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

Applicant
Name: Du Pont Kabushiki Kaisha
Minoru Amoh, President
Address: 2-11-1, Nagata-chou, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Soybean high oleic acid and tolerant to herbicide acetolactate synthase inhibitor (*gm-fad2-1*, *gm-hra*, *Glycine max* (L.) Merr.) (DP-305423-1, OECD UI: DP-305423-1)
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 with high oleic acid content and herbicide acetolactate synthase inhibitor[1)] tolerance (*gm-fad2-1*, *gm-hra*, *Glycine max* (L.) Merr.) (DP-305423-1, OECD UI: DP-305423-1) (hereinafter referred to as "this recombinant soybean") and the origins of component elements are shown in Table 1 (Page 2), and the nucleotide sequences are shown in Figures 3 and 4 in Annex 2.

2) Function of component elements

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

Functions of component elements of donor nucleic acid are shown in Table 1 (Page 2).

[1)] Acetolactate synthase inhibitors include thifensulfuron methyl and tribenuron methyl.

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

Component elements	Size (bp)	Origin and function
gm-fad2-1 gene expression cassette（PHP19340 A)		
KTi3 promoter	2,084	A promoter region of Kunitz trypsin inhibitor 3 gene derived from soybean to induce transcription. It allows the highest transcriptional activity in embryos during embryogenesis, which is approximately 1,000 times higher than the transcriptional activity in leaves (Jofuku and Goldberg, 1989; Jofuku *et al*., 1989).
gm-fad2-1	597	A DNA fragment (hereinafter referred to as "*gm-fad2-1*") containing the region from 399th to 995th nucleotides in the endogenous *FAD2-1* gene derived from soybean. The soybean endogenous *FAD2-1* gene encodes the ω-6 desaturase that catalyzes the biosynthesis from oleic acid to linoleic acid. The *gm-fad2-1* gene was transferred with the intent to induce gene silencing[2)] and thereby suppress the expression of ω-6 desaturase.
KTi3 terminator	196	A terminator region of Kunitz trypsin inhibitor 3 gene derived from soybean to terminate transcription (Jofuku and Goldberg, 1989; Jofuku *et al*., 1989).
gm-hra (modified *als*) gene expression cassette (PHP17752A)		
FRT1	51	Flp recombinant enzyme recognition sequence derived from yeast (*Saccharomyces cerevisiae*) (Broach *et al*., 1982).
SAMS promoter	645	A constitutive expression promoter region of S-adenosyl-L-methionine synthetase (SAMS) gene derived from soybean to initiate transcription (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), enhancing the level of gene expression and the stability of transcription products.
gm-hra (Modified *als*)	1,971	Modified gene (*gm-hra*) of acetolactate synthase gene (*als*) derived from soybean to eliminate the susceptibility to herbicide acetolactate synthase inhibitors, encoding the GM-HRA protein precursor. By modification, the 178th proline in the endogenous acetolactate synthase (ALS) has been substituted by alanine and the 555th tryptophan substituted by leucine. In addition, in the N-terminal region, five (5) amino acids (methionine-proline-histidine-asparagine-threonine) have been newly added (Falco and Li, 2003).
als terminator	652	A terminator region of *als* gene derived from soybean to terminate transcription (Falco and Li, 2003).
FRT6	51	Modified FRT1 sequence having 94% homology with FRT1, and constituting the recognition sequence of Flp recombinant enzyme.

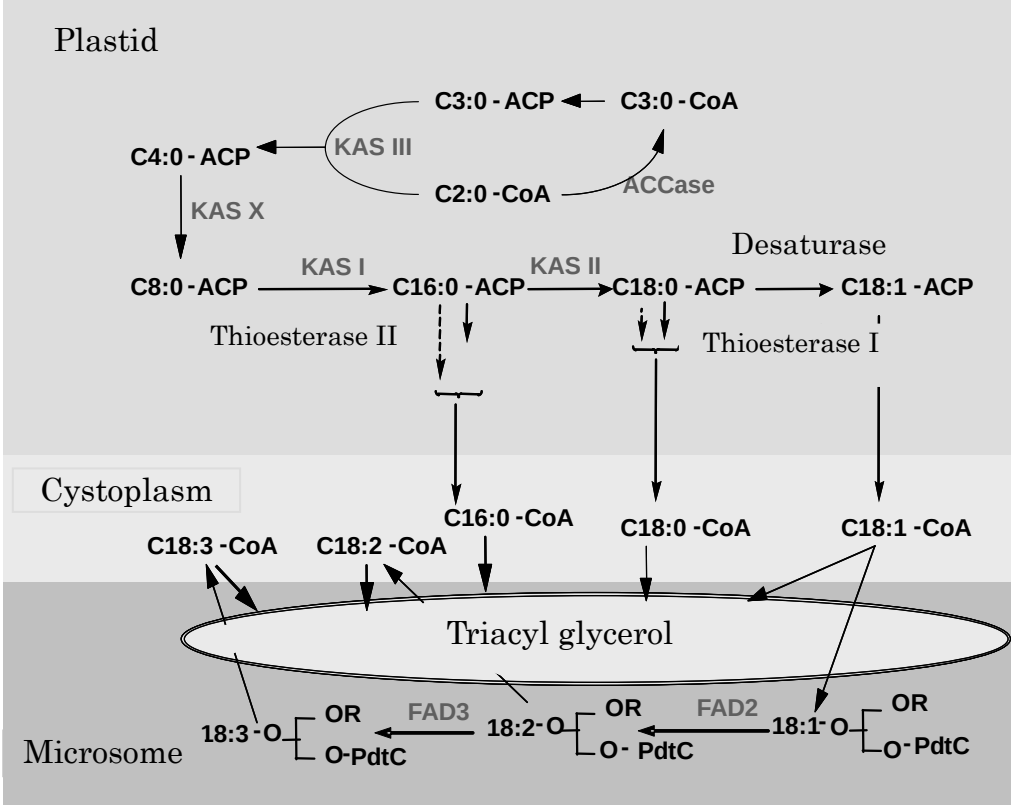
(b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

2) Gene silencing: A known phenomenon to suppress the expression of transferred genes and endogenous genes in the transformants which have the exogenous genes homologous with endogenous genes transferred to the nuclear genome of plant (Morino and Shimamoto, 1996).

In this recombinant soybean, the *gm-fad2-1* gene to confer the traits of high oleic acid and the *gm-hra* gene to confer the tolerance to acetolactate synthase inhibitors have been transferred.

gm-fad2-1 gene

In this recombinant soybean, the *gm-fad2-1* gene has been transferred. The *gm-fad2-1* gene constitutes a part of soybean endogenous *FAD2-1* gene which encodes the ω-6 desaturase that catalyzes the reaction for biosynthesis from oleic acid to linoleic acid in soybean (Figure 1 on Page 3). This gene has been transferred to induce gene silencing and thereby suppress the expression of ω-6 desaturase. As discussed later, in this recombinant soybean, the expression level of soybean endogenous *FAD2-1* gene is successfully suppressed as intended (Figure 7 on Page 21) and as a result, the linoleic acid content has been reduced while the oleic acid content has been increased to account for around 75% of the entire fatty acids (Table 2 on Page 7).



C18:1=oleic acid, C18:2=linoleic acid, C18:3=linolenic acid
FAD2＝ω-6 desaturase, FAD3＝ω-3 desaturase

Figure 1 Pathway for the biosynthesis of fatty acids in the oilseed crop

(All the rights pertinent to the diagram above and the responsibility for the contents rest upon Du Pont Kabushiki Kaisha.)

GM-HRA protein

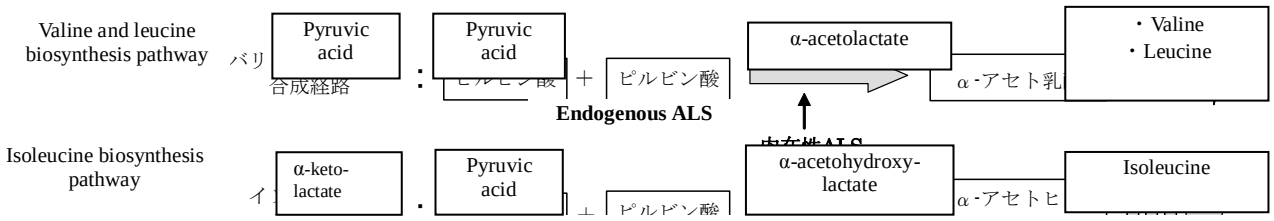
The GM-HRA protein precursor encoded by the *gm-hra* gene possesses the chloroplast transport sequence, which contains 47 amino acids following the five (5) amino acids in the N-terminal region. The chloroplast transport sequence is removed as the transport into chloroplast proceeds, and eventually the mature GM-HRA protein becomes 604 amino acids long with a molecular weight of 65kDa (hereinafter referred to as "GM-HRA protein") is formed (Figure 2 in Annex 2).

