was conducted with the specific primer to the *gat4601* gene expression cassette region to examine the presence of transferred gene and determine the crossing rate.

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

Figure 8 Layout of soybeans tested in the field in the US

Similarly, in order to examine the crossing rate between the non-recombinant soybean and the soybean variety 93M01, the seeds were collected from 30 individuals of soybean variety 93M01 in the non-recombinant test plot, and 300 seeds of them were sown in the field. From the primary leaf, leaf tissue was collected to extract the DNA. Using the DNA as template, the real-time PCR analysis was conducted with the DNA marker for the non-recombinant soybean to examine the presence of marker gene and determine the crossing rate.

As a result, the crossing rate between this recombinant soybean and the soybean variety 93M01 was found 1.0%, and the crossing rate between the non-recombinant soybean and the soybean variety 93M01 was found 0.7%.

It is generally reported that the rate of natural crossing of soybean is normally 0.5 to 3% (Garber and Odland, 1926; Caviness, 1966; Ahrent and Caviness, 1994; Poehlman and Sleper, 1995; Agricultural Technology System, 2002;

Encyclopedia of Agriculture, 1994). As a result of the crossability experiments in the field in the US, the crossability of this recombinant soybean was found 1.0%, which is similar to the crossability observed in the non-recombinant control soybean varieties, not exceeding the typical rate of natural crossing of soybean. Consequently, it was considered that the natural crossability of this recombinant soybean in the field is equivalent to that of conventional soybeans.

In the isolated field in Japan, the seeds were collected from the non-recombinant soybean (ridge spacing 80 cm) adjacent to this recombinant soybean in the plot for examination of morphological and growth characteristics to identify the possibility of crossing between this recombinant soybean and non-recombinant soybean (Annex 6). As a result, the crossing rate in this test was found 0%.

(g) Productivity of harmful substances

Substances secreted from the roots with a threat to affect other plants (succeeding crop test)

For soybeans, the secretion of any harmful substances from roots, which affect the surrounding plants, has not been reported. To confirm the possibility of producing any harmful substances (the substances secreted from the roots to affect other plants), the succeeding crop test was conducted.

After harvesting, rhizosphere soil was collected from three (3) points around the bottom of plants in each plot (3 repeats) in the test area, and it was filtered with a sieve to remove the roots and mixed uniformly then filled into 1/5000a Wagner pots by plot. As a test plant, 30 seeds of radish were sown per pot and after examination for the germination rate, each pot was assigned 10 plants. For about three weeks after sowing, the plants were raised in a glass greenhouse and then, plants were collected to determine the plant height, fresh weight and dry weight. As a result, regarding the germination rate, plant height, fresh weight and dry weight of radish, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 8).

In 2005, at the US Pioneer Hi-Bred International, Inc., examination was conducted based on the rhizosphere soil method (Iqbal *et al.*, 2004) using the T5 generation for productivity of intrinsic substances secreted from the roots with a threat to affect other plants. As a result, it was also confirmed that no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean.
(http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_356043_5ap.pdf).

Based on the above understanding, it was concluded that the ability of this recombinant soybean to produce any harmful substances (the substances secreted from the roots with a threat to affect other plants) is similar to that of the non-recombinant control soybean.

Substances contained in the plant with a threat to affect other plants after they die (plant body plow-in test)

For soybeans, productivity of harmful substances contained in plants with a threat to affect other plants after dying has not been reported. In this recombinant soybean, the GAT4601 protein and the GM-HRA protein are produced, though it is not reported that the proteins would have any adverse effects on the growth of plants. To confirm the possibility of producing any harmful substances (the substances contained in plants with a threat to affect other plants after they die), the plow-in test was conducted.

Aerial part of plants after harvesting was collected from each plot (3 repeats) to prepare dry powder. The dry powder was mixed with the soil from the field in an uncultivated area by 0.5%(w/w) and packed to 1/5000a Wagner pots. As a test plant, the seeds of radish were sown, 30 seeds per pot, and after examination of germination rate, each pot was assigned 10 plants. For about 3 weeks after sowing, the plants were raised in a glass greenhouse and then plants were collected to determine the plant height, fresh weight and dry weight. As a result, regarding the germination rate, plant height, fresh weight and dry weight of radish, no statistically significant difference was observed between the soils plowed with the plants of this recombinant soybean and the non-recombinant control soybean (Annex 6, Table 9).

In 2005, at the US Pioneer Hi-Bred International, Inc., examination was conducted based on the sandwich method (Fujii *et al.*, 2003 and 2004, National Institute for Agro-Environmental Sciences, Annual Report, 1997) using the T5 generation for productivity of intrinsic substances contained in plants with a threat to affect other plants after dying. As a result, it was also confirmed that no statistically significant difference was observed between this recombinant soybean and non-recombinant soybean.
(http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_356043_5ap.pdf).

Based on the above understanding, it was concluded that the ability of this recombinant soybean to produce any harmful substances (the substances contained in plants with a threat to affect other plants after dying) is similar to that of the non-recombinant control soybean.

Substances secreted from the roots with a threat to affect soil microorganisms (soil microflora test)

For soybeans, productivity of harmful substances secreted from the roots with a threat to affect surrounding microorganisms in soil has not been reported. To confirm the possibility of producing harmful substances (the substances secreted from the roots with a threat to affect microorganisms in soil), dilution plate technique was conducted.

