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

Name: Du Pont Kabushiki Kaisha
Akio Kobayashi, President
Address: Sanno Park Tower
2-11-1, Nagata-chou, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	High oleic acid soybean (*GmFad2-1, Glycine max* (L.) Merr.) (260-05, OECD UI：DD-Ø26ØØ5-3)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	－

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

High oleic acid soybean line 260-05 is genetically modified soybean produced by Optimum Quality Grains, L.L.C. in the US, a joint venture set up by DuPont in the US and Pioneer Hi-Bred International in the US. In the high oleic acid soybean line 260-05, the *GmFad2-1* gene is transferred which encodes the δ-12 desaturase that catalyzes the reaction for biosynthesis of linoleic acid derived from the soybean (*G. max*). By gene silencing[1], the expression of both the soybean intrinsic *Fad2-1* gene and the transferred gene is suppressed and consequently, the contents of the polyunsaturated fatty acids including linoleic acid and linolenic acid are decreased, and the oleic acid content is increased to account for 80% or more among the entire fatty acid.

The oil derived from the high oleic acid soybean line 260-05 virtually eliminates the need for hydrogenation typical for normal soybean oil even in the applications where high thermal stability is required, and they are also expected to delay deterioration of the prepared foods. The high oleic acid soybean oil will be predominantly used in the applications such as spraying for crackers, rice cookies and cornflakes to meet the requirements for oil of higher oxidization stability and frying for tempura (deep-fried fish and vegetables), deep-fried pork cutlets, potato chips and French fries. Besides, blended use with other vegetable oil is also anticipated for improvement of health, tastes and stability. Especially for the health reason, it is important to suppress excessive intake of saturated fatty acid or polyunsaturated fatty acid and thus, the oil derived from the high oleic acid soybean line 260-05 will be ideal to answer the application needs due to the low contents of the fatty acids.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

At early stages of production of the high oleic acid soybean line 260-05, production of such soybean was intended that features high contents of both oleic acid and lysine and then, both the plasmid pBS43, which contains the *GmFad2-1* gene expression cassette to confer high productivity of oleic acid, and the plasmid pML102, which contains the *dapA* gene expression cassette to confer high productivity of lysine, were used for transferring. As mentioned in 2-(4) in this section, it has been confirmed that the *dapA* gene transferred to this high oleic acid soybean line 260-05 is not expressed and thus, only the high oleic acid trait is conferred. Composition of the nucleic acid of plasmid pBS43 and plasmid pML102 that were used for the production of the high oleic acid soybean line 260-05 and the origins of component elements are shown in Table 1 and Table 2 respectively. In addition, the nucleotide sequences of plasmid pBS43 and plasmid pML102 are shown in Annex 2.

[1] Gene silencing: A phenomenon to suppress the expression of transferred genes or endogenous genes in the transformants which have exogenous genes transferred to the nuclear genome of plant

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, including target gene, expression regulatory region, localization signal, and selective marker, that was used for the production of the high oleic acid soybean line 260-05 are shown in Table 1 and Table 2 respectively.

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

As mentioned in 2-(4) in this section, it has been confirmed that the target genes, *GmFad2-1* gene and *dapA* gene, and the selective markers, *GUS* gene and amp^r gene, are not expressed (Annex 3). This means that in the high oleic acid soybean line 260-05, no protein is produced by the expression of the target genes and selective markers.

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

In the oilseed crops, the biosynthesis of polyunsaturated fatty acid during the formation of seeds is catalyzed by two membrane-associated desaturases (Kinny, 1994). At first, due to the δ-12 desaturase which is encoded by the *Fad2* gene, a second double bond is transferred at the δ-12 (n-6) position of the oleic acid (C18:1), the mono-unsaturated fatty acid to form the linoleic acid (C18:2) (Okuley *et al*., 1994; Heppard *et al*., 1996). Then, due to the δ-15 desaturase which is encoded by the *Fad3* gene, a third double bond is transferred at the δ-15 (n-3) position of linoleic acid to form the linoleic acid (C18:3) (Yadav *et al*., 1993) (Figure 1). This means that the suppression of the δ-12 desaturase encoded by the *Fad2* gene inhibits the reaction for biosynthesis from oleic acid to linoleic acid, thereby leading to the increased oleic acid content.

In soybean, there are two *Fad2* genes, but only the *GmFad2-1* gene is expressed specifically in the producing seed (Hepperd *et al*., 1996). The expression of this gene increases during the period of oil storage, starting around 19 days after flowering, and its gene product is responsible for the synthesis and storage of the polyunsaturated fatty acids in soybean seeds. The second *GmFad2-2* gene is expressed in the entire plant body and it is responsible for the synthesis of the polyunsaturated fatty acids required for the formation of cell membranes in plants. As a result of the Northern blotting analysis using the soybeans actually, the GmFad2-1 mRNA was detected only in the seeds, while the GmFad2-2 mRNA was detected in the leaves, roots and stems of soybean (Hepperd *et al*., 1996) (Figure 3). For this reason, for the production of the high oleic acid soybean line 260-05, among the two *Fad2* genes, the *GmFad2-1* gene was used which is only responsible for the synthesis and storage of polyunsaturated fatty acids in the producing soybean

seed.

In the production of the high oleic acid soybean line 260-05, an attempt was made to increase the oleic acid content by suppressing the production of δ-12 desaturase, which catalyzes the reaction for biosynthesis of linoleic acid through addition of double bond to the oleic acid. Then, the method was used that suppresses the expression of *GmFad2-1* gene in seeds by inducing the gene silencing through transferring of the *GmFad2-1* gene into the soybean genome (Morino *et al*., 1996; US Patent No. 5034323 by DNAP). As a result of the Northern blotting analysis, it was confirmed that in this line, the δ-12 desaturase, the protein expressed by the gene, is not produced (see Annex 3), and it was considered that as intended, the expression of both extraneous and endogenous *GmFad2-1* genes was suppressed by the gene silencing. As a result, the oleic acid content in the seed is increased to 80% or more compared to 17 to 30% in the conventional soybeans.