Acetolactate synthase inhibitors specifically inhibit the activity of endogenous acetolactate synthase for biosynthesis of branched-chain amino acids in plants and as a result, the branched-chain amino acids of valine, leucine and isoleucine are not synthesized in plants and plants would die (Figure 2 on Page 5). The GM-HRA protein exhibits the activity even in the presence of acetolactate synthase inhibitors and then the pathway for the biosynthesis of branched-chain amino acids is not inhibited, conferring the tolerance to acetolactate synthase inhibitors on plants (Figure 2 on Page 5).

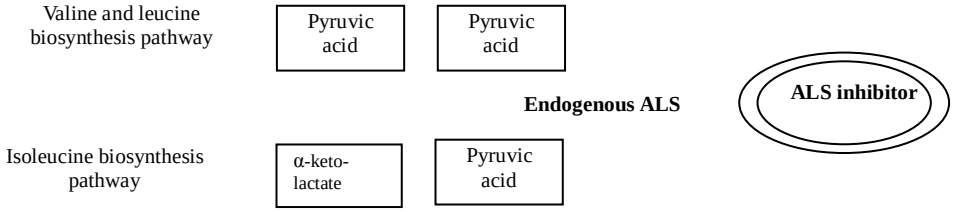
In order to identify the structural homology between the GM-HRA protein and any known allergens, an amino acid sequence homology search was conducted using the allergen database provided by the Food Allergy Research and Resource Program of University of Nebraska in the US (FARRP, 2006) and the FASTA34 algorithm (Pearson, 2000). This database holds the amino acid sequences of a total of 1,541 known allergens without any duplication. As a result of statistical analysis using the database, there were no known and estimated allergens observed which exhibited homology with the GM-HRA protein.

In addition, in order to examine the structural homology between GM-HRA protein and any known toxic proteins, an amino acid sequence homology search was conducted using the database available from the National Center for Biotechnology Information (NCBI) which holds the amino acid sequences of proteins and the BLASTP algorithm (NCBI, 2006; version 2.2.13). As a result of statistical analysis using the database, there were no known toxic proteins observed which exhibited homology with the GM-HRA protein.

i) When the non-recombinant soybean is not sprayed with any herbicide:



ii) When the non-recombinant soybean is sprayed with acetolactate synthase inhibitors:



iii) When this recombinant soybean is sprayed with acetolactate synthase inhibitors:

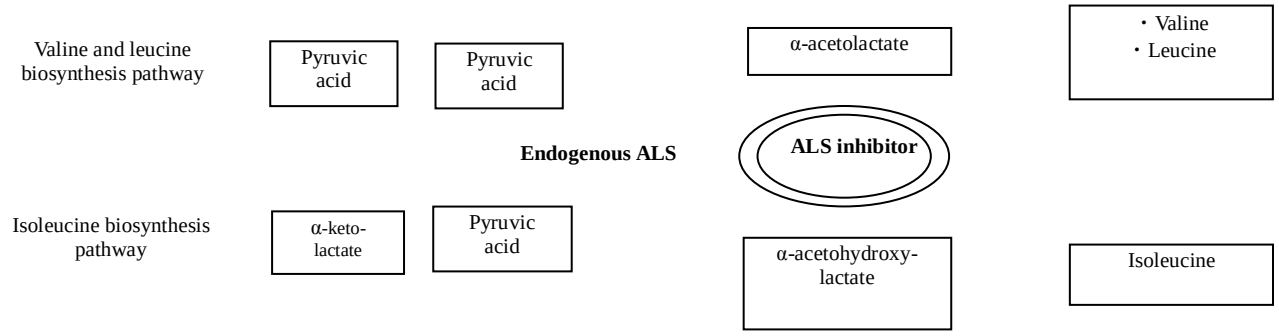


Figure 2 Mechanism of action of GM-HRA protein

i) Plant endogenous acetolactate synthase (ALS) synthesizes the branched-chain amino acids of valine, leucine and isoleucine.
ii) In the non-recombinant soybean, ALS is inhibited by the acetolactate synthase inhibitors (ALS inhibitors) and as a result, the biosynthesis of branched-chain amino acids of leucine, valine and isoleucine becomes impossible and plants would die out.
iii) In this recombinant soybean, the GM-HRA protein is produced and as a result, plants are not affected by the ALS inhibitors and thus can synthesize the branched-chain amino acids.

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

For any change caused to the metabolic system of recipient organism, examination was conducted for the *gm-fad2-1* gene and the GM-HRA protein individually and also for possible interaction between the both.

a *gm-fad2-1* gene

In this recombinant soybean, the expression level of soybean endogenous *FAD2-1* gene is suppressed due to the transferred *gm-fad2-1* gene and thus the synthesis of linoleic acid is suppressed and as a result, the content of oleic acid is increased (I. 2. (1). 2).(b) on Page 2).

The oleic acid content against the total amount of fatty acids in the seed of this recombinant soybean was increased to 76.5% from 21.1% in the non-recombinant soybean, and the linoleic acid content was decreased to 3.62% from 52.5% (Table 2 on Page 7). The contents of palmitic acid, stearic acid and linolenic acid exhibited statistically significant (P-value<0.05) decreases compared to those in the non-recombinant soybean, though they were found falling within the tolerances based on the analytical results of non-recombinant soybean or data from published literature (Table 2 on Page 7).

For the composition of main fatty acids in the leaf, a statistically significant difference (P-value<0.05) was observed in the contents of oleic acid and linolenic acid. The oleic acid content in the non-recombinant soybean was found 3.61%, while that in this recombinant soybean was 4.43%. In addition, the linolenic acid content was 50.0% in the non-recombinant soybean but 47.4% in this recombinant soybean (Table 2 on Page 7).

As a result of measurement of the lipid content in the seed, the lipid content was found 15.9% and 14.9% for this recombinant soybean and the non-recombinant soybean, respectively, showing no statistically significant difference (P-value<0.05) between the both plants.

Table 2 Composition of main fatty acids in the seed and leaf [1)]

Item to be analyzed	Seed					Leaf		
	This recombinant soybean [2)]	Non-recombinant soybean [2)]	P-value	Tolerance Interval [3)]	Literature range[4)]	This recombinant soybean [5)]	Non-recombinant soybean [5)]	P-value
Palmitic acid (C16:0)	6.28±0.16	10.3±0.2	0.0001	2.93 – 19.6	7 – 15.8	11.2±0.3	10.7±0.2	0.18
Stearic acid (C18:0)	4.36±0.16	4.98±0.16	0.0001	0.852 – 8.34	2 – 5.88	4.92±0.17	4.89±0.09	0.87
Oleic acid (C18:1)	76.5±1.3	21.1±1.3	0.0001	11.3 – 32.6	14.3 – 34	4.43±0.02	3.61±0.09	0.0001
Linoleic acid (C18:2)	3.62±0.91	52.5±0.9	0.0001	41.7 – 64.3	42.3 – 60	10.1±1.2	9.56±0.36	0.55
Linolenic acid (C18:3)	5.39±0.53	9.35±0.53	0.0001	1.15 – 14.7	2 – 12.5	47.4±0.4	50.0±0.8	0.04

1) Values are presented in percentage (%) of the total amount of fatty acids.
2) Values denote mean value ± standard error for the sample size n=18. The seeds of BC1F5 generation obtained from cultivation in 2005 in 3 plots in the fields at 6 sites in the North America (Figure 6 on Page 14) were used. Statistical analysis was based on the analysis of variance comparisons.
3) Tolerance interval refers to the interval between the upper and lower limits adjusted statistically to include 99% of analysis values based on the analytical results of four (4) commercial varieties of non-recombinant soybean cultivated at 6 sites in the North America in 2005.
4) Based on the data from published literature of ILSI (2004) and OECD (2001).
5) Values denote mean value ± standard error for the sample size n=4 for this recombinant soybean and n=8 for the non-recombinant soybean. The leaves of T4 generation cultivated in 2006 in a greenhouse in the US (Figure 6 on Page 14) were used. Statistical analysis was based on the t-test. For leaves, there are no tolerances and literature data available.

b GM-HRA protein

In order to identify whether or not the GM-HRA protein causes any change to the metabolic system of recipient organism, examination was conducted for possible effects on the amino acid synthesis first then the biosynthesis of fatty acids.

Regarding possible effects on amino acid synthesis:

The GM-HRA protein works in the biosynthetic pathway of branched-chain amino acids in the similar manner as the endogenous ALS protein does (Figure 2 on Page 5). In the biosynthetic pathway of valine and leucine among the branched-chain amino acids, the endogenous ALS protein is subject to the feedback control by valine. On the other hand, in the isoleucine biosynthetic pathway, it is generally known that in addition to the 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 endogenous ALS and the GM-HRA protein work in conjunction in the biosynthetic pathway of branched-chain amino acids, it is

considered that the content of branched-chain amino acids to be synthesized is regulated by the action of feedback control.

