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

Name: Du Pont Kabushiki Kaisha
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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Soybean with high oleic acid content and tolerances to herbicides acetolactate synthase inhibitor and glyphosate (*gm-fad2-1*, *gm-hra*, modified *cp4 epsps, Glycine max* (L.) Merr.) (305423×40-3-2, OECD UI: DP-305423-1×MON-04032-6)
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

The parent lines of this stack soybean line, DP-305423-1 (http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_305423_1_2009ap.pdf) was developed by Pioneer Hi-Bred International Inc. in the U.S., and MON-04032-6 (http://www.bch.biodic.go.jp/download/lmo/public_comment/40-3-2ap.pdf, and an application for approval of unlimited cultivation of the cultivar to the United States Department of Agriculture (USDA) (1993)) was developed by Monsanto Company in the U.S. In DP-305423-1, the *gm-fad2-1* gene to confer the traits of high oleic acid content and the *gm-hra* gene to confer the tolerance to acetolactate synthase inhibitors have been transferred. In MON-04032-6, the modified *cp4 epsps* gene to confer the tolerance to herbicide glyphosate has been transferred.

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of DP-305423-1 and MON-04032-6 are shown individually in Table 1 (p. 3) and Table 2 (p. 5).

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid are shown in Table 1 (p. 3) and Table 2 (p. 5).

Table 1 Composition of the donor nucleic acid and the origin and function of component elements used for the development of DP-305423-1 (1)

Component elements	Size (kb)	Origin and function
gm-fad2-1 gene expression cassette (PHP19340 A)		
KTi3 promoter	2.08	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	0.60	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[1)] and thereby suppress the expression of ω-6 desaturase.
KTi3 terminator	0.20	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	0.05	Flp recombinant enzyme recognition sequence derived from yeast (*Saccharomyces cerevisiae*) (Broach *et al*., 1982).
SAMS promoter	0.65	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	0.59	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*)	1.97	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	0.65	A terminator region of *als* gene derived from soybean to terminate transcription (Falco and Li, 2003).
FRT1	0.05	Same as above
FRT6	0.05	Modified FRT1 sequence having 94% homology with FRT1, and constituting the recognition sequence of Flp recombinant enzyme.

1) 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).

Table 1 Composition of the donor nucleic acid and the origin and function of component elements used for the development of DP-305423-1 (2)

Component elements	Size (kb)	Origin and function
Other component elements (This was not transferred to DP-305423-1.)		
T7 promoter	0.08	A promoter region derived from coliform bacteriophage T7 genome (GenBank Accession No. V01146). It initiates transcription of genes in *Escherichia coli* (*E. coli*).
hyg	1.03	Antibiotic hygromycin-resistant gene derived from *Escherichia coli* (*E. coli*) (GenBank Accession No. K01193). Used as a selectable marker for cloning in *E. coli*.
T7 terminator	0.12	A terminator region derived from coliform bacteriophage T7 genome (GenBank Accession No. V01146). It terminates transcription.
ori	0.37	The replication origin of *E. coli*, derived from colE1 plasmid.

Table 2 Composition of the donor nucleic acid and the origin and function of component elements used for the development of MON-04032-6

Component elements	Size (kb)	Origin and function
Modified *cp4 epsps* gene expression cassette controlled by P-CMoVa (E35S)		
CMoVa (E35S)	0.61	35S promoter of cauliflower mosaic virus (CaMV). Has a duplication enhancer region. Involved in the constitutive expression of the target gene in all tissues.
CTP	0.23	A nucleotide sequence in the *epsps* gene of *Arabidopsis thaliana*, which encodes the chloroplast transit peptide sequence located at N-terminal region of EPSPS protein. Transfers target proteins from cytoplasm to chloroplast.
Modified *cp4 epsps*	1.36	5-enol-pyruvyl-shikimate-3-phosphate synthase *gene* derived from *Agrobacterium* strain CP4.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation.
uidA gene expression cassette controlled by P-MAS (This was not transferred to MON-04032-6.)		
P-MAS	0.42	Promoter region of *mannopine synthase 2'* gene derived from *Agrobacterium tumefaciens*. Involved in the constitutive expression of the target gene.
uidA(GUS)	1.81	*uidA* gene derived from *Escherichia coli* (*E. coli*). Encodes GUS (β-D-glucuronidase) protein.
7S3'	0.43	3' terminal untranslated region of 7S seed storage protein α subunit of soybean.
Modified *cp4 epsps* gene expression cassette controlled by P-CMoVb (FMV) (This was not transferred to MON-04032-6.)		
CMoVb (FMV)	0.57	35S promoter of Figwort mosaic virus. Involved in the constitutive expression of the target gene in all tissues.
CTP	0.22	A nucleotide sequence in the *epsps* gene of *Arabidopsis thaliana*, which encodes the chloroplast transit peptide sequence located at N-terminal region of EPSPS protein. Transfers target proteins from cytoplasm to chloroplast.
Modified *cp4 epsps*	1.36	5-enol-pyruvyl-shikimate-3-phosphate synthase *gene* derived from *Agrobacterium* strain CP4.
NOS 3'	0.26	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation.
Other component elements (These were not transferred to MON-04032-6.)		
LAC	0.24	Consists of a partial sequence encoding a lac repressor, lac promoter, and a partial sequence encoding β galactosidase. Used as a selectable marker for cloning in *E. coli*.
ori-pUC	0.65	Replicator region from *E. coli* plasmid pUC119.

KAN(*nptII*)	1.32	A gene isolated from Tn5 transposon of *E. coli*. Encodes neomycin phosphotransferase type II (NPTII) protein.

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

***gm-fad2-1* gene**

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. This gene has been transferred to induce gene silencing and thereby suppress the expression of ω-6 desaturase. In DP-305423-1, the expression level of soybean endogenous *FAD2-1* gene is successfully suppressed as intended 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.

GM-HRA protein

The GM-HRA protein precursor encoded by the *gm-hra* gene possesses the chloroplast transport sequence in the N-terminal region. For the GM-HRA protein precursor, the chloroplast transport sequence is removed as the transport into chloroplast proceeds, and eventually the mature GM-HRA protein (hereinafter referred to as "GM-HRA protein") is formed.

Acetolactate synthase inhibitors specifically inhibit the activity of endogenous acetolactate synthase (hereinafter referred to as "ALS") 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. 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.

In order to identify the structural homology between the GM-HRA protein and any known allergens, an amino acid sequence homology search was conducted in 2010 using the allergen database (FARRP10). As a result, there were no known allergens observed which exhibited homology with the GM-HRA protein.

Modified CP4 EPSPS protein

The herbicide glyphosate inhibits the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), an aromatic amino acid biosynthesis pathway in plants, which interrupts the synthesis of aromatic amino acids in plants, causing the plants to die. The modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene exhibits the activity even in the presence of glyphosate, preventing the

shikimate pathway from being inhibited, thereby conferring glyphosate herbicide tolerance to the plant.

In order to identify the structural homology between the modified CP4 EPSPS protein and any known allergens, an amino acid sequence homology search was conducted in 2010 using the allergen database (AD_2010, TOX_2010 and PRT_2010). As a result, there were no known allergens observed which exhibited homology with the modified CP4 EPSPS protein.

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

For any changes caused to the metabolic system of recipient organism, examination was conducted for the *gm-fad2-1* gene, the GM-HRA protein and the modified CP4 EPSPS protein individually and also for possible interaction between them.

***gm-fad2-1* gene**

In DP-305423-1, 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), page 7).