On the other hand, as a result of analysis on the soybean seed storage proteins, it was observed that in the high oleic acid soybean line 260-05, α and α' subunits of β-conglycinin are decreased while the glycinin acid subunits and A2, B1A subunits precursors are increased compared to the parental soybean line A2396. For the profile of other storage proteins, no difference was observed (Annex 4). The reasons for the decreased α and α' subunits of β-conglycinin and the increased glycinin are estimated as that, by the transferring of β-conglycinin promoter connected to the *GmFad2-1* gene, the expression of soybean endogenous β-conglycinin gene is suppressed by the gene silencing. Also in the soybean variety improved by the crossbreeding method (Nordlee, 1995) and the mutation induced soybean (Takahashi *et al*., 1994；Kitamura, 1995), the decrease in the α and α' subunits of β-conglycinin and the increase in the glycinin acid subunits or glycinin A2, B1A subunits precursors have been observed in some cases; therefore, the decrease in the α and α' subunits of β-conglycinin and the increase in the glycinin content in this high oleic acid soybean line 260-05 was considered to fall within the range for conventional soybeans.

For the high oleic acid soybean line 260-05, field tests were carried out by DuPont in the US from 1995 to 1996 at 24 sites in the US and one site in Puerto Rico to analyze the components of fatty acid, amino acid, isoflavone, raffinose, stachyose, phytic acid and trypsin inhibitor. As a result, except the findings that the oleic acid content was increased to 80% or more, all the components analyzed were observed to be well within the range for conventional soybeans.

Table 1 Composition of the nucleic acid of plasmid pBS43 and the origins and functions of the component elements

Gene	Size (kbp)	Origin	Function
GmFad2-1 gene expression cassette			
β-conglycinin promoter	0.630	A promoter derived from α'-subunit of β-conglycinin seed storage protein of *Glycine max*.	It is a seed specific promoter that allows high level gene expression during seed production.
GmFad 2-1	1.490	A cDNA encoding sequence of the δ-12 fatty acid desaturase derived from *Glycine max*.	It comprises a partial 5' and 3' untranslated regions and the protein coding region. The enzyme produced adds a second double bond at the δ-12 (n-6) position of oleic acid, thus converting it to linoleic acid.
Phaseolin 3' terminator	1.186	The 3' terminator region derived from the phseolin seed storage protein of *Phaseolus vulgaris.*	It contains signals for termination of transcription and directs polyadenylation.
GUS gene expression cassette			
35S promoter	1.380	A promoter derived from cauliflower mosaic virus (CaMV) (Harpster *et al*., 1988).	It is a promoter of high level constitutive gene expression in plant tissues.
Cab 22L untranslated leader	0.059	The 5' untranslated leader from the photosynthetic *22L* chlorophyll a/b binding protein (*Cab 22L*) promoter of *Petunia hybrida* var. *Mitchell*.	The untranslated leader sequence helps to stabilize mRNA and improve translation.
GUS	1.856	The β-glucronidase enzyme (*uidA* gene) sequence encoding gene derived from the *uidA* gene of *E. coli*.	It comprises a partial 3' untranslated region and the protein coding region. An enzyme to act on the β-glycoside of *D*-glucuronic acid to hydrolyze the glucuronide bonding. Used for selection of transgenic plants based on the colorimetric analysis.
NOS 3'	0.796	The 3' terminator region of the nopaline synthase gene derived from *Agrobacterium tumefaciens*.	It contains signals for termination of transcription and directs polyadenylation.
Others			
lac	0.231	A partial Lac l coding sequence, the promoter Plac and a partial β-D-galactosidase (*lacZa*').	The gene is not intact and no longer function in *E. coli*.
ori	0.599	The replication origin of plasmid pUC19 derived from *E. coli.*	It initiates replication of plasmid in *E. coli*.
amp^r	1.008	A gene encoding for the enzyme β-lactamase derived from *E. coli.*	It confers ampicillin resistance to *E. coli*.
f1 ori	0.461	The replication origin derived from bacteriophage f1.	The origin of replication recognized by bacteriophage f1 to produce single stranded DNA. The replication origin is not recognized unless a phage f1 is present.
lacZa'3'	0.166	The 3' terminal of *lacZa'* gene.	The gene is not intact and no longer function.

Table 2 Composition of the nucleic acid of plasmid pML102 and the origins and functions of the component elements

Gene	Size (kbp)	Origin	Function
dapA gene expression cassette			
pTZ18R	0.035	A plasmid derived from pBR322 of *Escherichia coli* (*E. coli*).	It is used for cloning and proliferation in *E. coli*.
Kti3 promoter	2.091	A promoter from Kunitz trypsin inhibitor of *Glycine max.*	It is a seed specific promoter that allows high level gene expression during seed production.
ssu CTC	0.167	The N-terminal chloroplast transit peptide sequence derived from the small subunit of Rubisco of *Glycine max.*	It is a sequence to direct the protein into the chloroplast.
dapA	0.923	A gene encoding the lysine insensitive version of the enzyme dihyropicolinic acid synthase derived from *Corynebacterium glutamicum.*	It catalyzes the reaction for synthesis of 2,3-dihydro-dipicolinic acid from pyruvic acid and aspartic acid β-semialdehyde in the lysine biosynthesis pathway. (See Figure 2)
Kti3 3' terminator	0.195	The 3' terminator region derived from Kunitz trypsin inhibitor gene 3 from *Glycine max.*	It contains signals for termination of transcription.
Others			
lac	0.231	A partial *Lac* l coding sequence, the promoter *Plac* and a partial β -D-galactosidase (*lacZa'*).	The gene is not intact and no longer function in *E. coli*.
ori	0.599	The replication origin of plasmid pUC19 derived from *E. coli.*	It initiates replication of plasmid in *E. coli*.
amp^r	1.008	A gene encoding for the enzyme β-lactamase derived from *E. coli.*	It confers ampicillin resistance to *E. coli*.
f1 ori	0.461	The replication origin derived from Bacteriophage f1.	The origin of replication recognized by bacteriophage f1 to produce single stranded DNA. The replication origin is not recognized unless a phage f1 is present.
lacZa'3'	0.166	The 3' terminal of *lacZa'* gene.	The gene is not intact and no longer functions.