In fact, as a result of composition analysis of amino acids in the seeds and leaves of this recombinant soybean, a statistically significant difference ($P < 0.05$) from the non-recombinant control soybean was observed for threonine and glutamate in the seeds, though it was found falling within the range of tolerance based on the analysis values of non-recombinant soybean or literature data. In addition, in the leaves, a statistically significant difference was observed for leucine, though its content was 9.60% in the non-recombinant soybean and 9.97% in this recombinant soybean. For the other amino acids, no statistically significant difference ($P<0.05$) was observed between this recombinant soybean and the non-recombinant soybean (Table 3 on Page 8).

Table 3 Composition of amino acids in the seed and leaf

Item to be analyzed	Seed					Leaf		
	This recombinant soybean [1)]	Non-recombinant soybean [1)]	P-value	Tolerance interval [2)]	Literature range[3)]	This recombinant soybean [4)]	Non-recombinant soybean [4)]	P-value
Aspartic acid	4.91±0.08	5.01±0.08	0.07	3.67 – 6.33	3.81 – 5.12	11.0±0.1	10.8±0.1	0.28
Threonine	1.95±0.03	1.91±0.03	0.02	1.57 – 2.21	1.14 – 1.89	5.07±0.13	5.09±0.13	0.99
Serine	2.28±0.04	2.26±0.04	0.35	1.85 – 2.71	1.11 – 2.48	4.37±0.28	4.97±0.29	0.24
Glutamate	7.92±0.11	7.69±0.11	0.02	6.04 – 9.54	5.84 – 8.72	12.1±0.1	12.3±0.1	0.47
Proline	2.32±0.04	2.27±0.04	0.18	1.85 – 2.70	1.69 – 2.61	4.70±0.10	4.55±0.10	0.40
Glycine	1.93±0.03	1.89±0.03	0.37	1.54 – 2.18	1.46 – 2.02	5.04±0.07	4.99±0.07	0.68
Alanine	1.73±0.04	1.66±0.04	0.22	1.35 – 2.07	1.49 – 2.10	5.59±0.07	5.62±0.07	0.73
Cystine	0.614 ± 0.018	0.638 ± 0.017	0.13	0.378 – 0.869	0.370 – 0.808	1.27±0.04	1.31±0.04	0.38
Valine	1.87±0.04	1.84±0.04	0.36	1.58 – 2.18	1.50 – 2.44	6.37±0.07	6.37±0.07	0.98
Methionine	0.712± 0.011	0.714± 0.011	0.92	0.488 – 0.852	0.431 – 0.681	2.09±0.04	2.05±0.04	0.55
Isoleucine	1.79±0.03	1.78±0.03	0.57	1.56 – 2.09	1.46 – 2.12	5.36±0.03	5.28±0.03	0.14
Leucine	2.99±0.03	2.97±0.03	0.54	2.53 – 3.52	2.20 – 4.00	9.97±0.07	9.60±0.08	0.01
Tyrosine	1.36±0.04	1.34±0.04	0.67	0.908 – 1.69	1.02 – 1.62	4.71±0.05	4.71±0.05	0.94
Phenylalanine	2.10±0.04	2.07±0.04	0.57	1.74 – 2.43	1.60 – 2.35	5.98±0.09	5.90±0.09	0.57
Histidine	1.21±0.03	1.17±0.03	0.29	0.897 – 1.41	0.878 – 1.22	2.48±0.02	2.44±0.02	0.25
Lysine	2.58±0.05	2.56±0.05	0.77	1.98 – 3.10	2.29 – 2.86	7.23±0.14	7.36±0.14	0.51
Tryptophan	0.507± 0.014	0.496±0.014	0.36	0.359 – 0.632	0.356 – 0.670	- [5)]	- [5)]	
Arginine	2.99±0.08	2.81±0.08	0.07	2.01 – 3.60	2.29 – 3.49	6.61±0.11	6.69±0.11	0.65

1) Values denote mean value ± standard error for the sample size n=18. Values for the seed are presented in percentage (%) per dry weight. The BC1F5 generation cultivated in 2005 in 3 plots in the fields at 6 sites in the North America (Figure 6 on Page 14) was used. Statistical analysis was based on the analysis of variance comparisons.

2) Tolerance interval refers to the interval between the upper and lower limits adjusted statistically to include 99% of analysis values based on the analytical results of four (4) commercial varieties of non-recombinant soybean cultivated at 6 sites in the North America in 2005.

3) Based on the data from published literature of ILSI (2004), OECD (2001) and Taylor *et al*. (1999).

4) Values denote mean value ± standard error for the sample size n=5. Values for the leaf are presented in percentage (%) of the total amount of amino acids. The T4 generation cultivated in 2006 in a greenhouse in the US (Figure 6 on Page 14) was used. Statistical analysis was based on the analysis of variance comparisons. For the leaf, there are no tolerance and literature data available.

5) Not analyzed

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Regarding possible effects on the biosynthesis of fatty acids:

As a result of analysis of fatty acid composition in the seeds of this recombinant soybean, a statistically significant increase ($P<0.05$) from the non-recombinant control soybean was observed in heptadecanoic acid and heptadecenoic acid. However, the percentage of heptadecanoic acid and heptadecenoic acid against the total amount of fatty acids was found 0.798% and 1.19%, respectively (Table 4 on Page 9).

As presented in Figure 2 (Page 5), the endogenous ALS uses pyruvic acid and α-ketobutyric acid as substrates in the biosynthetic pathway of branched-chain amino acids. These acids also work as substrates in the biosynthetic pathway of fatty acids (Annex 3). The GM-HRA protein has lower substrate affinity for α-ketobutyric acid compared to the endogenous ALS. Then, it was estimated that in this recombinant soybean, the concentration of α-ketobutyric acid would become increased compared to the pyruvic acid and the contents of heptadecanoic acid and heptadecenoic acid synthesized from the α-ketobutyric acid would be increased. This is detailed in Annex 3. According to the Standard Tables of Food Composition in Japan (2005) published by the Ministry of Education, Culture, Sports, Science and Technology, heptadecanoic acid and heptadecenoic acid are typical fatty acids contained in many animals and plants (Table 5 on Page 10 and Table 6 on Page 11).

For fatty acids other than oleic acid, linoleic acid, heptadecanoic acid and heptadecenoic acid, there was no statistically significant difference ($P<0.05$) observed between this recombinant soybean and the non-recombinant control soybean, or if any statistically significant difference was observed, it was found falling within the tolerance interval based on the analysis values of non-recombinant soybean or literature range.

Table 4 Percentage (%) of heptadecanoic acid and heptadecenoic acid in the seed against the total amount of fatty acids

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

Table 5 Percentage (%) of heptadecanoic acid (C17:0) in animals and plants against the total amount of fatty acids

Vegetables	Eggplant	1.4
	Celery	0.9
	Carrot	0.7
	Garlic	0.6
	Cabbage	0.4
	Japanese mountain yam	0.4
Fruits	Persimmon	0.8
	Apple	0.7
	Kumquat	0.6
	Lemon	0.6
Kinds of mushrooms	Hypsizigus marmoreus	0.8
	Mushroom	0.8
Algae	Hizikia fusiforme	0.5
Fishery products	Corbicula	2.9
	Clam	1.9
	Fleshy prawn	1.8
	Yellowtail	1.6
	Dried sardine	1.5
	Oyster	1.4
Meats	Beef	1.0
	Pork	0.3
Eggs	Hen egg	0.3

Based on the data in the Standard Tables of Food Composition in Japan (2005) published by the Ministry of Education, Culture, Sports, Science and Technology

Table 6 Percentage (%) of heptadecenoic acid (C17:1) in animals and plants against the total amount of fatty acids

Vegetables	Onion	0.7
	Crown daisy	0.3
	Garlic	0.3
	Pea bean	0.2
	Pumpkin	0.2
Fruits	Persimmon	0.7
	Citrus unshiu	0.4
	Kumquat	0.2
Kinds of mushrooms	Hen of the woods	0.3
	Tree ear	0.3
Algae	Hizikia fusiforme	0.4
	Japanese kelp	0.3
Fishery products	Sweetfish	1.5
	Fleshy prawn	1.5
	Oyster	1.3
	Sea urchin	1.2
	Yellowtail	1.0
	Tuna	0.8
Meats	Beef	1.0
	Pork	0.3
Eggs	Hen egg	0.2

Based on the data in the Standard Tables of Food Composition in Japan (2005) published by the Ministry of Education, Culture, Sports, Science and Technology

c Any interaction between expressions of *gm-fad2-1* gene and *gm-hra* gene

The expressions of *gm-fad2-1* gene and *gm-hra* gene take part in the synthesis from oleic acid to linoleic acid and the biosynthesis of branched-chain amino acids, respectively, though the both biosynthetic pathways constitute independent metabolic pathways from each other in plants and involve different substrates, therefore, it is considered unlikely that expressions of the both genes would interact with each other.