The oleic acid content against the total amount of fatty acids in the seed of DP-305423-1 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%. The oleic acid content in the leaf was found 4.43% for DP-305423-1 compared to 3.61% for the non-recombinant soybean (P-value <0.05). The linolenic acid content was found 47.4% compared to 50.0% for the non-recombinant soybean (P-value <0.05). In this way, DP-305423-1 showed an increase in the oleic acid content due to the *gm-fad2-1* gene.

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 endogenous ALS protein works in the biosynthetic pathway of branched-chain amino acids. 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. In addition, in the isoleucine

biosynthetic pathway, 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, 1998). Therefore, it is considered that also in the GM-HRA protein which works on the biosynthetic pathway of branched-chain amino acids, the feedback control would work similarly as in the endogenous ALS protein.

In fact, as a result of analysis of amino acid composition in the seeds and leaves of DP-305423-1, 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 interval or literature data. In addition, leucine in the leaves showed a statistically significant increase ($P<0.05$) from the non-recombinant control soybean, though the analysis value was 9.97% for DP-305423-1 and 9.60% for the non-recombinant soybean, showing a slight difference. For valine synthesized in the same pathway, no significant increase was observed. Thus, the significant difference was considered unlikely to result from the lack of feedback control.

Regarding possible effects on the biosynthesis of fatty acids:
As a result of analysis of fatty acid composition in the seeds of DP-305423-1, a statistically significant increase ($P<0.05$) from the non-recombinant control soybean was observed in heptadecanoic acid and heptadecenoic acid. However, these are fatty acids generally contained in many animals and plants (Ministry of Education, Culture, Sports, Science and Technology (MEXT), 2005), and the percentage of heptadecanoic acid and heptadecenoic acid against the the total amount of fatty acids was found 0.798% and 1.19%, respectively.

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 biosynthesis pathway of fatty acids. The GM-HRA protein has lower substrate affinity for α-ketobutyric acid compared to the endogenous ALS. Then, it was estimated that in DP-305423-1, 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.

Modified CP4 EPSPS protein

It has been suggested that the EPSPS is not a rate-determining enzyme in the shikimate pathway for biosynthesis of aromatic amino acids, and as such it is considered that the concentration of aromatic amino acids, the final product of this pathway, would not be increased even if the total activity of the

EPSPS is increased due to the production of the modified CP4 EPSPS protein. In fact, it has been reported that plant cells producing a 40-times or more amount of the EPSPS protein than normal cannot excessively synthesize aromatic amino acids as a final product. In addition, it has been confirmed that there is no difference in the content of amino acids between the genetically modified crops by U.S. Monsanto Company for tolerance to herbicide glyphosate (soybean, oilseed rape, cotton, and maize) and the original non-recombinant crops.

The EPSPS is known to react specifically with its substrates of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P). The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P. However, the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate acts as the substrate of EPSPS in the living body.

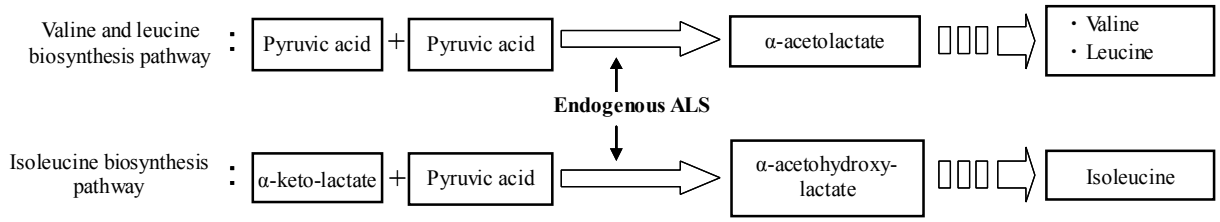
Any interaction between expressions of transferred genes

Metabolic system and substrate:
The expressions of the *gm-fad2-1* gene, the *gm-hra* gene and the modified *cp4 epsps* gene take part in the synthesis from oleic acid to linoleic acid, the biosynthesis of branched-chain amino acids (Figure 1, p. 11) and the biosynthesis of aromatic amino acids (
Figure 2, p.12), respectively. These biosynthetic pathways constitute independent metabolic pathways from each other in plants and involve different substrates; therefore, it is considered unlikely that expressions of the above-mentioned transferred genes would interact with each other.

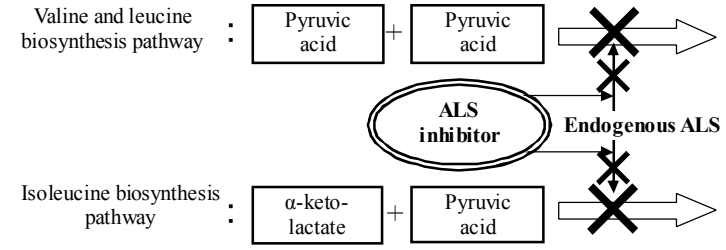
Amino acid composition:
Actually, analysis of amino acid composition in the seeds of this stack soybean line, the parent lines DP-305423-1 and MON-04032-6, and the non-recombinant control soybean was conducted. As a result, some items analyzed showed statistically significant differences ($P<0.05$) between this stack soybean line and the parent lines (DP-305423-1 and MON-04032-6), though the differences were found falling within the range of values based on tolerance interval or literature data (Table 3, p.13). Therefore, it was confirmed that expressions of the transferred genes in this stack soybean line would not have any effects on the amino acid composition.

Based on the above understanding, it was considered unlikely that expressions of the transferred genes in this stack soybean line would interact with each other.

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 stack soybean line is sprayed with acetolactate synthase inhibitors:

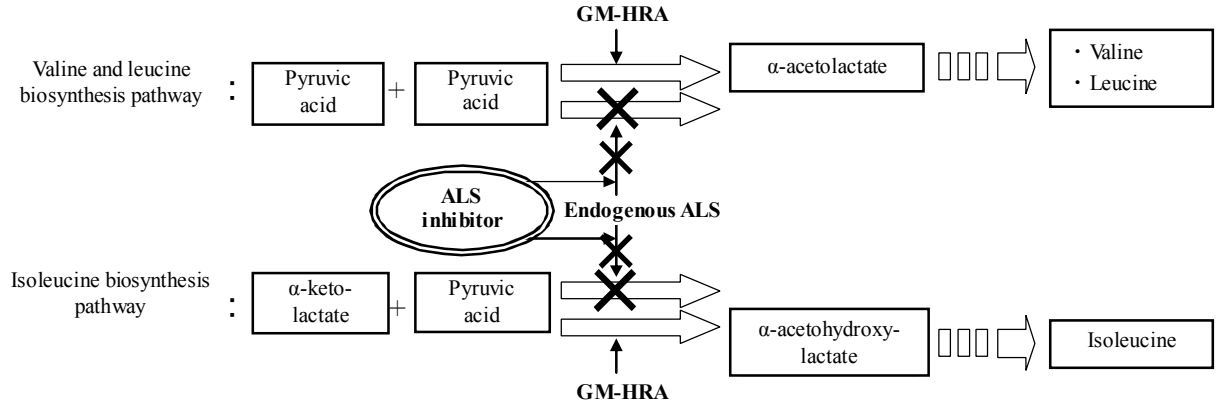
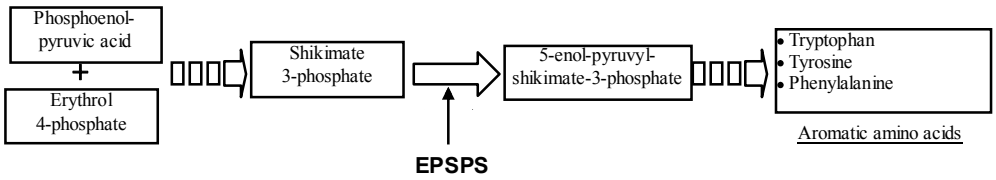


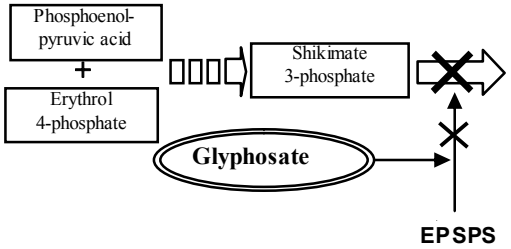
Figure 1 Mechanism of action of the GM-HRA protein

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 becomes impossible and plants would die out.
iii) In this stack soybean line, 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.