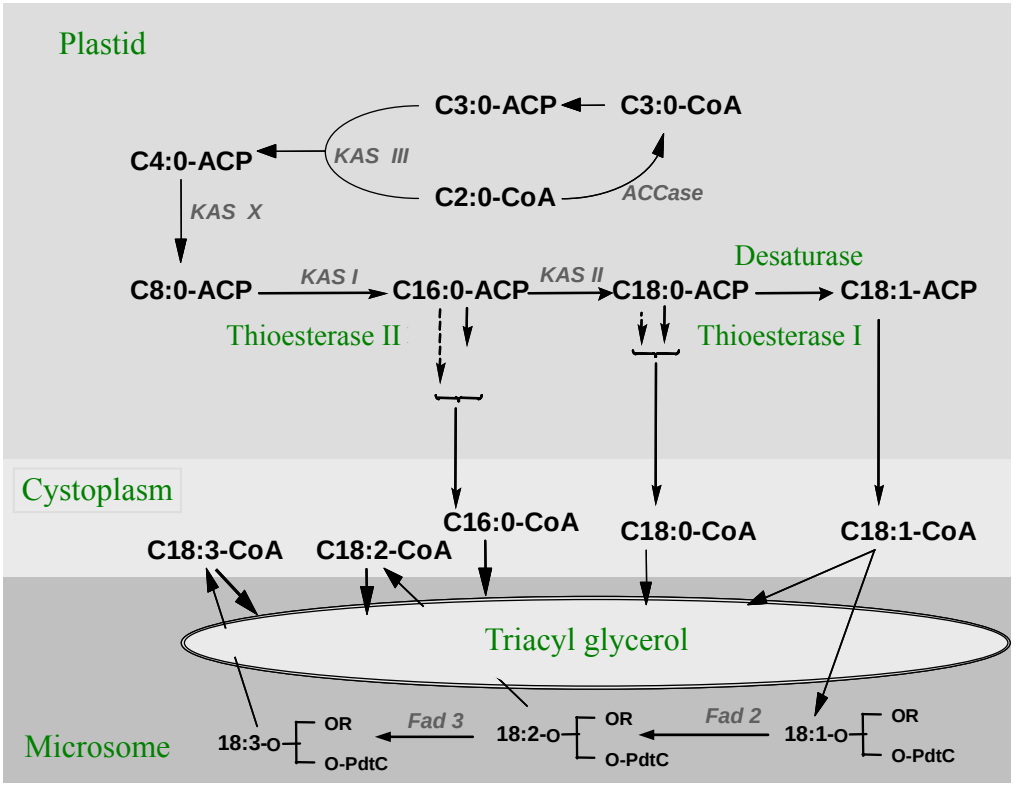

Figure 1　Pathway for the biosynthesis of fatty acids in the soybean seed

C18:1・・・Oleic acid, C18:2・・・Linoleic acid, C18:3・・・Linolenic acid
Fad2・・・Gene encoding the δ-12 desaturase, *Fad3*・・・Gene encoding the δ-15 desaturase

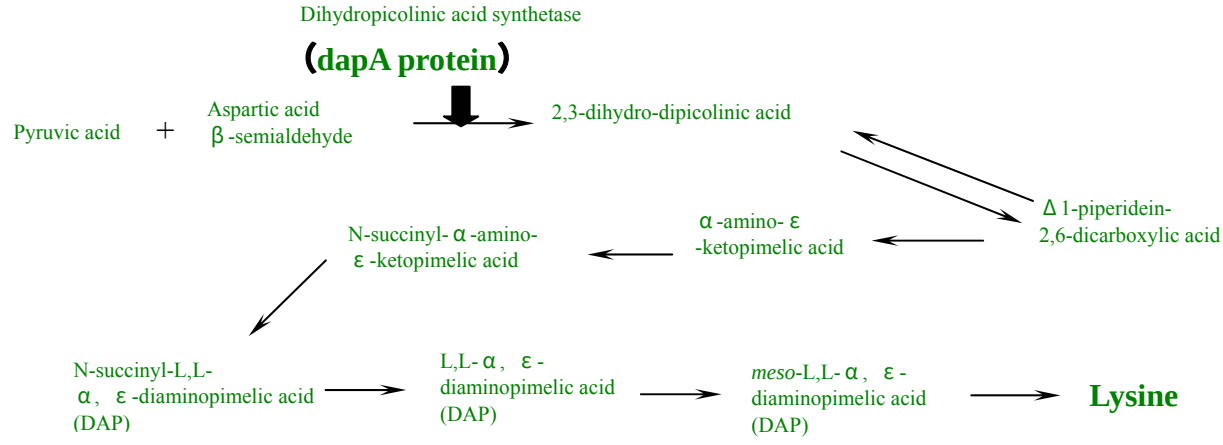

Figure 2　Lysine biosynthesis pathway

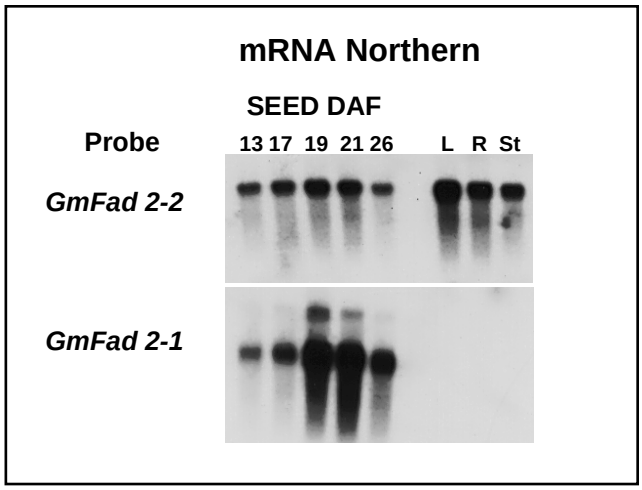

Figure 3 The Northern blotting analysis to identify the expression of *GmFad2-1* mRNA and *GmFad2-2* mRNA in the soybean tissues

In the above diagram, L refers to leaf, R refers to root, and St refers to stem, and DAF stands for Days after flowering (the number of days after flowering) (Hepperd *et al*., 1996).

(2) Information concerning vectors

1) Name and origin

The plasmid pBS43（Figure 4）and the plasmid pML102（Figure 5）used to produce this high oleic acid soybean line 260-05 are both derived from pBR322, which is a synthetic plasmid from *Escherichia coli* (*E. coli*).

2) Properties

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

The total number of base pairs of plasmid pBS43 and plasmid pML102 used to produce the high oleic acid soybean line 260-05 are 10,303 bp and 6,247 bp respectively. The entire nucleotide sequences of the both plasmids are shown in Annex 2.