(2) Information concerning vectors

1) Name and origin

The plasmid PHP19340 (Figure 3 on Page 12) and the plasmid PHP17752 (Figure 4 on Page 12) were used as vectors to produce this recombinant soybean. These plasmids were prepared from the plasmid pUC19 derived from *Escherichia coli* (*E. coli*). From the plasmid PHP19340 and the plasmid PHP17752, the linear DNA fragments PHP19340A and PHP17752A were cleaved respectively by the restriction enzyme and transferred into this recombinant soybean.

2) Properties

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

The total number of base pairs of plasmids PHP19340 and PHP17752 is 5,438bp and 7,026bp respectively. In addition, the total number of base pairs of linear DNA fragments PHP19340A and PHP17752A is 2,924bp and 4,512bp respectively. The entire nucleotide sequences of the individual linear DNA fragments are shown in Figure 3 and Figure 4 in Annex 2.

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

For the plasmids PHP19340 and PHP17752, in the backbone region, the antibiotic hygromycin resistant marker gene, *hyg* gene is present. This gene works as an essential marker to select the microorganisms which contain the transformed plasmids during the proliferation of vectors in the microorganisms. It has been confirmed that the antibiotic resistant gene has not been transferred in the recipient organism (Annex 4).

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

There is no infectivity of vector.

(3) Method of preparing living modified organisms

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

Composition of the nucleic acid in the PHP19340A and PHP17752A used for the transferring and the restriction enzyme cleavage sites are shown in Figure 3 (Page 12) and Figure 4 (Page 12).

Figure 3 Composition of nucleic acid in the plasmid PHP19340 and linear DNA fragment PHP19340A and the restriction enzyme cleavage sites

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

Figure 4 Composition of nucleic acid in the plasmid PHP17752 and linear DNA fragment PHP17752A and the restriction enzyme cleavage sites

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

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). Process of transferring the nucleic acid into the recipient organism is shown in Figure 5 (Page 14).

3) Processes of rearing of living modified organisms

Process of selection and rearing of this recombinant soybean is as described below (Figure 5 on Page 14).

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

The transformed cells containing the transferred nucleic acid were selected by culturing the somatic embryo callus in the media containing the acetolactate synthase inhibitors.

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

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

Process of rearing of this recombinant soybean is shown in Figure 6 (Page 14). The scope of application for approval of this recombinant soybean includes the progeny lines of T0 and later generations shown in the process of rearing in Figure 6.

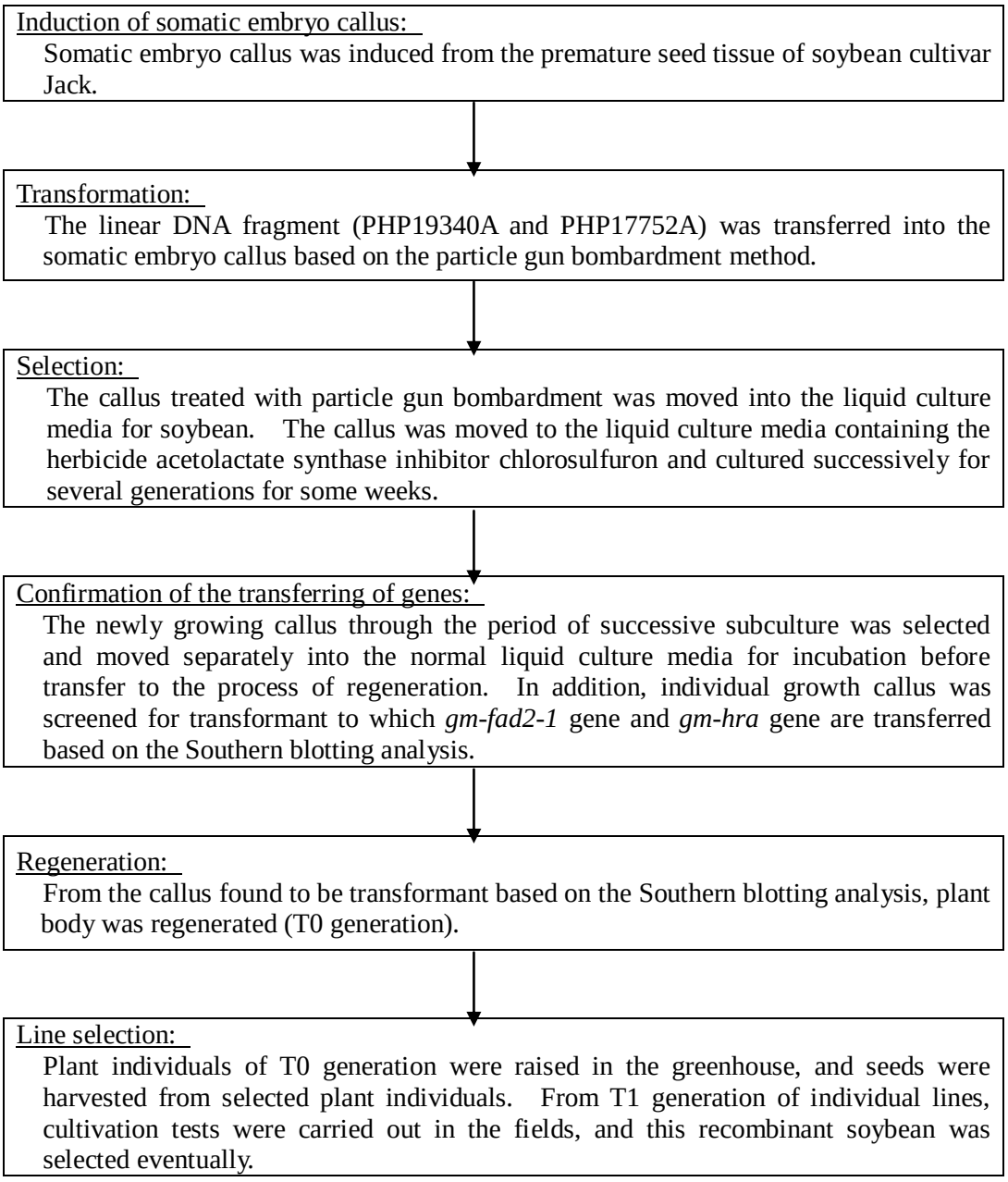

Figure 5 Processes of transferring the nucleic acid into the recipient organism and of selecting this recombinant soybean

(All the rights pertinent to the diagram above and the responsibility for the contents rest upon Du Pont Kabushiki Kaisha.)

Figure 6 Process of rearing of this recombinant soybean

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

(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

gm-fad2-1 gene: This recombinant soybean of T1, T2 and T3 generations (Figure 6 on Page 14) was examined for segregation ratio of *gm-fad2-1* gene with the high oleic acid trait defined as an indicator. As a result, in all the generations examined, the ratio of the number of individuals that have an oleic acid content of around 75% of the total amount of fatty acids to the number of individuals that have an oleic acid content of around 20% was found corresponding to the expected value of 3:1 (Table 7 on Page 15).

gm-hra gene: This recombinant soybean of BC1F2 generation (Figure 6 on Page 14) was examined for the number of individuals that contain the *gm-hra* gene based on the PCR method and for segregation ratio of *gm-hra* gene. As a result, the segregation ratio was found corresponding to the expected value of 3:1 (Table 8 on Page 16).

As such the transferred genes are both found stably inherited in the progeny in accordance with the Mendel's laws, therefore, it is confirmed that the replication products of transferred nucleic acid exist on the genome of soybean chromosome.

Table 7 Analysis for segregation ratio based on the oleic acid content in T1, T2 and T3 generations

Generations tested	Actual measured value of the number of seeds		Expected value of the number of seeds		P-value
	Seed with high oleic acid content	Seed without high oleic acid content	Seed with high oleic acid content	Seed without high oleic acid content	
T1	26	4	22.5	7.5	0.14
T2	30	7	27.75	9.25	0.39
T3	78	23	75.75	25.25	0.61

In 2002 and 2003 in a greenhouse in the US, one plant individual for each generation tested was cultivated and the fatty acid composition in the harvested seeds was determined based on the gas chromatography. The number of individuals that have an oleic acid content of around 75% and around 20% of the total amount of fatty acids was counted. With an expected value of segregation ratio set at 3:1, chi-square (χ^2) test was conducted.