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



ii) When the non-recombinant soybean is sprayed with herbicide glyphosate:



iii) When this recombinant soybean is sprayed with herbicide glyphosate:

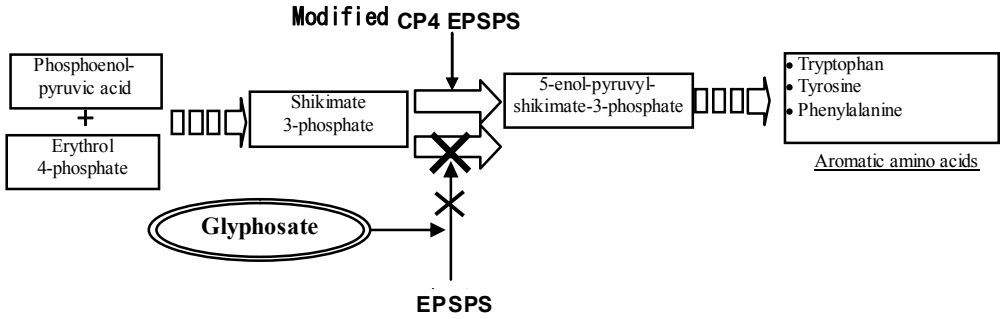


Figure 2 Mechanism of action of the modified CP4 EPSPS protein

i) EPSPS is an enzyme involved in the synthesis of 5-enol-pyruvyl-shikimate-3-phosphate, and the 5-enol-pyruvyl-shikimate-3-phosphate is used for biosynthesis of the aromatic amino acids of tryptophan, tyrosine and phenylalanine.
ii) In the non-recombinant soybean, the activity of EPSPS is inhibited by the herbicide glyphosate and as a result, the biosynthesis of aromatic amino acids becomes impossible and plants would die.
iii) In this stack soybean line, the modified CP4 EPSPS protein is produced and as a result, plants are not affected by the herbicide glyphosate and thus can synthesize the aromatic amino acids.

Table 3 Composition of amino acids in the seeds of this stack soybean line and the parent lines (1)

Item to be analyzed		% Dry weight					
		This stack line (BC1 F5 generation)	Control; Parent line (DP-305423-1)	Control; Parent line (MON-04032-6)	(Reference) Non-recombinant soybean (BC1F6 null generation)	Tolerance interval [1]	Literature range
Methionine	Mean value	0.695	0.713	0.661	0.714	0.322 – 0.911	0.431[4] – 0.681[4]
	Standard deviation	0.0443	0.0395	0.0582	0.0510		
	P value		0.231	0.0294[3]	0.206		
	FDR [2]		0.260	0.222	0.265		
Cystine	Mean value	0.614	0.613	0.594	0.638	0.429 – 0.820	0.370[4] – 0.808[4]
	Standard deviation	0.0513	0.0465	0.0615	0.0615		
	P value		0.974	0.254	0.171		
	FDR		0.974	0.508	0.237		
Lysine	Mean value	2.45	2.58	2.53	2.56	2.06 – 3.14	2.29[4] – 2.86[5]
	Standard deviation	0.211	0.176	0.157	0.109		
	P value		0.0466[3]	0.227	0.0798		
	FDR		0.0839	0.508	0.161		
Tryptophan	Mean value	0.471	0.508	0.482	0.496	0.356 – 0.668	0.356[4] – 0.67[6]
	Standard deviation	0.0345	0.0481	0.0337	0.0393		
	P value		0.00395[3]	0.328	0.0343[3]		
	FDR		0.0119[3]	0.509	0.154		
Threonine	Mean value	1.85	1.95	1.87	1.91	0.743 – 2.67	1.14[4] – 1.89[6]
	Standard deviation	0.133	0.0877	0.101	0.0723		
	P value		0.00604[3]	0.396	0.0769		
	FDR		0.0155[3]	0.509	0.161		
Isoleucine	Mean value	1.68	1.79	1.78	1.78	1.52 – 2.08	1.46[5] – 2.12[5]
	Standard deviation	0.115	0.0865	0.0975	0.0658		
	P value		0.00215[3]	0.00598[3]	0.00492[3]		
	FDR		0.00966[3]	0.108	0.0443[3]		
Histidine	Mean value	1.16	1.21	1.17	1.17	0.680 – 1.49	0.878[4] – 1.22[6]
	Standard deviation	0.0886	0.100	0.112	0.103		
	P value		0.116	0.698	0.708		
	FDR		0.149	0.785	0.749		
Valine	Mean value	1.77	1.87	1.84	1.84	1.59 – 2.15	1.5[6] – 2.44[6]
	Standard deviation	0.127	0.0982	0.105	0.0825		
	P value		0.00802[3]	0.0720	0.0449[3]		
	FDR		0.0180[3]	0.324	0.161		
Leucine	Mean value	2.83	2.99	2.93	2.97	2.37 – 3.54	2.2[6] – 4.0[6]
	Standard deviation	0.172	0.105	0.135	0.0751		
	P value		0.00187[3]	0.0370[3]	0.00476[3]		
	FDR		0.00966[3]	0.222	0.0443[3]		
Arginine	Mean value	2.71	3.00	2.76	2.81	1.50 – 3.75	2.29[4] – 3.49[5]
	Standard deviation	0.166	0.250	0.175	0.144		
	P value		0.000243[3]	0.370	0.103		
	FDR		0.00437[3]	0.509	0.174		
Phenylalanine	Mean value	2.00	2.10	2.06	2.07	1.26 – 2.67	1.6[6] – 2.35[4]
	Standard deviation	0.145	0.0959	0.106	0.0980		
	P value		0.0218[3]	0.120	0.0806		
	FDR		0.0436[3]	0.432	0.161		

* For explanation of footnotes, refer to the next page.

Table 3 Composition of amino acids in the seeds of this stack soybean line and the parent lines (2)