(b) Types of any nucleotide sequence having specific functions

For the both plasmids, in the region outside of nucleic acid, the antibiotic (ampicillin) resistant marker gene (*amp*^{r} gene) derived from pBR322 is present to select the microorganisms which contain the transformed plasmids during the proliferation of plasmids in the microorganisms. The *amp*^{r} gene is under the control of promoter derived from *E. coli* and it is not expressed in any plants. Besides, it was actually confirmed based on the Northern blotting analysis that the *amp*^{r} gene is not expressed in the high oleic acid soybean line 260-05 (Annex 3).

(c) Presence or absence of infectivity of vector

For the both plasmids, the entire nucleotide sequences have not been clarified, and no sequence is contained that allows transfer to other microorganisms; therefore, there is no infectivity of vector.

(3) Method of preparing living modified organisms

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

The structure of the entire nucleic acid transferred in the recipient organism is shown in Figures 9 and 10 in Annex 5.

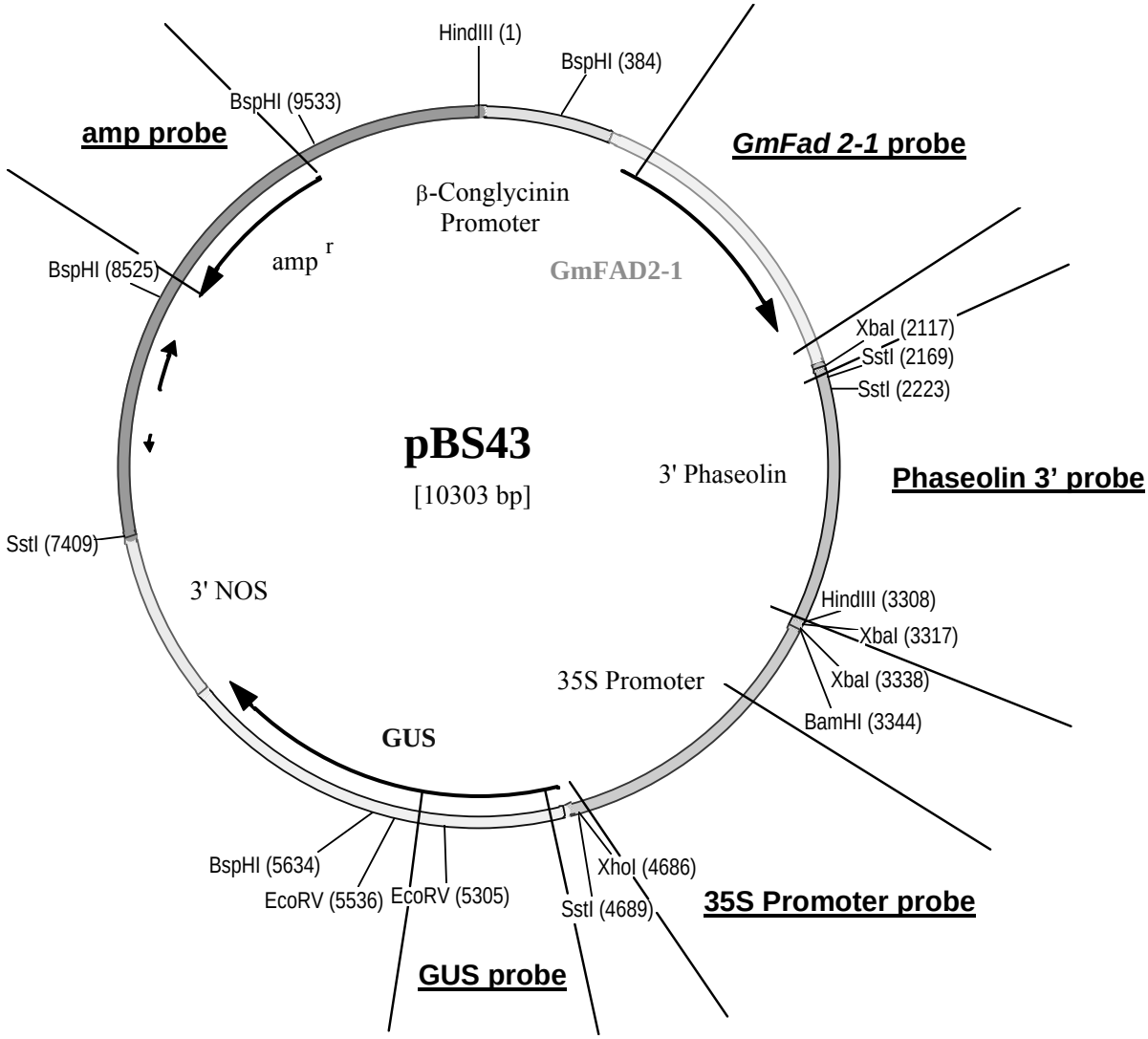

Figure 4 Physical Map of plasmid pBS43

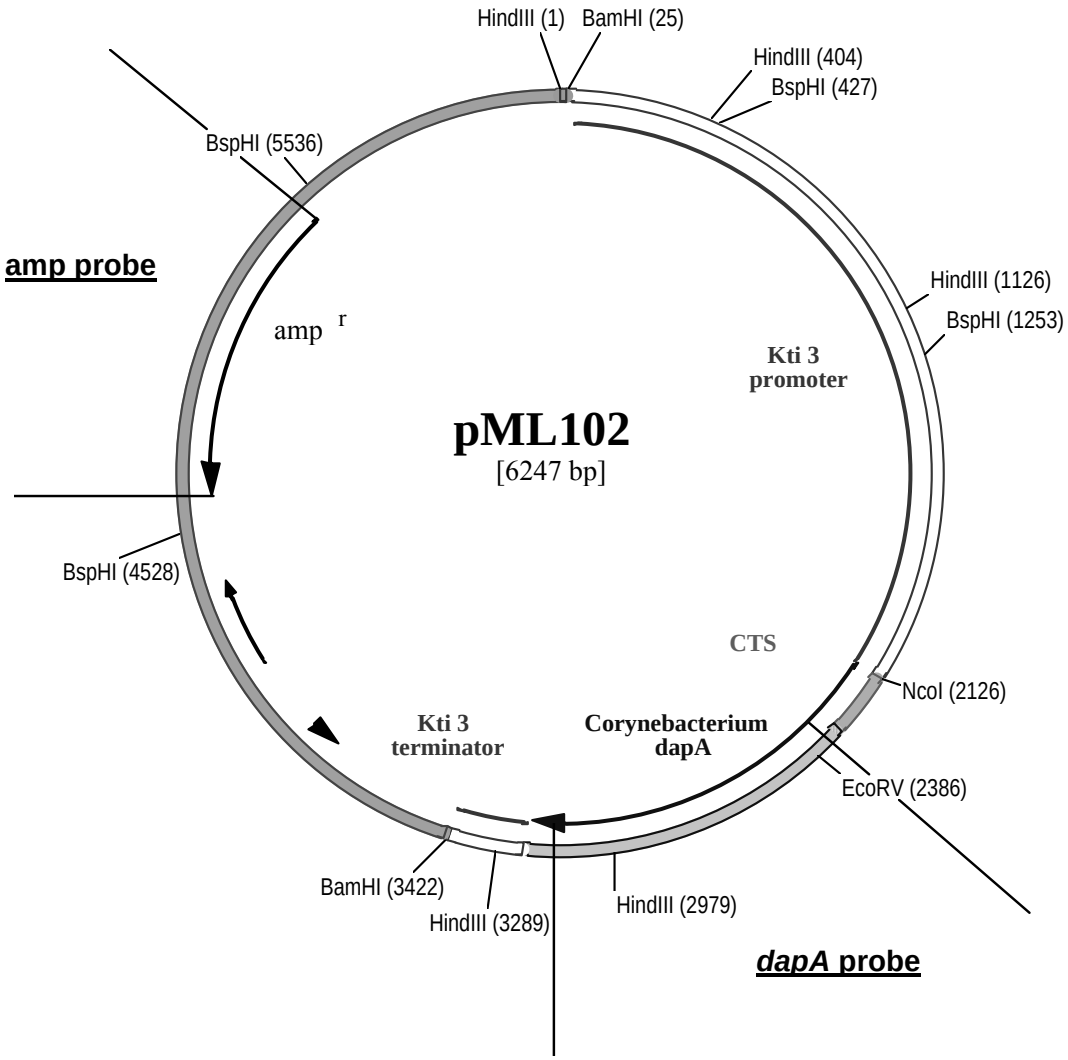

Figure 5 Physical Map of plasmid pML102

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

For transferring the nucleic acid to the recipient organism, particle gun bombardment was used to transfer the plasmid pBS43 and the mixture with the plasmid pML102 to the variety A2396, which belongs to the soybean mature group II.

3) Processes of rearing of living modified organisms

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

The transformed cells containing the transferred nucleic acid were selected by the GUS activity assay.

(b) Presence of any residual body cell of *Agrobacterium*

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(c) Process of rearing and genealogical tree

Among the plant bodies regenerated from a selected cell (R0 generation), a recombinant plant was selected which exhibited GUS activity and which was also shown, using the PCR method, to contain the *GmFad2-1* gene. Then the R1 generation was selected for fatty acid composition and lysine content, but no recombinant plant was obtained which possessed both traits, high oleic acid content and high lysine level, so R1 and later generations were selected for high oleic acid trait. The process of rearing the R0 and later generations of line 260-05 selected as elite soybean lines is shown in Figure 6.