Table 8 Analysis for the segregation ratio of *gm-hra* gene based on the PCR method in BC1F2 generation

Generations tested	Actual measured value of the number of individuals		Expected value of the number of individuals		P-value
	gm-hra gene - present	*gm-hra* gene - absent	*gm-hra* gene - present	*gm-hra* gene - absent	
BC1F2	111	33	108	36	0.63

In 2006 in a greenhouse in the US, the seeds of BC1F2 generation were sown. Leaves were collected from each individual and subjected to the PCR analysis to count the individuals that contain the *gm-hra* gene. With an expected value of segregation ratio set at 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

The number of copies of replication products of transferred nucleic acid

The number of copies of replication products of transferred nucleic acid in this recombinant soybean was identified based on the Southern blotting analysis. The analysis was conducted using the genome DNA extracted from the leaves of T4 and T5 generations (Figure 6 on Page 14) with several combinations of restriction enzymes and probes (Annex 4).

As a result, it was confirmed that several copies including fragments are transferred in this recombinant soybean (Annex 4). Then, the transferred regions were cloned to detect the nucleotide sequences and as a result, it was found that the genes were transferred in four (4) regions. The structure of individual regions is as described below.

- Region 1: One intact copy of *gm-fad2-1* gene expression cassette (PHP19340A), one intact copy of *gm-hra* gene expression cassette (PHP17752A), three (3) PHP19340A fragments and one KTi3 promoter fragment are transferred (Figure 7 in Annex 4).
- Region 2: One PHP19340A fragment is transferred (Figure 8 in Annex 4).
- Region 3: One KTi3 promoter fragment and the plasmid backbone region of 495 bp are transferred (Figure 9 in Annex 4).
- Region 4: The PHP19340A fragment is transferred in the inverted repeat sequence (Figure 10 in Annex 4).

The stability of inheritance of replication products of transferred nucleic acid through multiple generations

This recombinant soybean of T4, T5 and F2 generations was used to conduct the Southern blotting analysis. The analysis included seven (7) individuals each for T4 and T5 generations and 100 individuals for F2 generation. In the 7 plant individuals of each of T4 and T5 generations, the detected band patterns are all found identical (Figure 11 and Figure 12 in Annex 4). In the F2 generation, the detected band patterns are found identical to the results of T4 and T5 generations except one plant individual.

Consequently, it was confirmed that the transferred genes to this recombinant soybean are stably inherited through multiple generations.

For one plant individual which exhibited different band patterns in the F2 generation, it was considered that among the transferred genes to this recombinant soybean, the *gm-hra* gene expression cassette of transferred gene 1 and the adjacent KTi3 promoter fragment had dropped out (Figure 11 in Annex 4).

Then, in order to identify that such gene dropout is not any easily occurring phenomenon, additional 1,000 plant individuals of F2 generation were examined for presence of genes based on the PCR method and as a result, there was no dropout of gene observed.

Based on the above results, it was confirmed that the transferred genes to this recombinant soybean are stably inherited in progeny.

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

As mentioned in 2) (Page 16), it was found that the replication product of transferred genes to this recombinant soybean is transferred in the four (4) regions on the chromosome, and there was no dropout of genes observed also in the 1,000 plant individuals.

Based on the above understanding, it was considered that the four transferred regions identified in this recombinant soybean are strongly linked with each other and present in the same gene locus.

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

(a) Expression stability of high oleic acid trait due to the *gm-fad2-1* gene

In order to identify the high oleic acid trait given by the *gm-fad2-1* gene, fatty acid composition was analyzed using the seeds harvested from 14 to 15 plants of 2 generations T4 and T5 (Figure 6 on Page 14).

As a result, the oleic acid content in the both generations was found around 75%, and it was confirmed that the conferred high oleic acid trait is stably expressed across individuals and generations (Table 9 on Page 17).

Table 9 Composition of main fatty acids in this recombinant soybean of T4 and T5 generations

Generations tested	Composition of fatty acids (percentage (%) of the total amount of fatty acids) [1)]				
	Palmitic acid (C16:0)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)
T4 generation [2)]	8.23±0.22 (7.00 -10.42)	2.53±0.05 (2.20 - 3.03)	78.8± 0.7 (74.8 - 83.5)	1.25 ±0.08 (0.681 - 1.86)	3.61±0.17 (2.90 - 5.05)
T5 generation [3)]	7.19±0.08 (6.73 - 7.74)	2.98±0.06 (2.62 - 3.41)	78.3± 0.3 (76.4 - 80.2)	1.31 ±0.06 (0.970 - 1.80)	5.88±0.21 (4.46 - 7.23)

1) Values denote mean value ± standard error (range between the lowest and highest individual values). The

contents of fatty acids in the seeds harvested from cultivation in a greenhouse in the US in 2005 were measured based on the gas chromatography.

2) n=15
3) n=14

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(b) Expression stability of *gm-hra* gene

In order to identify that the GM-HRA protein is stably produced in this recombinant soybean, the herbicide spraying test and the ELISA analysis were conducted using T6 generation and BC1F5 generation of this recombinant soybean (Figure 6 on Page 14).

Herbicide spraying test

Acetolactate synthase inhibitors were sprayed and the severity of herbicide injury was visually evaluated based on the scale from 0% (no impact) through 100% (complete death).

As a result (Table 10 on Page 18), the non-recombinant soybean exhibited the herbicide injury on the scale of about 60%, though in this recombinant soybean, no herbicide injury was observed in all the individuals of both T6 and BC1F5 generations. Therefore, it was confirmed that the trait of tolerance to acetolactate synthase inhibitors is stably expressed across the generations and individuals.

Table 10 Results of acetolactate synthase inhibitor spraying test

Generations tested	Sprayed or not sprayed with herbicides	This recombinant soybean	Non-recombinant soybean
T6 generation	Sprayed with herbicides	0±0	58±3
	Not sprayed with herbicides	0±0	0±0
BC1F5 generation	Sprayed with herbicides	0±0	59±4
	Not sprayed with herbicides	0±0	0±0

Values denote mean value ± standard error for the sample size n=30. The test was conducted in a greenhouse in the US in 2005. Ten (10) seeds each of this recombinant soybean and the non-recombinant soybean were sown for 3 repeats, and 12 days after sowing, acetolactate synthase inhibitor [Chlorimuron (33.4 g ai/ha) + Thifensulfuron (10.7 g ai/ha)] were sprayed. Two (2) weeks after spraying of herbicides, the severity of herbicide injury was visually evaluated for each individual based on the scale from 0 (intact) to 100% (complete death). The herbicide spraying test was conducted with 6-times higher dosage than the pesticide registration usage standard in the US.

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Determination of GM-HRA protein in the leaves based on the ELISA analysis

For this recombinant soybean, the ELISA analysis was conducted using the leaves from three (3) individuals of each generation for production of GM-HRA protein. As a result, in all the individuals of this recombinant soybean, the GM-HRA protein is found expressed, and regarding the production, no statistically significant difference was observed between the T6 generation and the BC1F5 generation. In addition, in the non-recombinant soybean, no expression of the protein was observed. Consequently, it was confirmed that the GM-HRA protein is stably produced across the individuals and generations (Table 11 on Page 19).

Table 11 Amount of GM-HRA protein produced in this recombinant soybean based on the ELISA analysis

Amount of protein produced	T6 generation	BC1F5 generation	P-value
Production of GM-HRA protein (ng/mg dry weight)	1.1±0.0 (1.0 - 1.1)	0.9±0.1 (0.7 - 1.1)	0.18

Values denote mean value ± standard error (range between the lowest and highest individual values) for the sample size n=3. ELISA analysis was conducted for the leaves collected 5 weeks after seed sowing, 10 mg per individual, from three (3) individuals of each of this recombinant soybean and non-recombinant soybean cultivated in a greenhouse in the US in 2005.

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 through virus infection and/or other routes.

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

1) Methods of detection and identification

Genome DNA of 100 ng extracted from seeds was used as a template for the real-time PCR analysis in the following conditions.

- Primers: Primer pair amplifying the borderline region between KTi3 promoter and soybean genome DNA. Base sequences of primers and probes are shown in Figure 5 in Annex 2.
- Annealing temperature: 60ºC
- The number of cycles: 45

This method allows amplification specific to this recombinant soybean.

2) Sensitivity

When the genome DNA of 100 ng is used as a sample, the limit of determination is found 0.09%.

3) Reliability

This recombinant soybean was analyzed at two different locations in eight (8) repeats to identify the reproducibility of this method.

(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

Characteristics conferred by the *gm-fad2-1* gene

The *gm-fad2-1* gene constitutes a part of soybean endogenous *FAD2-1* gene that encodes the ω-6 desaturase. This gene was transferred for the purpose to induce gene silencing and thereby suppress the expression of ω-6 desaturase.