Item to be analyzed		% Dry weight					
		This stack line (BC1 F5 generation)	Control; Parent line (DP-305423-1)	Control; Parent line (MON-04032-6)	(Reference) Non-recombinant soybean (BC1F6 null generation)	Tolerance interval [1]	Literature range
Glycine	Mean value	1.85	1.93	1.91	1.89	1.18 – 2.33	1.46[4] – 2.02[5]
	Standard deviation	0.116	0.0831	0.112	0.0966		
	P value		0.0665	0.166	0.378		
	FDR [2]		0.0921	0.497	0.426		
Alanine	Mean value	1.60	1.73	1.65	1.66	1.40 – 2.02	1.49[5] – 2.10[4]
	Standard deviation	0.118	0.139	0.116	0.0983		
	P value		0.00286[3]	0.205	0.106		
	FDR		0.0103[3]	0.508	0.174		
Aspartic acid	Mean value	4.73	4.92	4.83	5.01	3.88 – 6.14	3.81[4] – 5.12[4]
	Standard deviation	0.368	0.220	0.324	0.214		
	P value		0.0643	0.287	0.00810[3]		
	FDR		0.0921	0.509	0.0486[3]		
Glutamate	Mean value	7.44	7.92	7.53	7.69	6.04 – 9.36	5.84[4] – 8.72[5]
	Standard deviation	0.513	0.286	0.450	0.396		
	P value		0.00149[3]	0.484	0.0664		
	FDR		0.00966[3]	0.581	0.161		
Proline	Mean value	2.28	2.31	2.26	2.27	1.15 – 3.05	1.69[4] – 2.61[5]
	Standard deviation	0.203	0.137	0.134	0.109		
	P value		0.428	0.749	0.934		
	FDR		0.453	0.793	0.934		
Serine	Mean value	2.19	2.28	2.23	2.26	1.10 – 3.09	1.11[4] – 2.48[4]
	Standard deviation	0.182	0.0999	0.126	0.134		
	P value		0.0526	0.363	0.149		
	FDR		0.0860	0.509	0.224		
Tyrosine	Mean value	1.30	1.35	1.31	1.34	0.523 – 1.84	1.02[4] – 1.62[5]
	Standard deviation	0.119	0.102	0.107	0.111		
	P value		0.142	0.848	0.316		
	FDR		0.170	0.848	0.379		

Individual lines, n=18 for each, were cultivated in the fields at 6 sites in the North America in 2005, and the seeds were harvested from 3 plots in each field at the time of ripening (R8 stage). Testing was performed based on the linear mixed model.

1) The interval between the upper and lower limits adjusted statistically to include 99% of analysis values based on the analytical results of a total of eight (8) commercial varieties of non-recombinant soybean cultivated at a total of 12 sites in the North America in 2005 and 2007
2) P-value in consideration of FDR (False Discovery Rate; Benjamini and Hochberg, 1995). When a significance test for $P<0.05$ is conducted for multiple analysis items regarding a given sample, the proportion of false positives increases as the number of analysis items increases, causing more items to be defined in error as having a significant difference. In this case, application of FDR method helps control the proportion of false positives for proper significance test.
3) A statistically significant difference (P-value< 0.05) from this stack line was observed.
4) ILSI (2006)
5) Taylor *et al*. (1999)
6) OECD (2001)

(2) Information concerning vectors

1) Name and origin

The plasmid vectors used for the development of the parent lines are as follows.

DP-305423-1: Plasmid PHP19340 (Figure 3, p.17) and PHP17752 (Figure 4, p.18) constructed based on the plasmid pSP72 derived from *Escherichia coli* (*E. coli*).

MON-04032-6: Plasmid PV-GMGT04 (Figure 5, p.19) constructed based on the plasmid pUC119 and others derived from *Escherichia coli* (*E. coli*).

2) Properties

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

The total number of base pairs in the plasmid vectors used for the development of parent lines is as follows.

DP-305423-1 PHP19340: 5,438bp
PHP17752: 7,026bp

MON-04032-6 PV-GMGT04: 10,505bp

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

As the selectable markers for the development of the parent lines DP-305423-1 and MON-04032-6, the following genes were used. It has been confirmed that these marker genes have not been transferred in the parent lines.

DP-305423-1 PHP19340: *hyg* gene (antibiotic hygromycin resistant marker gene)
PHP17752: *hyg* gene

MON-04032-6 PV-GMGT04: *nptII* gene (antibiotic kanamycin resistant marker gene) and LAC (used as a selectable marker for cloning in *E. coli*)

(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 donor nucleic acid in the linear DNA fragments PHP19340A and PHP17752A and the plasmid PV-GMGT04 used for the transferring and the restriction enzyme cleavage sites are shown in Figure 3 to Figure 5 (p.17 - p.19).

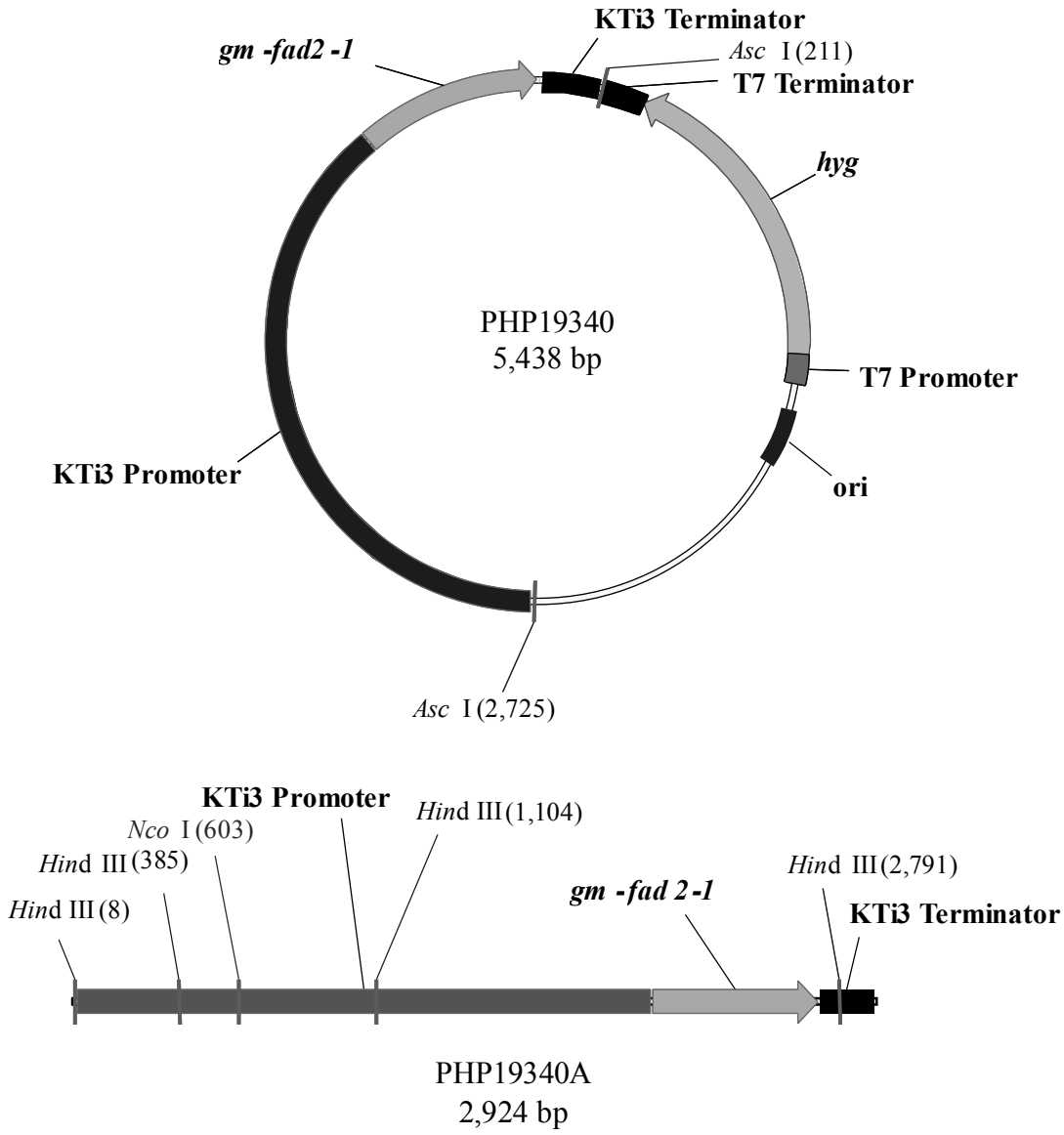


Figure 3 Composition of the donor nucleic acid in the plasmid PHP19340 and linear DNA fragment PHP19340A and the restriction enzyme cleavage sites