In the generations R0 and R1, the *GmFad2-1* gene expression cassette transferred gene locus, the *dapA* gene expression cassette transferred gene locus, and the *GUS* gene expression cassette transferred gene locus were present in the heterozygote form. In the process of inbreeding these generations, the *GUS* gene expression cassette transferred gene locus, which expressed the GUS protein, was separated and dropped out. Then the individuals G168, G94-1 and G94-19 were selected as sub lines, which contained only the *GmFad2-1* gene expression cassette transferred gene locus and the *dapA* gene expression cassette transferred gene locus in the homozygote form. The *GUS* gene was present in the *GUS* gene expression cassette transferred gene locus, while as mentioned in 1-(4)-(b) , it was present also in the *GmFad2-1* gene expression cassette transferred gene locus as an intact copy of *GUS* gene expression cassette which is not expressed and a truncated copy of *GUS* gene expression cassette which is not expressed. In addition, the genes in the *GmFad2-1* gene expression cassette transferred gene locus and the *dapA* gene expression cassette transferred gene locus were found not expressed and thus, these gene loci are to be hereinafter referred to as the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus and the *dapA* gene expression cassette (not expressed) transferred gene locus.

The sub lines are derived from one individual in the R0 generation of line

260-05 and have been raised in the similar process of rearing, so the transferred genes are precisely identical. The three selected sub lines are referred to as the generic name of high oleic acid soybean line 260-05, and the inbred posterities of the individual sub lines are commercialized. The generations of the high oleic acid soybean line 260-05 subjected to various evaluation tests are summarized in Table 3.

For the high oleic acid soybean line 260-05, the intended use in Japan in any open system was approved in May 1999 as conforming to the "Guideline for the use of recombinant in agriculture, forestry and fisheries" (hereinafter referred to as "Guideline"). In addition, in May 2000, the safety for use as food and feed was approved (the safety as food and feed was re-approved in March 2001 and March 2003 respectively when the review was designated as legal obligation).