In order to confirm that the expression level of endogenous *FAD2-1* gene has been suppressed, Northern blotting analysis was conducted using the seeds of this recombinant soybean. As a result, it was confirmed that in the seeds of this recombinant soybean, the expression of endogenous *FAD2-1* gene has been significantly suppressed (Figure 7 on Page 21). In addition, as a result of Northern blotting analysis using the leaves, in the both of this recombinant soybean and the non-recombinant control soybean, there was no mRNA band detected which is derived from soybean endogenous *FAD2-1* gene and *gm-fad2-1* gene (Figure 8 on Page 22). Therefore, it was found that the expression of the both genes is seed-specific.

In fact, it was confirmed that the oleic acid content per the total amount of fatty acids in the seed of this recombinant soybean is increased to around 75% (Table 2 on Page 7). Ingestion of oleic acid is reported to decrease the human blood LDL-cholesterol (generally known as "bad" cholesterol) (Wollet and Dietschy, 1994). In addition, in the production of commercially available edible oil and fat, hydrogenation is conducted to increase the oxidation stability, though it is known that during the hydrogenation, trans fatty acids are produced. Ingestion of the trans fatty acids increases the blood LDL cholesterol (Food Safety Commission, 2007). Oil from this recombinant soybean has a high content of oleic acid that features higher oxidation stability and then the need for hydrogenation is reduced. Therefore, utilizing oil derived from this recombinant soybean is expected to suppress the ingestion of trans fatty acids.

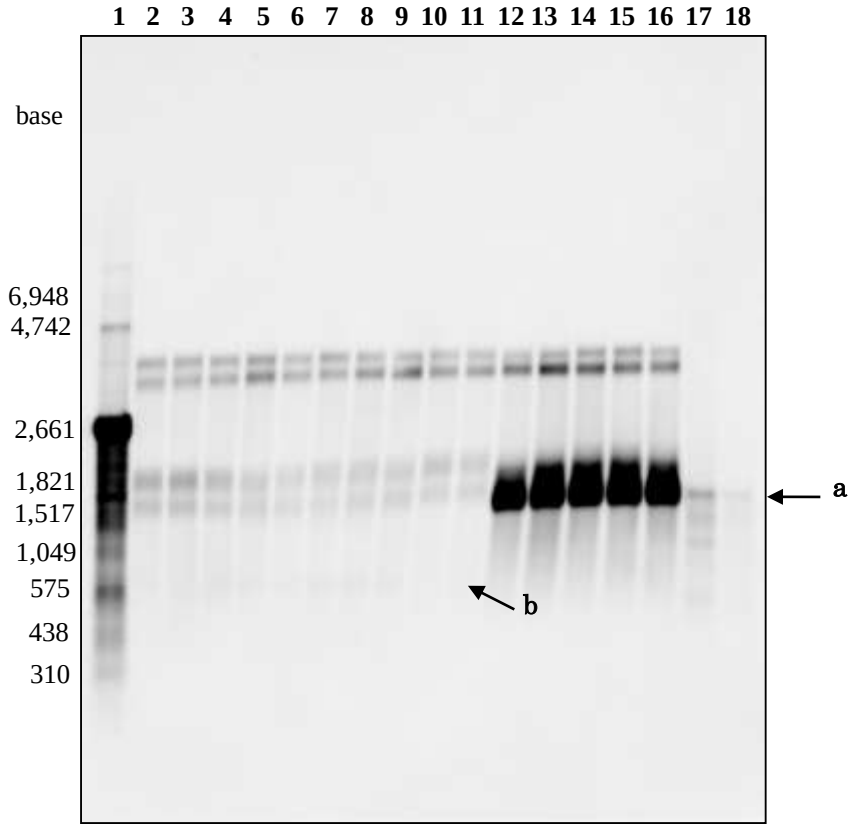


Figure 7 Northern blot analysis of seeds using the *gm-fad2-1* gene probe

Lane	Samples tested
1	Molecular weight marker (DIG RNA I)
2	This recombinant soybean -1
3	This recombinant soybean -2
4	This recombinant soybean -3
5	This recombinant soybean -4
6	This recombinant soybean -5
7	This recombinant soybean -6
8	This recombinant soybean -7
9	This recombinant soybean -8

Lane	Samples tested
10	This recombinant soybean -9
11	This recombinant soybean -10
12	Non-recombinant soybean -1
13	Non-recombinant soybean -2
14	Non-recombinant soybean -3
15	Non-recombinant soybean -4
16	Non-recombinant soybean -5
17	Positive control* (25 pg *FAD2-1* gene transcription product)
18	Positive control* (5 pg *FAD2-1* gene transcription product)

* The *in vitro* transcription products of soybean endogenous *FAD2-1* gene were individually resolved electrophoretically.

T4 generation was cultivated in a greenhouse in the US and 30 days after flowering, the seeds were collected. In the analysis, 200 ng mRNA per lane was electrophoresed. In the non-recombinant soybean, a band of approximately 1,500 bases ("a" in the diagram), a transcription product of soybean endogenous *FAD2-1* gene, was detected. On the other hand, the transcription level of soybean endogenous *FAD2-1* gene in this recombinant soybean was found considerably suppressed compared to the non-recombinant soybean, and also the detected band of transcription product of *gm-fad2-1* gene (approx. 700 bases, "b" in the diagram) was faint.

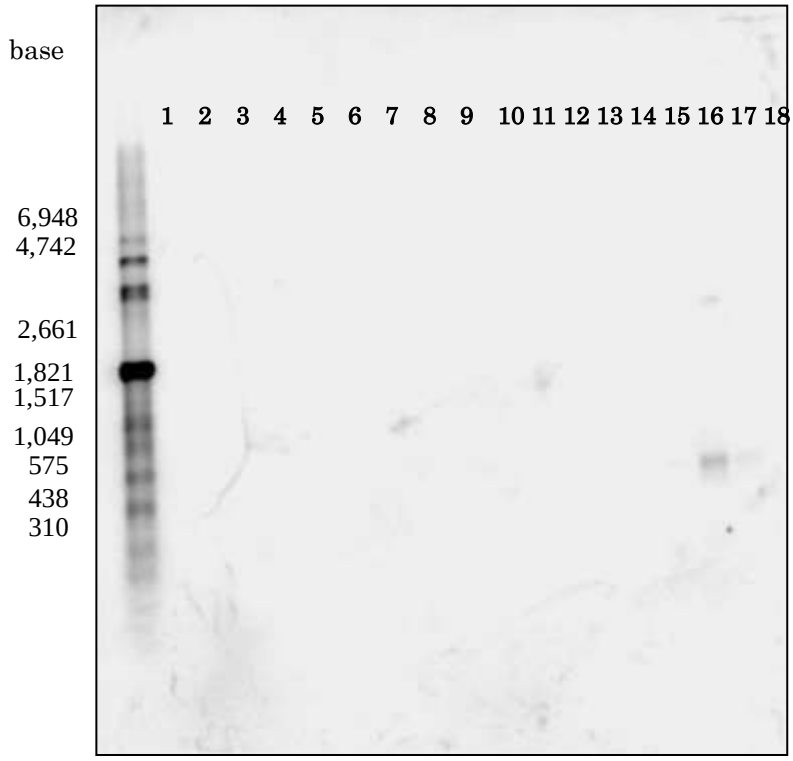


Figure 8 Northern blot analysis of leaves using the *gm-fad2-1* gene probe

Lane	Samples tested
1	Molecular weight marker (DIG RNA I)
2	This recombinant soybean -1
3	This recombinant soybean -2
4	This recombinant soybean -3
5	This recombinant soybean -4
6	This recombinant soybean -5
7	This recombinant soybean -6
8	This recombinant soybean -7
9	This recombinant soybean -8

Lane	Samples tested
10	This recombinant soybean -9
11	This recombinant soybean -10
12	Non-recombinant soybean -1
13	Non-recombinant soybean -2
14	Non-recombinant soybean -3
15	Non-recombinant soybean -4
16	Non-recombinant soybean -5
17	Positive control* (25 pg *FAD2-1* gene transcription product)
18	Positive control* (5 pg *FAD2-1* gene transcription product)

* The *in vitro* transcription products of soybean endogenous *FAD2-1* gene were individually resolved electrophoretically.

The analysis used the leaves from T4 generation cultivated in a greenhouse in the US. In the analysis, 200ng mRNA per lane was electrophoresed. In the non-recombinant soybean, there was no band of approximately 1,500 bases, a transcription product of soybean endogenous *FAD2-1* gene, detected. In this recombinant soybean, the band (approximately 1,500 bases) of transcription product of soybean endogenous *FAD2-1* gene and the band of transcription product (approximately 700 bases) of *gm-fad2-1* gene were not detected.

Characteristics conferred by the *gm-hra* gene

This recombinant soybean has the trait to be tolerant to acetolactate synthase inhibitors due to the transferred *gm-hra* gene (Table 10 on Page 18).