Top diagram: Plasmid PHP19340

Bottom diagram: The linear DNA fragment PHP19340A cleaved by the restriction enzyme *Asc* I from the plasmid PHP19340

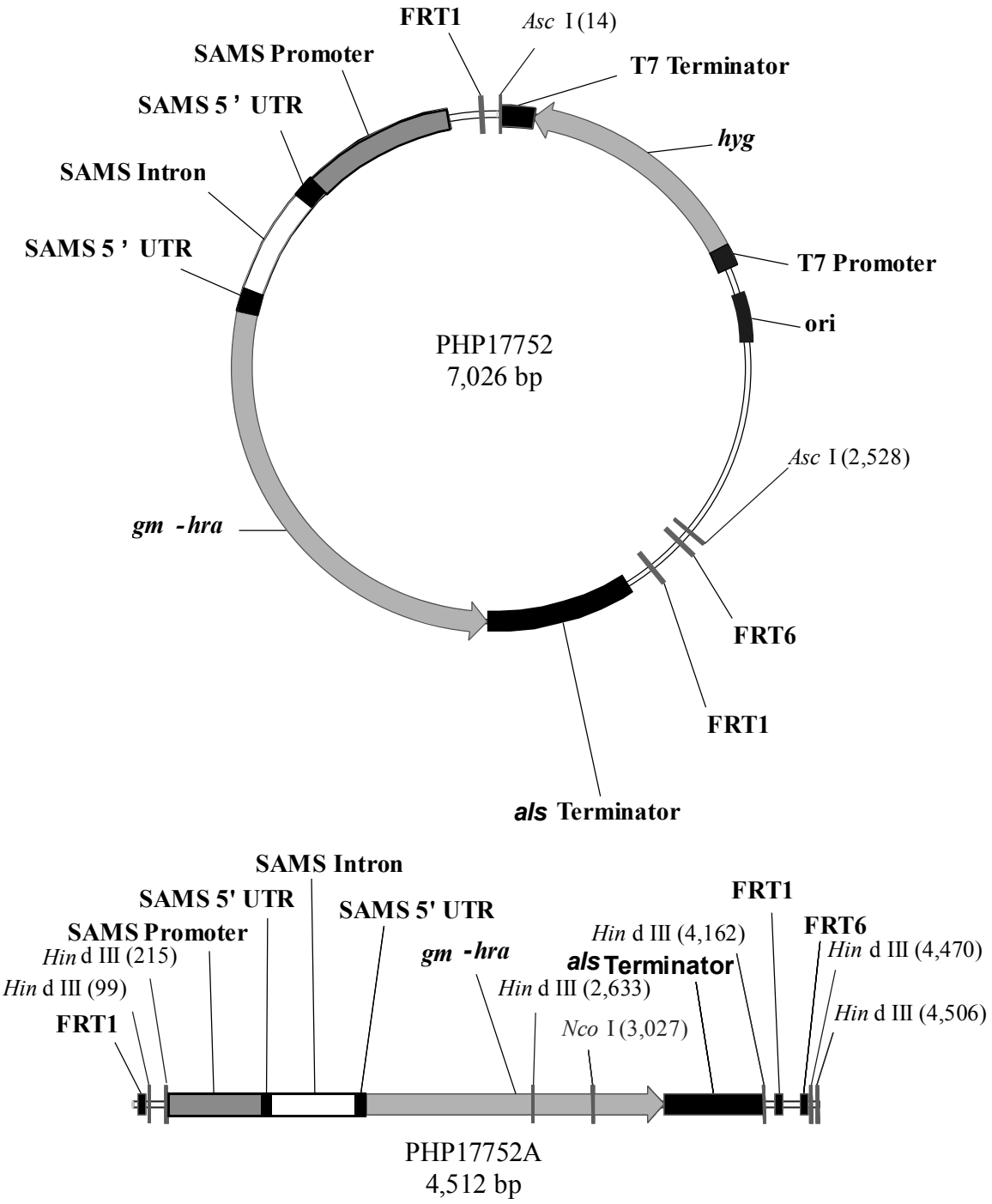


Figure 4 Composition of the donor nucleic acid in the plasmid PHP17752 and linear DNA fragment PHP17752A and the restriction enzyme cleavage sites

Top diagram: Plasmid PHP17752

Bottom diagram: The linear DNA fragment PHP17752A cleaved by the restriction enzyme *Asc* I from the plasmid PHP17752

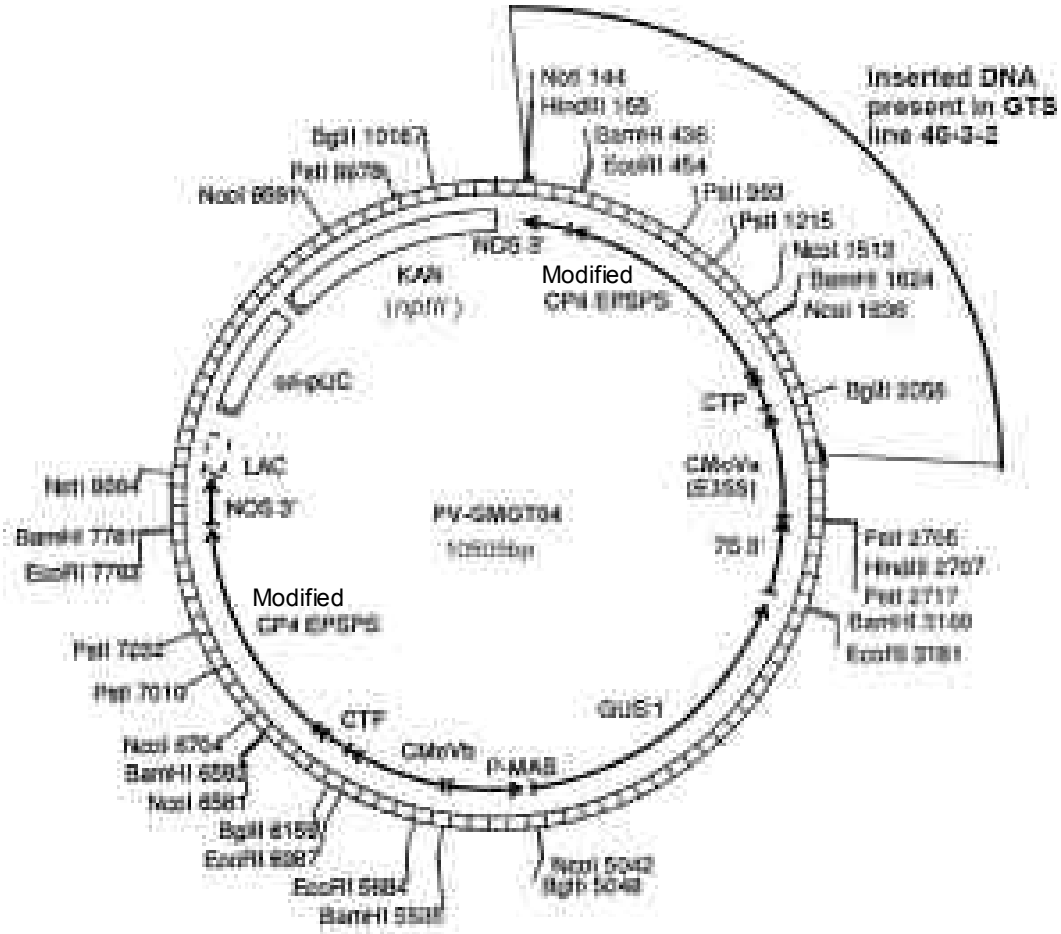


Figure 5 Composition of the donor nucleic acid in the plasmid PV-GMGT04 and the restriction enzyme cleavage sites

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

For transferring the nucleic acid to the recipient organism, particle gun bombardment method was used in both cases of DP-305423-1 and MON-04032-6.