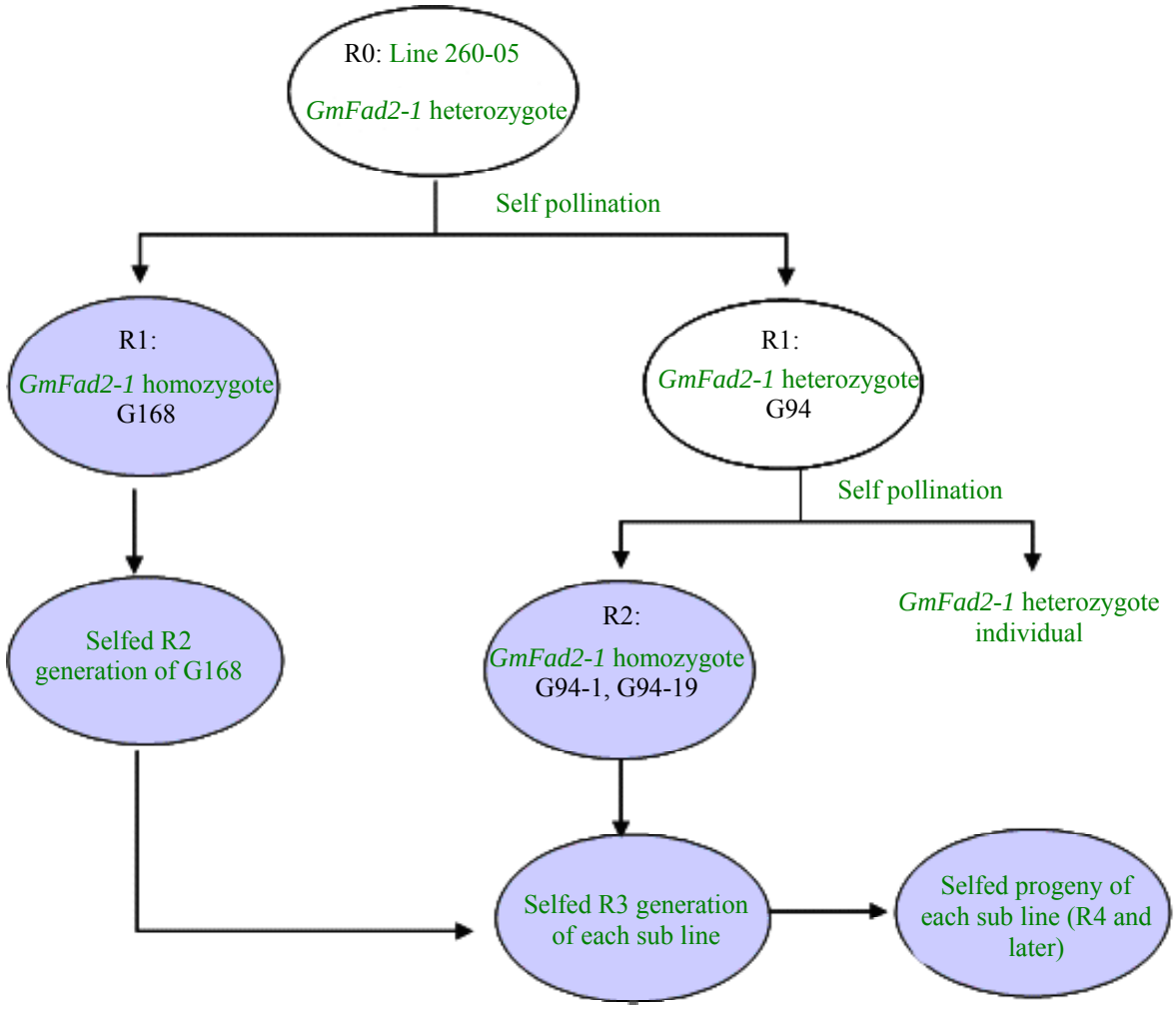

Figure 6 Breeding diagram of the high oleic acid soybean line 260-05

The R0 generation of line 260-05 contains the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus, the *dapA* gene expression cassette (not expressed) transferred gene locus, and the *GUS* gene expression cassette transferred gene locus in the heterozygote form.

In the R1 generation obtained from the R0 generation, the individual G168 was selected which contained the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus and the *dapA* gene expression cassette (not expressed) transferred gene locus in the homozygote form without the *GUS* gene expression cassette transferred gene locus that had been separated and dropped out. On the other hand, in the R2 generation obtained from the heterozygote individual in the R1 generation, the individuals G94-1 and G94-19 were selected which contained the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus and the *dapA* gene expression cassette (not expressed) transferred gene locus in the homozygote form without the *GUS* gene expression cassette transferred gene locus that had been separated and dropped out. In the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus, one intact copy of *GUS* gene expression cassette (not expressed) and one truncated copy of *GUS* gene expression cassette (not expressed) are present.

The selected G168, G94-1 and G94-19 all contain the *GmFad2-1* gene expression cassette (not expressed) transferred gene and the *dapA* gene expression cassette (not expressed) transferred gene locus in the homozygote form, and the three selected sub lines are referred to as “high oleic acid soybean line 260-05”.

Table 3 Generations of high oleic acid soybean line 260-05 used for different evaluation tests

Generations tested	Description of evaluation	Reference
R2	To confirm based on the Northern blotting analysis that the *GmFad2-1*, *GUS*, *dapA* and *amp*^{r} genes are not expressed (for GUS, additionally confirm using the R6 generation that the genes are not expressed in the leaves).	Annex 3
R6	To analyze the seed proteins in the high oleic acid soybean line 260-05.	Annex 4
R6	To evaluate the transferred genes based on the Southern blotting analysis.	Figs. 1 to 8 of Annex 5
R1, R2 and R6	To confirm the stability of the transferred genes based on the Southern blotting analysis.	Figs. 11 & 12 of Annex 5
R4, R5, R6 and R7	To confirm the stability of high-oleic acid trait based on the fatty acid composition analysis.	Annex 6
R4 and R5	Field tests in the US and Puerto Rico	Annex 8
R8	Isolated field tests at the National Agricultural Research Center for Hokkaido Region	Annex 9

(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

Based on the Southern blotting analysis, it was confirmed that the transferred nucleic acid was transferred in the genome of soybean.

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

In order to identify the number of copies of the transferred genes to the high oleic acid soybean line 260-05 and whether individual expression cassettes are entirely transferred, the Southern blotting analysis was conducted. In the analysis, the R6 generation of high oleic acid soybean line 260-05 raised in 1996 by Pioneer Hi-Bred International in the US and the parental soybean line A2396 were used. As a result, the high oleic acid soybean line 260-05 was shown to contain the *GmFad2-1* gene expression cassette (not expressed) transferrer gene locus and the *dapA* gene expression cassette (not expressed) transferred gene locus (Figures 1 to 8 of Annex 5).

In the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus, two intact copies of *GmFad2-1* gene expression cassette, one intact copy of *GUS* gene expression cassette, one truncated copy of *GUS* gene expression cassette, two intact copies of amp^r gene expression cassette, and one truncated copy of amp^r gene expression cassette were transferred.

On the other hand, in the *dapA* gene expression cassette (not expressed) transferred gene locus, one truncated copy of *dapA* gene expression cassette was transferred. Mapping of the transferred genes to the both gene loci is presented in Figures 9 and 10 of Annex 5 respectively.