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

In 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 2007 in Utsunomiya Site of Du Pont Kabushiki Kaisha in Japan using this recombinant soybean of BC1F6 generation (Figure 6 on Page 14) to examine the items (a) through (g) described below (Annex 5). As a non-recombinant control soybean, the BC1F6 null, which was obtained with the transferred genes segregated during the process of rearing of this recombinant soybean, was used (Figure 6 on Page 14). The test was conducted based on the block design in a total of 4 repeats with one repeat composed of a pair of plots of this recombinant soybean and the non-recombinant control soybean.

(a) Morphological and growth characteristics

Differences in morphological and growth characteristics were examined between this recombinant soybean and the non-recombinant control soybean in four (4) repeats for 12 plants per plot with reference to the items of soybean morphological and growth characteristics listed in the criteria for investigation on classification of soybean characteristics issued by the Ministry of Agriculture, Forestry and Fisheries with respect to a total of 18 items: uniformity of germination, germination rate, time of flower initiation, shape of leaflet, pubescence density, maturation period, plant shape, main stem length, height of the lowest node of pod formation, the number of main stem nodes, the number of branches, the total number of pods per plant, the total weight of seeds per plant, the number of mature seeds per plant, the weight of ripe seeds per plant, 100 seeds weight, difficulty in pod shattering, and shape of seed. For any items which allows statistical treatment, analysis of variance comparisons was applied with the lines and replications defined as factors between this recombinant soybean and the non-recombinant control soybean.

As a result, in all the items examined, no statistically significant difference ($P < 0.05$) or difference was observed between this recombinant soybean and the non-recombinant control soybean (Table 12 on Page 23).

Table 12 Morphology and growth characteristics

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

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

Soybean does not grow in winter and then, examination was conducted for the

cold-tolerance of this recombinant soybean at the early stage of growth.

The harvested seeds from this recombinant soybean and the non-recombinant control soybean were sown in pots, 12 seeds per plot. After germination, the pots were assigned four (4) plants each and placed in a greenhouse to raise the plant individuals up to V2 to V3 stage. Then, the plants were moved to an isolated field (an average temperature of 3℃ and an average lowest temperature of -6℃ during the test period) and they were visually examined for any damage due to low temperatures on the 8th, 15th, and 22nd days based on the scale of 7 levels. The test was conducted in four (4) repeats with one plot in the field defined as one repeat, and analysis of variance comparisons was made between this recombinant soybean and the non-recombinant control soybean.

As a result, the plant individuals of this recombinant soybean and the non-recombinant control soybean all withered 22 days after exposure to the low temperature conditions, and no statistically significant difference ($P<0.05$) was observed between the both plants (Table 13 on Page 24).

Table 13 Comparison of damage due to low temperatures (cold-tolerance)

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

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

The plant individuals of this recombinant soybean and the non-recombinant control soybean were left in the field for cultivation even after the harvesting time, during which the growth conditions were visually examined to identify the wintering ability of matured plants.

As a result, the plant individuals of this recombinant soybean and the non-recombinant control soybean all died down in mid-December, and no difference was observed between the both plants regarding the growth conditions (death).

(d) Fertility and size of the pollen

Flowers of this recombinant soybean and the non-recombinant control soybean were collected immediately before flower initiation, one flower per plant from a total of five (5) plants per plot. Pollens collected from the flowers were stained with acetocarmine solution and photographed. Percentage of the pollens stained with the solution among 300 pollens per plot was determined. With respect to the size of pollen, measurement of diameter was made for 12 pollens per plot. The tests were conducted in four (4) repeats with one plot in the field defined as one repeat, and analysis of variance comparisons was made between this recombinant soybean and the non-recombinant control soybean.

As a result, regarding the pollens of this recombinant soybean and the non-recombinant control soybean, no statistically significant difference ($P<0.05$)

was observed between the both plants in both the percentage of stained pollens and the pollen diameter (Table 14 on Page 25).

Table 14 Percentage of stained pollens and pollen diameter

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

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

Seed production: In the examination described in 2). (a) (Page 23), the total number of pods 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 result, in all the items examined, no statistically significant difference ($P < 0.05$) was observed between this recombinant soybean and the non-recombinant control soybean (Table 12 on Page 23).

Shattering habit: In the examination described in 2). (a) (Page 23), shattering habit of the seed was examined in terms of difficulty in pod shattering and as a result, this recombinant soybean and the non-recombinant control soybean both exhibited difficult pod shattering, showing no difference between the both plants regarding shattering habit of the seed (Table 12 on Page 23).

Germination rate: Fifty (50) seeds per plot were collected and sown in pots within a few hours after harvesting then cultivated in a greenhouse (set at 20℃) and the germination rate was examined 20 days after sowing. The test was conducted in four (4) repeats with one plot in the field defined as one repeat, and analysis of variance comparisons was made between this recombinant soybean and the non-recombinant control soybean. As a result, for the seeds of both of this recombinant soybean and the non-recombinant control soybean, the germination rates were 98% or more, and no statistically significant difference ($P<0.05$) was observed between the both plants (Table 15 on Page 25). Also at the time of seed sowing in the isolated field test, germination rate was examined (Table 12 on Page 23) and as a result, no statistically significant difference ($P<0.05$) was observed between this recombinant soybean and the non-recombinant control soybean.

Table 15 Germination rate of harvested seeds

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

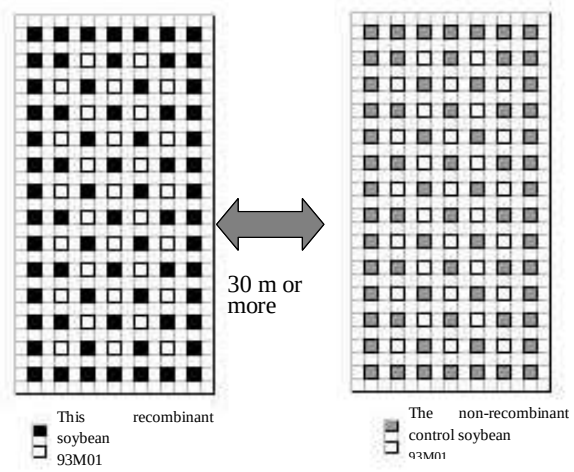
Dormancy: Soybean is reported to possess little dormancy of the seeds (OECD, 2000). In the examination for germination rate described above, the seeds were sown within a few hours after harvesting to examine the germination rate and as a result, for the seeds of the both plants of soybean, the germination rates were 90% or more (Table 15 on Page 25). Consequently, it was considered that this recombinant soybean also possesses no dormancy of the seeds similarly as the non-recombinant control soybean.

(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 the field in the US using the T6 generation (Figure 6 on Page 14).

Sixty eight (68) seeds of this recombinant soybean and 30 seeds of low linolenic acid soybean variety 93M01 were sown 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", Figure 9 (A) on Page 26). In addition, with a buffer zone provided 30 m or more distant from the recombinant test plot, 68 seeds of the non-recombinant control 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 9 (B) on Page 26). From the individual test plots, the seeds of soybean variety 93M01 were harvested to identify the crossing rate of this recombinant soybean or the non-recombinant control soybean with the soybean variety 93M01.

As a result, the crossing rate between this recombinant soybean or the non-recombinant control soybean and soybean variety 93M01 was found 0.3% or 0.7% respectively.



(A) Recombinant test plot (B) Non-recombinant test plot

Figure 9 Layout of crossability test plots in the field in the US

The test was conducted in 2006 for a total of 98 plants of soybean (30 pollination parent plants and 68 pollen parent plants) planted in 7 ridges × 14 plants per ridge with a planting distance of 30 cm and a ridge spacing of 80 cm.

(A) The seeds were collected from 30 individuals of soybean variety 93M01 in the recombinant test plot in the maturation period, and 300 seeds of them were sown in the field. At the one-leaf stage, the leaves were collected to extract the DNA. Using the DNA as template, the real-time PCR analysis was conducted with the specific primer to the *gm-fad2-1* gene expression cassette region to examine the presence of the transferred genes and determine the crossing rate.

(B) In order to examine the crossing rate between the non-recombinant control 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, the leaves were 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 control soybean to examine the presence of marker gene and determine the crossing rate.

(All the rights pertinent to the diagram above and the responsibility for the contents rest upon Du Pont Kabushiki Kaisha.)

In addition, in the isolated field test in Japan, a total of 1,600 seeds were collected from the individuals of the non-recombinant control soybean (planting distance of 30 cm) adjacent to this recombinant soybean and they were sown. The plants were raised in a greenhouse up to the one-leaf stage and then sprayed with an acetolactate synthase inhibitor (Tribenuron methyl, dosage of 4.5 g ai/ha) and as a result, all the plants were found suffering herbicide injury (average herbicide injury of 74±2%). In this recombinant soybean simultaneously raised and sprayed with the herbicide, no herbicide injury was observed (herbicide injury of 0±0%). Therefore, in this test, crossing between this recombinant soybean and the non-recombinant control soybean was not observed (crossing rate of 0%).