From each of 4 points in each plot, 100 mL of soil was collected with a core sampler and mixed with each other. The soil was added with sterilized water and then stirred and mixed with a blender to prepare the dilution solutions from 10^{-3} to 10^{-7}. Based on the dilution plate technique, bacteria and actinomycete were left to stand for 7 days on the PTYG media for incubation, and filamentous fungi was left to stand for 3 days on the rose bengal media at 25 for incubation. Sampling of soil was conducted July 24 and October 16. The test was conducted in 3 repeats to identify the number of bacteria, the number of filamentous fungi and the number of actinomycete in the cultivation soil of this recombinant soybean and non-recombinant soybean, and the t-test was conducted between this recombinant soybean and non-recombinant soybean.

As a result, regarding the number of bacteria, the number of filamentous fungi and the number of actinomycete in the soil taken from the plots for this recombinant soybean and the control plant at the both points of sampling time, no statistically significant difference was observed (Annex 6, Table 10). Based on the above understanding, it was concluded that the ability of this recombinant soybean to produce any harmful substances secreted from the roots with a threat to affect microorganisms in soil is similar to that of the non-recombinant control soybean.

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

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

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

(1) Competitiveness

The plant of soybean (*Glycine. max* (L.) Merr.) to which the recipient organism belongs, has been cultivated for a long time in Japan, but there is no report that it grows voluntarily in Japan.

This recombinant soybean is given the traits to be tolerant to herbicide glyphosate due to the transferred *gat4601* gene and to be tolerant to acetolactate synthase inhibitors due to the transferred *gm-hra* gene. However, it is expected unlikely that the glyphosate and the acetolactate synthase inhibitor would exert pressure for selection under a natural environment. Therefore, it is considered unlikely that these traits cause this recombinant soybean to become competitive.

In the isolated field in Japan, various characteristics relating to the competitiveness of this recombinant soybean were examined and as a result, regarding only the main stem length, a statistically significant difference was observed between this recombinant soybean and the control plant. However, as a result of additional examination for main stem length conducted for re-confirmation, no statistically significant difference was observed. Consequently, it is considered unlikely that the significant difference observed regarding the main stem length would cause Adverse Effect on Biological Diversity relating to the competitiveness.

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

(2) Productivity of harmful substances

Regarding the plant of soybean to which the recipient organism belongs, there is no report that it produces a harmful substance to wild animals and wild plants.

In this recombinant soybean, the GAT4601 protein and the GM-HRA protein are produced, though there is no report that the both proteins have any adverse effects on the growth of plants, and homology of amino acid sequences with known allergens and toxic proteins has not been observed.

In the isolated field in Japan, the ability of this recombinant soybean to produce any harmful substances (the substances secreted from the roots to affect other plants, the substances secreted from the roots to affect soil microorganisms, and the substances contained in plant bodies to affect other plants after dying) was examined, and no significant difference between this recombinant soybean and the non-recombinant soybean 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 soybean poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

1) Identification of wild animals and wild plants likely to be affected

Since it is known that if the *Glycine soja* Sieb. et Zucc. (*G. soja*) that grows voluntarily in Japan is crossed with soybean (*G. max*), it produces fertile seeds, the *G. soja* was specified as a wild plant likely to be affected, to perform the following examination.

2) Evaluation of concrete details of adverse effect

Existing documents do not show any obstacle to the growth and reproduction of the hybrid obtained from soybean and *G. soja*. So, in the case where this recombinant soybean and *G. soja* are crossed with each other in the Japanese natural environment, there is possibility that the hybrid grows and that the gene is transferred into this recombinant soybean through the back crossing from the hybrid to *G. soja* diffuses among the population of *G. soja* without remaining at a low level.

3) Evaluation of likelihood of adverse effect

G. soja grows voluntarily and widely throughout Japan in sunny fields, on the roadsides and the like. So, in the case where this recombinant soybean grows near *G. soja*, it cannot be denied that there are chances where both plants cross with each other. However:

(a) Both *G. max* and *G. soja* are typical autogamous plants engaged in cleistogamy.

(b) It is generally known that the flowering time of *Glycine max* and *Glycine soja* is unlikely to match with each other, and even in the case when the both plants are cultivated alternately at a planting distance of 50 cm by matching the flowering time, the rate of crossability is reportedly 0.7%.

(c) As a result of examination in the field in the US, the rate of crossing between this recombinant soybean and conventional soybean cultivars did not exceed the crossability between conventional soybean varieties.

Consequently, it was judged that the rate of crossing between this recombinant soybean and *Glycine soja* is as low as the crossability between conventional soybean varieties and *Glycine soja*.

4) Judgment of existence of Adverse Effect on Biological Diversity

Based on the above understanding, it was judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability 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 soybean 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.

Reference

(Confidential: Not made available or disclosed to unauthorized person)

Annex list

Annex 1 Reference material 5-1 for the second meeting on the "Guideline for Experimental Cultivation of Genetically Modified Crops approved for Use as Type I Use Regulations": Concept on the isolation distance by crop for cultivation experiments 2. Soybean

Annex 2 Nucleotide sequences of the linear DNA fragment PHP20163A used for the transferring and amino acid sequences of GAT4601 protein and GM-HRA protein (Confidential: Not made available or disclosed to unauthorized person)

Annex 3 Evaluation results on the substrate specificity using the GAT4601 protein (Confidential: Not made available or disclosed to unauthorized person)

Annex 4 Free amino acid content in seeds (Confidential: Not made available or disclosed to unauthorized person)

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

Annex 6 Cultivation test report in isolated field (Confidential: Not made available or disclosed to unauthorized person)