3) Processes of rearing of living modified organisms

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

DP-305423-1: The somatic embryo callus was cultured successively for several generations on the media containing the acetolactate synthase inhibitor chlorosulfuron and then, individual growth callus was screened for transformant to which the *gm-fad2-1* gene and the *gm-hra* gene are found transferred (T0 generation).

MON-04032-6: The shoot apex cells were grown on the medium containing cytokinin and auxin to induce and redifferentiate adventitious buds. Among redifferentiated individuals, those individuals showing positivity to the GUS reaction were selected as individual transformants (=R0 generation).

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

-

(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 development of this stack soybean line from the parent lines and selection and rearing is shown below.

DP-305423-1:
Plant individuals of T0 generation were raised in the greenhouse, and seeds (T1 generation) were harvested. Based on the cultivation tests carried out in the fields using T1 and later generations, DP-305423-1 was selected eventually as a commercial pedigree.

MON-04032-6:
After the R0 generation, the estimation of tolerance to glyphosate, the estimation of hereditary mode of tolerance, the estimation of agricultural traits and the analysis of transferred genes were conducted. Finally based on the results of field tests conducted in the U.S. and Puerto Rico, MON-04032-6 was selected as a commercial pedigree.

This stack soybean line was produced by crossing DP-305423-1 and MON-04032-6 with use of the crossbreeding method. Process of rearing of this stack soybean line is shown in Figure 6 (p. 21; Not made available or disclosed to unauthorized person). The scope of application for approval of this recombinant soybean includes the progeny lines of F1 and later generations shown in the process of rearing. Status of application and approval of DP-305423-1 and MON-04032-6 in Japan is summarized in Table 4 (p.21).

(Not made available or disclosed to unauthorized person)

Figure 6 Process of rearing this stack soybean line

Table 4 Status of application and approval of DP-305423-1 and MON-04032-6

Competence authority	Contents of application	Status of application and approval	
		DP-305423-1	MON-04032-6
Ministry of Health, Labour and Welfare	Safety as food	Applied in April 2009	Approved in March 2001
Ministry of Agriculture, Forestry and Fisheries	Safety as feed	Applied in April 2009	Approved in March 2003
Ministry of Agriculture, Forestry and Fisheries, and Ministry of the Environment	Type 1 Use, etc. (Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them)	Hearing from experts (Comprehensive review meeting: March 2009) and completion of Public Comment Solicitation (May to June 2009)	Approved in May 2005

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

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

For analysis of segregation ratio, DP-305423-1 was examined for presence of high oleic acid trait and the *gm-hra* gene, and MON-04032-6 was examined for presence of herbicide tolerance. As a result, all the items examined were found corresponding to the expected value of 3:1. As such the transferred genes are confirmed to be inherited in accordance with the Mendel's law; therefore it is considered that the replication products of transferred nucleic acid exist on the genome of soybean chromosome.

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

DP-305423-1:
As a result of detection of nucleotide sequences based on the cloning of the transferred regions, it was found that the genes were transferred in four (4) regions listed below.

- Region 1: Composed of one intact copy of the *gm-fad2-1* gene expression cassette (PHP19340A), one intact copy of the *gm-hra* gene expression cassette (PHP17752A), three (3) PHP19340A fragments and one KTi3 promoter fragment.
- Region 2: Composed of one PHP19340A fragment.
- Region 3: Composed of one KTi3 promoter fragment and the plasmid backbone region of 495 bp.
- Region 4: Composed of the PHP19340A fragment transferred in the inverted repeat sequence.

In order to identify whether these transferred genes are stably inherited in progeny, Southern blotting analysis was conducted using seven (7) individuals each for T4 and T5 generations and 100 individuals for F2 generation. As a result, for one plant individual in the F2 generation, it was considered that the *gm-hra* gene expression cassette of the transferred gene region 1 and the adjacent KTi3 promoter fragment had dropped out. Then, additional 1,000 plant individuals of F2 generation were examined for presence of genes and as a result, there was no dropout of gene observed. Therefore, it was confirmed that the transferred genes to DP-305423-1 are stably inherited in progeny.

MON-04032-6:
In addition to one copy of the modified *cp4 epsps* gene expression cassette region, 250bp and 72bp fragments of the modified *cp4 epsps* gene were found transferred. However, it was confirmed by Northern blotting analysis and Western blotting analysis that these gene fragments did not function. Southern blotting analysis indicated that these transferred genes were stably inherited in

the progeny.

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

As mentioned in 2) (p. 22), it was found that the replication products of nucleic acid transferred to DP-305423-1 and MON-04032-6 are stably inherited in progeny. Based on the above understanding, it was considered that the several transferred regions identified in DP-305423-1 and MON-04032-6 are strongly linked with each other.

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

Expression stability of the parent lines of this stack soybean line was identified based on the methods listed below.

- The *gm-fad2-1* gene transferred into DP-305423-1: Analysis of main fatty acid composition in the seed

- The *gm-hra* gene transferred into DP-305423-1: Determination of expression level of the GM-HRA protein based on the herbicide acetolactate synthase inhibitor-spraying test and ELISA analysis

- The modified *cp4 epsps* gene transferred into MON-04032-6: Herbicide glyphosate-spraying test

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

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) Method of detection

Specific detection method for individual parent lines (DP-305423-1 and MON-04032-6) based on the real-time quantitative PCR analysis is available from the Web site of European Commission (Joint Research Centre, 2009a &

2009b). The proportion of the number of copies of detected DP-305423-1 or MON-04032-6 against the number of copies of soybean endogenous gene (lectin gene) is determined as a content of individual parent lines.

2) Sensitivity

The detection sensitivity is 0.09% for DP-305423-1 and 0.1% for MON-04032-6.

3) Reliability

For the method for detection of DP-305423-1 and MON-04032-6 mentioned above in 1), the reliability has been verified based on the tests conducted in 4 repeats at 12 and 14 member test laboratories of the European Network of GMO Laboratories, respectively.

In order to detect and identify this stack line, one seed or plant body needs to be examined by the two methods mentioned above, and this stack line can be confirmed when the results of the both analyses are found positive.

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

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

This stack soybean line is given the traits to have the oleic acid content increased in the seeds because of the *gm-fad2-1* gene derived from DP-305423-1, to be tolerant to acetolactate synthase inhibitors due to the *gm-hra* gene, and to be tolerant to herbicide glyphosate due to the modified *cp4 epsps* gene derived from MON-04032-6.

These genes were examined for the possibility of functional interaction with each other.

The *gm-fad2-1* gene suppresses the expression level of soybean endogenous *FAD2-1* gene and thus biosynthesis to linoleic acid, a fatty acid, is inhibited and as a result, the oleic acid content in seeds is increased. The GM-HRA protein encoded by the *gm-hra* gene possesses the acetolactate synthase activity in the biosynthesis pathway of branched-chain amino acids (valine, leucine and isoleucine) and uses the pyruvic acid and α-ketobutyric acid for the substrate. The modified CP4 EPSPS protein encoded by the modified *cp4 epsps* gene possesses the activity of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) in the shikimate pathway, the biosynthesis pathway for aromatic amino

acids (tryptophan, tyrosine and phenylalanine). The substrate for EPSPS includes phosphoenolpyruvate (PEP), shikimate-3-phosphate (S3P) and shikimic acid.