In order to confirm that the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus required to confer the high oleic acid trait is stably inherited in offspring, the Southern blotting analysis was conducted (restriction enzyme: *Bam*HI, probe: *Phaseolin* 3', Figures 11 and 12 of Annex 5). The samples subjected to the analysis were from the R1 and R2 generations cultivated in the US from 1993 to 1995 by Pioneer Hi-Bred International in the US, R6 generation of high oleic acid soybean line 260-05 cultivated in the US in 1996, and the parental soybean line A2369. As a result, it was confirmed that the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus required to confer the high oleic acid trait is stably inherited in offspring.

The Northern blotting analysis was conducted to confirm the expression of the transferred genes. The samples subjected to the analysis were the seeds from the R2 generation of high oleic acid soybean line 260-05 cultivated in the US from 1993 to 1995 by Pioneer Hi-Bred International in the US and the parental soybean line A2396, and the leaf tissues from the R6 generation of high oleic acid soybean line 260-05 cultivated in the US in 1996 and the parental soybean line A2396. As a result, it was confirmed that the *GmFad2-1* gene, the *dapA* gene, and the selective

markers *GUS* gene and *amp*^{r} gene were not expressed (Annex 3).

In addition, in 2005, the high oleic acid soybean line 260-05 was cultivated in the US field by the contract farmer using the R10 seeds to produce the seeds of the high oleic acid soybean cultivar. Then based on the result that the high content of oleic acid remained unchanged, it was evidenced that the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus is stably inherited up to the R10 generation.

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

As mentioned above, as a result of the Southern blotting analysis, it was demonstrated that two copies of the *GmFad2-1* gene expression cassette (not expressed) are transferred in tandem with each other to the different gene locus from the *dapA* gene expression cassette. Besides, as a result of the Southern blotting analysis on the R6 generation, no separation was observed between the *GmFad2-1* gene expression cassette (not expressed) transferred gene locus and the *dapA* gene expression cassette (not expressed) transferred gene locus; therefore, it was confirmed that these two gene loci are both homogenized and fixed in the final selection line (line 260-05).

Moreover, it was confirmed that onto the *GmFad2-1* gene expression cassette (not expressed) gene locus, two copies of the vector pBS43 are partially transferred adjacent to each other in the inverted repeat sequence. The map of the individual genes in the transferred sites is shown in Figure 9 of Annex 5.

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

As a result of fatty acid composition analysis on the seeds of R4, R5, R6 and R7 generations obtained from the field tests conducted in the US and Puerto Rico from 1995 to 1997, the high oleic acid soybean line 260-05 exhibited the higher contents of oleic acid of 80% or more compared to 17 to 30% of the oleic acid content of the control parental soybean line A2396. Consequently, it was confirmed that the transferred trait is stably inherited across several generations (see Annex 6).

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

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

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

For the detection and identification of this high oleic acid soybean line 260-05, a qualitative PCR method has been developed by DuPont in the US where two primers (18 bp and 19 bp respectively) are used based on the flanking sequence of the transferred DNA transferred to the high oleic acid soybean line 260-05 (Annex 7). The PCR method is found to make it possible to specifically detect the high oleic acid soybean line 260-05 by using the flanking sequence as primer when additional PCR is concurrently conducted using two primers (both 18 bp) based on the soybean genome DNA sequence. This method allows detection from the 20ng DNA samples. In addition, this method is confirmed to ensure high reproducibility through repeated tests.

(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

As detailed in 1-(1)-2) in this section, due to the gene silencing by the transferring of *GmFad2-1* gene, the expression of extraneous and endogenous *GmFad2-1* genes is suppressed, and the production of the δ-12 desaturase which catalyzes the reaction for biosynthesis to linoleic acid from oleic acid is inhibited and as a result, the oleic acid content in seeds is increased. In addition, it was observed that in the high oleic acid soybean line 260-05, compared to the parental soybean line A2396, α and α' subunits of β-conglycinin is decreased and glycinin acid subunit and glycinin A2, B1A subunits precursors are concomitantly increased (Annex 4). This is estimated to result from the fact that due to the transferring of β-conglycinin promoter connected to the *GmFad2-1* gene, the expression of the soybean endogenous β-conglycinin gene was suppressed by the gene silencing. The changes of decreasing amount of β-conglycinin and increasing glycinin or glycinin precursor are also observed in the soybean varieties improved by crossbreeding methods (Julie Nordlee, 1995) and the mutation induced soybeans (Takahashi *et al*., 1994；Kitamura, 1995); therefore, the changes were considered well within the ranges recognized for conventional soybeans (see Annex 4).

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

From 1995 to 1996, field tests were carried out at 24 sites in the US and one site in Puerto Rico on the high oleic acid soybean line 260-05 to evaluate the seed germination rate, plant body appearance, flowering time, seed setting mode, degree of maturity, yield, and sensitivity to disease and insect damage. In all items examined, no difference was observed that is caused by any effects of the transferred genes. In addition, as a result of monitoring of the 5 cultivation fields in the US conducted from May to June in the next year of the cultivation tests, there was no individual observed that survived the winter (Annex 8). Based on the above results, it was considered that there was no difference between the high oleic

acid soybean line 260-05 and the taxonomic species to which the recipient organism belongs except the increased oleic acid content and glycinin level.

Moreover, in consideration of possible environmental impacts due to the unlikely event of spilled seeds, isolated field test was carried out at Hokkaido Agricultural Research Center (the present National Agricultural Research Center for Hokkaido Region) to evaluate estimated environmental impacts. Results of the isolated field test are summarized below (Annex 9). The wintering ability of the matured plant and the productivity of harmful substances are described based on the results of tests and observations conducted in the US.