It is generally reported that the rate of natural crossing between 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). The crossability of this recombinant soybean tested in the crossability experiments in the US and Japan was found not exceeding the typical rate of natural crossing rate of soybean.

(g) Productivity of harmful substances

There is no report that soybean produces any substances that would adversely affect the living or growing of wild plants. In this recombinant soybean, the high oleic acid trait is conferred due to the transferred *gm-fad2-1* gene and the GM-HRA protein is expressed due to the transferred *gm-hra* gene. However, there is no report that they would adversely affect the growth of plants or soil microorganisms. Then, to confirm that there is no possibility of any unintended production of harmful substances, a succeeding crop test, plow-in test, and soil microflora test were conducted.

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

At the time of harvesting, rhizosphere soil was collected from within approximately 20 cm around the bottom of plants in each plot. It was filtered with a sieve to remove the plant residues and mixed uniformly then filled into a total of 25 pots by plot (measuring 6 cm×6 cm and a depth of 5.5 cm). As a test plant, one seed of radish (cultivar: Icicle) was sown per pot and raised in a greenhouse (set at 20℃) to examine the germination rate for a unit of 25 pots. In addition, 14 days after sowing, plants were collected to determine the dry weight, and the mean value of dry weight of germinated plants in units of 25 pots was defined as a value for one

repeat. The test was conducted in four (4) repeats with one plot in the field defined as one repeat, and analysis of variance comparisons was made between the soils used for cultivation of this recombinant soybean and the non-recombinant control soybean.

As a result, regarding the germination rate and dry weight of radish, no statistically significant difference ($P<0.05$) was observed between the soils used for cultivation of this recombinant soybean and the non-recombinant control soybean (Table 16 on Page 28).

Table 16 Germination rate and dry weight of radish in the succeeding crop test
[Confidential: Not made available or disclosed to unauthorized person]

Also in the US, examination was conducted based on the rhizosphere soil method (Iqbal *et al.*, 2004) using the lettuce germination rate, radicle length and hypocotyl length as indicators for productivity of intrinsic substances secreted from the roots with a threat to affect other plants. As a result, similarly as confirmed from the succeeding crop test, regarding the individual indicators, no statistically significant difference ($P<0.05$) was observed between the soild for cultivation of this recombinant soybean and the non-recombinant control soybean [Outline of the Biological Diversity Risk Assessment Report for soybean with high oleic acid content and herbicide acetolactate synthase inhibitor tolerance (*gm-fad2-1, gm-hra, Glycine max* (L.) Merr.) (DP-305423-1, OECD UI: DP-305423-1), http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_305423_1ap.pdf].

Based on the above understanding, it was considered that there is no difference between this recombinant soybean and the non-recombinant control soybean regarding the ability to produce any substances secreted from the roots with a threat to affect other plants.

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

In this recombinant soybean, the oleic acid content in the seed is increased and the linoleic acid content is decreased (I. 2. (1). 2). (c) on Page 6). Therefore, the plow-in test was conducted using the aerial part of plants other than seeds (hereinafter referred to as "forage") and the seeds.

At the time of harvesting, forage and seed of plants were collected from each plot to prepare dry powder. The dry powder was mixed with the nursery bed soil for seedlings by 0.5% (w/w) and packed to a total of 25 pots per plot (diameter of 6.5 cm and depth of 8 cm). As a test plant, the seeds of radish (cultivar: Icicle) were sown, one seed per pot, and raised in a greenhouse (at 20℃) to determine the germination rate by 25 pots. In addition, 14 days after seed sowing, plants were collected to determine the dry weight, and the mean value of dry weight of germinated plants by 25 pots was defined as a value for one repeat. The test was conducted in four (4) repeats with one plot in the field defined as one repeat, and

analysis of variance comparisons was made between the soils plowed with this recombinant soybean and the non-recombinant control soybean.

As a result, in both of forage and seed, no statistically significant difference ($P < 0.05$) was observed between the soils plowed with this recombinant soybean and the non-recombinant control soybean with respect to the germination rate and dry weight of radish (Table 17 on Page 29).

Table 17 Germination rate and dry weight of radish in the plow-in test
[Confidential: Not made available or disclosed to unauthorized person]

In addition, in the US, 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 leaves of this recombinant soybean for productivity of intrinsic substances with a threat to affect other plants with the lettuce germination rate, radicle length and hypocotyl defined as indicators. As a result, regarding all the indicators, no statistically significant difference ($P<0.05$) was observed between the leaves of this recombinant soybean and the non-recombinant control soybean [Outline of the Biological Diversity Risk Assessment Report for soybean with high oleic acid content and herbicide acetolactate synthase inhibitor tolerance (*gm-fad2-1, gm-hra, Glycine max* (L.) Merr.) (DP-305423-1, OECD UI: DP-305423-1),
http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_305423_ 1ap.pdf].

Based on the above understanding, it was considered that there is no difference between this recombinant soybean and the non-recombinant control soybean regarding the ability to produce any intrinsic substances with a threat to affect other plants after dying.

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

At the time of harvesting, soil was collected from the bottom of 4 plants per plot and mixed by plot. The soil was added with sterilized water and then stirred and mixed to prepare the dilution solutions from 10^{-3} to 10^{-6}. Based on the dilution plate technique, the dilution solutions were left to stand at 25℃ for incubation. For media, the PTYG media for bacteria and actinomycete and the rose bengal media for filamentous fungi were used, and they were left to stand for 7 days and 3 days respectively for incubation. The test was conducted in four (4) repeats with one plot in the field defined as one repeat to identify the number of bacteria, the number of filamentous fungi and the number of actinomycete in the soils used for cultivation of this recombinant soybean and the non-recombinant control soybean, and analysis of variance comparisons was made between this recombinant soybean and the non-recombinant control soybean.

As a result, regarding the number of bacteria, the number of filamentous fungi and the number of actinomycete in soils used for cultivation of this recombinant

soybean and the non-recombinant control soybean, no statistically significant difference ($P < 0.05$) was observed (Table 18 on Page 30).

Based on the above understanding, it was considered that there was no difference between this recombinant soybean and the non-recombinant control soybean regarding the ability to produce any harmful substances secreted from the roots with a threat to affect microorganisms in soil.

Table 18 The number of bacteria, the number of filamentous fungi and the number of actinomycete in soil taken from the plots

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

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 has the oleic acid content increased in the seeds because of the transferred *gm-fad2-1* and *gm-hra* genes, and it is given the trait to be tolerant to acetolactate synthase inhibitors. However, there is no report that the oleic acid is especially useful for energy supply at the time of germination. In addition, it is expected unlikely that the acetolactate synthase inhibitors would exert pressure for selection under a natural environment. Therefore, it is considered unlikely that these traits cause this recombinant soybean to become competitive.

Furthermore, based on the results of investigations in the isolated fields in Japan, with regard to various traits relating to the competitiveness of this recombinant soybean, no significant difference from the non-recombinant control soybean has been 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 competitiveness is reasonable.

(2) Productivity of harmful substances

Regarding the plant species 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 GM-HRA protein is produced, though there is no report that the protein has any adverse effect on the growth of plants, and homology of amino acid sequences with known allergens and toxic proteins has not been observed.

In addition, in the isolated field in Japan, to examine 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), succeeding crop test, plow-in test, and soil microflora test were conducted and as a result, no statistically significant difference was observed between this recombinant soybean and the non-recombinant control

soybean.

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

(3) Crossability

Since it is known that if the *Glycine soja* Sieb. et Zucc. (*G. soja*) that grows naturally 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.

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 would grow and that the gene transferred into this recombinant soybean through the back crossing from the hybrid to *G. soja* could diffuses among the population of *G. soja* without remaining at a low level.

G. soja grows naturally and widely throughout Japan in river beaches and banksides, in the vicinity of farmlands, and orchards 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:

(i) It is generally known that the flowering time of soybean and *G. 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 artificially matching the flowering time, the rate of crossability is reportedly 0.73%.

(ii) There is a report that no genetic marker has been detected suggesting any crossing between soybean and *G. soja*.

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

(iv) As a result of crossability test in which the flowering time of herbicide glyphosate tolerant soybean line 40-3-2 and *G. soja* was matched with each other and the both plants were cultivated adjacent to each other and raised with *G. soja* wound around soybean, one grain of 32,502 harvested seeds of *G. soja* is reportedly crossed with soybean.

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

If this recombinant soybean crosses with *G. soja*, the obtained hybrid is considered to possess the trait to be tolerant to acetolactate synthase inhibitor due to the *gm-hra* gene. However, this trait is considered unlikely to increase the competitiveness. In addition, even if such a hybrid is produced that is given the trait to tolerant to acetolactate synthase inhibitors, it is considered unlikely that the hybrid dominate the population of *G. soja*.

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.