As mentioned above, the individual genes are involved in different metabolic pathways which are independent from each other, and the GM-HRA protein and the modified CP4 EPSPS protein differ from each other in the substrate and the mechanism of action. Thus, it is considered unlikely that any unintended metabolites would be produced.

Actually, in order to confirm whether the genes derived from the parent lines interact with each other in this stack line soybean, analysis of main fatty acid composition and spraying test of herbicides acetolactate synthase inhibitor and glyphosate were carried out using this stack line soybean, the parent line (DP-305423-1 or MON-04032-6) and the non-recombinant control soybean.

As a result of analysis of main fatty acid composition in the seeds, no statistically significant difference ($P<0.05$) was observed between this stack soybean line and its parent line (DP-305423-1) (Table 5, p. 26). In addition, as a result of spraying test of herbicides acetolactate synthase inhibitor and glyphosate, no statistically significant difference ($P<0.05$) was observed between this stack soybean line and the parent line (DP-305423-1 or MON-04032-6) at all dosages tested, normal, 16-time and 32-time concentrations (Table 6, p. 27).

Based on the above results, it was found that the traits given to the parent lines DP-305423-1 and MON-04032-6 remain unchanged in this stack soybean line and thus, it was considered unlikely that any interaction would take place due to the expression of the genes from the parent lines in this stack soybean line.

Consequently, with regard to the differences in physiological or morphological characteristics between this stack soybean line and the soybean, the taxonomic species to which the recipient organism belongs, the evaluation was conducted based on the results of the isolated filed tests of the parent lines DP-305423-1 and MON-04032-6 (http://www.bch.biodic.go.jp/download/lmo/public_comment/DP_305423_1_2009ap.pdf and http://www.bch.biodic.go.jp/download/lmo/public_comment/40-3-2ap.pdf).

Table 5 Comparison of main fatty acid composition in the seeds of this stack line soybean and its parent lines

Soybean line tested	Fatty acid content (% total fatty acid)				
	Palmitic acid (C16:0)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)
This stack line (BC1F5 generation)	6.5 a* ±0.4	4.3 a ± 0.2	76.0 a ± 4.1	3.9 a ± 2.6	5.6 a ± 1.4
Control; Parent line (DP-305423-1)	6.3 a ± 0.5	4.4 a ± 0.3	76.5 a ± 3.7	3.6 a ± 2.6	5.4 a ± 1.1
(Reference) Non-recombinant soybean (BC1F6 null generation)	10.3 b ± 0.2	5.0 b ± 0.4	21.1 b ± 2.4	52.5 b ± 1.6	9.4 b ± 1.4

n=18, mean value ± standard deviation. Individual lines were cultivated in the fields at 6 sites in the U.S. in 2005 and at the time of ripening (R8 stage), the seeds were harvested from 3 plots in each field. Testing was performed based on the linear mixed model and Tukey method.

* Values with different alphabetical characters refer to presence of a statistically significant difference between them ($P<0.05$).

Table 6 Comparison of herbicide injury due to spraying of herbicides acetolactate synthase inhibitor and glyphosate between this stack line soybean and its parent lines

Herbicide sprayed[1]	Soybean line tested	Severity of herbicide injury by concentration of herbicide sprayed (%)			
		Not sprayed with herbicides	Standard concentration [1)]	16-time concentration	32-time concentration
Acetolactate synthase inhibitor	This stack line (BC1F6 generation)	0.0 ± 0.0	0.0 a[2)] ± 0.0	0.0 a ± 0.0	0.0 a ± 0.0
	Control; Parent line (DP-305423-1)	0.0 ± 0.0	3.3 a ± 6.1	0.0 a ± 0.0	0.0 a ± 0.0
	(Reference) Non-recombinant soybean (BC1F6 null generation)	0.0 ± 0.0	3.3 a ± 5.2	10.8 b ± 6.7	13.3 b ± 5.2
Glyphosate	This stack line (BC1F6 generation)	0.0 ± 0.0	0.0 a[2)] ± 0.0	18.3 a ± 6.8	34.2 a ± 10.7
	Control; Parent line (MON-04032-6)	0.0 ± 0.0	4.2 a ± 10.2	30.8 a ± 11.6	38.3 a ± 10.3
	(Reference) Non-recombinant soybean (BC1F6 null generation)	0.0 ± 0.0	60.0 b ± 12.2		

n=3, mean value ± standard deviation. Ten (10) plant individuals for each line were cultivated in 3 repeats in the greenhouse and sprayed with herbicide at the 3-leaf stage (V3 stage). Fourteen (14) and 21 days after spraying of herbicide, the severity of herbicide injury in individuals was evaluated by each repeat based on the scale from 0% (no damage) to 100% (complete death). Multiple comparisons based on variance analysis and Sidak method (Westfall *et al.,* 2006) by the concentration of herbicide sprayed (standard, 16-time, 32-time) were conducted.

1) Acetolactate synthase inhibitor: Chlorimuron 11.2 g active ingredient (a.i.)/ha + Thifensulfuron 3.6 g a.i./ha.
   Glyphosate: Glyphosate 1.7 kg acid equivalent (a.e.)/ha.
2) Values with different alphabetical characters refer to presence of a statistically significant difference between them ($P<0.05$).

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

(a) Morphological and growth characteristics

For the morphological and growth characteristics, examination was conducted for the items listed in Table 7 (p. 29), using the non-recombinant

soybean BC1F6 null, which was produced by segregation and dropout of transferred genes in the process of rearing of the parent lines DP-305423-1 and DP-305423-1, and using the recipient organism cultivar A5403 of the parent lines MON-04032-6 and MON-04032-6. As a result, it was confirmed that there was no difference observed between the lines tested in all the items examined.

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

DP-305423-1 and MON-04032-6 withered or died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control soybean.

(c) Wintering ability and summer survival of the mature plant

The plant bodies of DP-305423-1 were left in the field for cultivation even after the harvesting time and as a result, they were all found dead similarly as the non-recombinant control soybean. In addition, in either MON-04032-6 or the non-recombinant control soybean, the formation of new buds from the aerial parts after harvesting was not observed.

(d) Fertility and size of the pollen

Pollens were sampled from DP-305423-1 and MON-04032-6, and the samples were stained to observe their fertility and size under a microscope for investigation. As a result, it was confirmed that there was no difference from the non-recombinant control soybean observed regarding both fertility and size of the pollen.

Table 7 Investigational results of morphological and growth characteristics

	DP-305423-1	MON-04032-6
Uniformity of germination	○	○
Germination rate	○	○
Germination time	—	○
Time of flower initiation	—	○
Flowering time	○	○
Full flowering time	—	○
Time of flower completion	—	○
Leaf yellowing time	—	○
Leaf falling time	—	○
Pod yellowing time	—	○
Maturation time	○	○
Shape of leaflet	○	—
Leaf color	—	○
Leaf size	—	○
Trichome color	—	○
Trichome quantity	○	○
Trichome length	—	○
Shape of trichome	—	○
Stiffness of trichome	—	○
Number of flowers	—	○
Color of flowers	—	○
Plant shape	○	○
Elongation type	—	○
Growth habit	—	○
Main stem length	○	○
Thickness of stem	—	○
The lowest main stem node height of podding	○	○
The lowest main stem node position of podding	—	○
Number of main stem nodes	○	○
Number of branches	○	○
Total weight of subterranean part	—	○
Total weight of plant	—	○
Weight of pod seeds per plant	—	○
Number of ripe pods per plant	—	○
Weight of ripe pods per plant	—	○
Total number of pods per plant	○	—
Total weight of seeds per plant	○	○
Number of ripe (perfect) seeds per plant	○	○
Weight of ripe (perfect) seeds per plant	○	○
Color of pods	—	○
Number of seeds per pod	—	○
Difficulty in pod bursting	○	○
100-kernel weight	○	○
Shape of seed	○	—
Seed hull color	—	○
Uniformity of seeds	—	○
Hilum color	—	○

○: Examined -: Not examined

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

Seed production: In the examination described in 2). (a) (p. 27), examination was conducted for 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 regarding DP-305423-1, and for the number of ripe pods per plant, weight of ripe pods per plant, weight of seeds per plant, the number of perfect seeds per plant, weight of perfect seeds per plant, the number of seeds per pod and weight of 100 seeds regarding MON-04032-6. As a result, it was confirmed that there was no difference from the non-recombinant control soybean observed in all the items examined.

Shedding habit: In the examination described in 2). (a) (p. 27), shedding habit of the seed was examined in terms of difficulty in pod bursting and as a result, DP-305423-1 and the non-recombinant control soybean both exhibited difficult pod bursting, showing no difference between the both plants regarding shedding habit of the seed. In addition, it has been confirmed regarding the both plants of MON-04032-6 and non-recombinant control soybean that there was no dropped pod or seed observed one month after the maturing time.

Dormancy and germination rate: Soybean is reported to possess little dormancy of the seeds (OECD, 2000). The seeds harvested from DP-305423-1 and MON-04032-6 were examined for the germination rate and as a result, the germination rate was found 98% or more and 95% or more, respectively, showing there was no statistically significant difference from the non-recombinant control soybean observed for the both plants.

(f) Crossability

DP-305423-1 or the non-recombinant control soybean and the non-recombinant soybean variety were cultivated adjacently to each other in the fields in the U.S. to evaluate the crossability and as a result, the crossing rate between DP-305423-1 or the non-recombinant control soybean and the non-recombinant soybean variety was found 0.3% and 0.7% respectively. In addition, in the isolated field test in Japan, crossing between DP-305423-1 and the non-recombinant control soybean grown adjacent to DP-305423-1 was not observed.

In the investigation for the crossability between the *Glycine soja* regulated in flowering time and MON-04032-6 in isolated fields and non-containment greenhouses, crossing between MON-04032-6 and *Glycine soja* was not

observed similarly as with the non-recombinant soybean.

(g) Production of harmful substances

As a result of plow-in tests, succeeding crop tests and soil microflora tests for DP-305423-1 and MON-04032-6, it has been confirmed that there was no statistically significant difference from the non-recombinant control soybean observed in all the tests conducted.

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

This stack soybean line was developed by crossing between the soybean with high oleic acid content and herbicide acetolactate synthase inhibitor tolerance (DP-305423-1) and the soybean tolerant to herbicide glyphosate (MON-04032-6). These parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when used in line with Type 1 Use described in the application for this stack soybean line.

The metabolic pathways in which the transferred genes in this stack soybean line (*gm-fad2-1* gene, *gm-hra* gene, and modified *cp4 epsps* gene) are involved are independent from each other, and the GM-HRA protein and the modified CP4 EPSPS protein differ from each other in the substrate and mechanism of action; therefore, it is considered unlikely that any unexpected metabolites would be produced.

In addition, the main fatty acid composition and the tolerances to acetolactate synthase inhibitors and glyphosate in this stack soybean line are found at similar levels as exhibited by the parent lines. Thus, it is considered low that the above-described proteins derived from the individual parent lines would interact with each other in the plant body of this stack soybean line, and it is considered unlikely that notable changes in traits have occurred in this stack soybean line except for the traits it received from the parent lines.

(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.

In the isolated field tests, DP-305423-1 and MON-04032-6, the parent lines of this stack soybean line, were examined for various characteristics relating to competitiveness. As a result, in all the items examined, no significant difference was

observed between the parent lines and the control cultivar.

In this stack soybean line, the oleic acid content in the seeds is found increased to around 75% due to the transferred *gm-fad2-1* gene, though there is no report that the oleic acid especially affects the energy supply at the time of germination.

In addition, this stack soybean line is given the traits to be tolerant to herbicides acetolactate synthase inhibitor and glyphosate due to the transferred *gm-hra* gene and the modified *cp4 epsps* gene. However, it is considered unlikely that these traits cause this stack soybean line to enhance the competitiveness under the natural environment usually expected not to suffer spraying of the herbicides.

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 stack soybean line 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 (*G. max*) to which the recipient organism belongs, there is no report that it produces a harmful substance to wild animals and wild plants.

In this stack soybean line, the GM-HRA protein and the modified CP4 EPSPS protein are produced, though there is no report that these proteins are harmful substances, and homology of amino acid sequences with any known allergens has not been observed. In DP-305423-1, the parent line of this stack soybean line, in addition to the intended increase in the oleic acid content, a statistically significant increase was observed in the heptadecanoic acid and heptadecenoic acid in the seeds and in the leucine in the leaves compared to the non-recombinant control soybean. However, these fatty acids and leucine are also contained in many specifies of animals and plants, and there is no report that they are harmful substances.

In addition, in the isolated field in Japan, to examine the ability of DP-305423-1 and MON-04032-6, the parent lines of this stack soybean line, 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 the parent lines and their 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 cannot be specified and that

the use of this stack soybean line poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

It is known that *Glycine soja* is closely related to soybean and the both plants have the same chromosome number 2n=40 and thus they can cross with each other. Then, *Glycine soja* was specified as a wild plant likely to be affected, and the following examination was performed.

Since there is no specific obstacle identified to the growth of any hybrid produced by artificial crossing between soybean and *Glycine soja*, there is a possibility that the hybrid, if produced by crossing between this recombinant soybean and *Glycine soja* in the natural environment in Japan, would grow and that the gene transferred in this recombinant soybean through backcross of the hybrid with *Glycine soja* would spread without remaining in a low content in the population of *Glycine soja*.

In addition, since *Glycine soja* ranges widely throughout the country and grows voluntarily in river beaches, banks, in the vicinity of farmlands, orchards and other places, it can cross with this recombinant soybean when it is raised adjacent to the recombinant soybean.

However,

1) It is generally known that the flowering time of soybean 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 artificially matching the flowering time, the rate of crossability is reportedly 0.73%.

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

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

4) As a result of examination for crossability between DP-305423-1, the parent line of this stack soybean line, and the non-recombinant control soybean, which were cultivated adjacent to each other, there was no crossing with the non-recombinant control soybean observed in the isolated field test in Japan. In addition, as a result of examination in the U.S., the rate of crossing did not exceed the generally known outcrossing rate between soybean varieties (3% or less).

Furthermore, as a result of examination for the traits relating to reproduction (pollen fertility and size, and seed production) for the parent lines DP-305423-1 and MON-04032-6, no difference was observed between the parent lines and the non-recombinant control soybean.

Based on the above results, it was estimated that the rate of crossing between the parent lines DP-305423-1 and MON-04032-6 and *Glycine soja* is as low as the crossability between conventional soybean varieties and *Glycine soja*, and it was considered that the possibility that this stack soybean line and *Glycine soja* would cross with each other to produce the hybrid is similarly low.

If this stack soybean line crosses with *Glycine soja*, the obtained hybrid is considered to possess the traits to have high oleic acid content and to be tolerant to herbicides acetolactate synthase inhibitor and glyphosate due to the *gm-fad2-1* gene, the *gm-hra* gene and the modified *cp4 epsps* gene. However, as mentioned in 1-(1) above, these traits are considered unlikely to increase the competitiveness; therefore, it is considered unlikely that the hybrid could dominate the population of *Glycine soja*.

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 stack soybean line poses no significant 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 stack 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.