(a) Morphological and growth characteristics

Differences in morphological and growing characteristics were examined between the high oleic acid soybean line 260-05 and the control parental soybean line A2396 with respect to the following items: germination rate; time of flower initiation; flowering period; flower color; main stem length; the number of main stem nodes; thickness of stem; the number of branches; leaf color; trichome color; trichome quantity; growth habit; plant type; difficulty in pod bursting; above-ground weight of a plant; weight of pod seeds per plant; weight of ripe pods per plant; pod color; seed weight per plant; number of perfect seeds per plant; weight of perfect seeds per plant; number of seeds per pod; weight of 100 seeds; seed hull color; and hilum color. As a result, except the number of main stem nodes in which a statistically significant difference was observed (23.2 in the high oleic acid soybean line 260-05 compared to 20.1 in the parental soybean line A2396), in all other items examined, no difference was observed between the high oleic acid soybean line 260-05 and the parental soybean line A2396 (Annex 9). The number of main stem nodes observed in this isolated field test is found to fall within the typical variations in the number of main stem nodes for soybeans (8 to 33 nodes) (A Compendium of Farming System, 2002).

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

There is no data available for the direct measurement of cold-tolerance at the early stage of growth. Then, the seeds of both the high oleic acid soybean line 260-05 and the parental soybean line A2396 were collected in the isolated field and dried up then placed in the earth in the isolated field (early December) and recovered 60 days later (early February) for visual observation. As a result, no seed was found germinated, and all the seeds were decayed 60 days later (Annex 9).

Besides, in the field test in the US, researchers visited the field where the high oleic acid soybean line 260-05 was cultivated in the next year of the cultivation for observation and as a result, no individual was observed that survived the winter and germinated and grew. Based on the above results, it was estimated that even if the seeds spill during the transportation, the possibility that the seeds of the high oleic acid soybean line 260-05A could germinate and grow at low temperatures is equivalent to that in the case of conventional soybeans and thus, it was considered extremely unlikely that the seed buds possess the

cold-tolerance and overwinter.

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

Soybean is an annual cultivated plant, and it naturally dies in winter after ripening and it is not known to overwinter. In addition, it does not re-grow and propagate vegetatively nor produce seeds after harvesting. Observation was not made in the isolated field test in Japan, but in the field test in the US, researchers visited the field where the high oleic acid soybean line 260-05 was cultivated in the next year of the cultivation for observation. As a result, no individual was observed that re-grew after harvesting.

(d) Fertility and size of the pollen

There is no data available for the direct measurement of fertility and size of the pollen. Instead, as a result of examination in the isolated field on the number of ripe pods per plant, the number of perfect seeds per plant, and the number of seeds per pod, no significant difference was observed between the high oleic acid soybean line 260-05 and the parental soybean line A2396. In the event if the fertility of pollens varies, a difference may be observed in these items. Based on the findings that no difference from the parental soybean line A2396 was observed in the above items, it was considered that there is no difference in fertility of the pollen between the high oleic acid soybean line 260-05 and conventional soybeans.

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

For the production of seeds, the following items were examined; the number of ripe pods per plant, weight of seeds per plant, number of perfect seeds per plant, weight of perfect seeds per plant, number of seeds per pod, and weight of 100 seeds. As a result, in all the items examined, no difference was observed between the high oleic acid soybean line 260-05 and the parental soybean line A2396. In addition, with regard to the shedding habit, difficulty in pod bursting was evaluated and as a result, the high oleic acid soybean line 260-05 and the parental soybean line A2396 both exhibited the difficult pod bursting.

Regarding the dormancy and germination rate, the seeds of the high oleic acid soybean line 260-05 and the parental soybean line A2396 used in the tests were sown and on the 19th day after sowing, the germination rate was investigated. As a result, the germination rate was found 91% for the high oleic acid soybean line 260-05 and 88% for the parental soybean line A2396, showing the equivalent germination rates. Consequently, the dormancy was also considered equivalent between the high oleic acid soybean line 260-05 and the parental soybean line A2396.

Based on the above results, it was considered that there is no difference between the high oleic acid soybean line 260-05 and conventional soybeans with regard to the characteristics including the production of seeds.

(f) Crossability

In the isolated field test, conventional soybean cultivar Tsurumusume and the *Glycine soja* Sieb. et Zucc. were cultivated adjacent to the high oleic acid soybean line 260-05 to investigate the natural crossing between them. As a result, the natural crossing rate of the seeds obtained from Tsurumusume was found 0%, which did not exceed the crossability rate of 0.5 to 1% in the existing findings (A Compendium of Farming System, 2002; Encyclopedia of Agriculture, 1994).

In the above test, the actual crossability rate could not be evaluated for *Glycine soja* Sieb. et Zucc. due to the different flowering time. However, the crossability between the high oleic acid soybean line 260-05 and the non-recombinant control soybean was found equivalent to the conventional natural crossability rate, therefore, the crossability was estimated at similar level as the crossability between *Glycine soja* Sieb. et Zucc. and conventional soybean cultivars (0.7%) in the existing findings (Nakayama Y. and Yamaguchi H., 2002).

(g) Productivity of harmful substances

For soybeans, the secretion of any harmful substances from roots, which affect the surrounding plants or microorganisms in soil, has not been reported. In addition, the production of any allelochemicals, which affect other plants after they die, has not been reported.

To confirm the possibility of producing any harmful substances (the substances secreted from the roots to affect other plants, the substances secreted from the roots to affect microorganisms in soil, and the substances contained in the plant bodies to affect other plants after dying out), additional test was carried out using the samples cultivated in a screened greenhouse at Pioneer Hi-Bred International in the US in 2005 using the high oleic acid soybean line 260-05 and the non-recombinant control soybean (Annex 10). As a result, regarding the productivity of harmful substances, the high oleic acid soybean line 260-05 exhibited no significant difference from the non-recombinant control soybean.

Moreover, in the field test in the US, researchers visited the field where the high oleic acid soybean line 260-05 was cultivated in the next year of the cultivation for observation (Annex 8), and there was no decisive evidence reported about the effects on any succeeding crops due to the cultivation of the high oleic acid soybean line 260-05. Consequently, it is considered unlikely that the high oleic acid soybean line 260-05 would newly produce any substances due to unintended changes, which affect other plants after they die.

Based on all the factors discussed above, it was considered that there is no difference between the high oleic acid soybean line 260-05 and conventional soybeans with regard to the productivity of harmful substances.

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 *GmFad2-1* gene. However, 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 including the germination rate of seeds, 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 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 addition, the ability of this recombinant soybean to produce harmful substances (the substances secreted from the roots to affect other plants, the substances secreted from the roots to affect microorganisms in soil, and the substances contained in the plant bodies to affect other plants after dying out) was investigated, and no significant difference between this recombinant soybean and the non-recombinant control soybean was observed.

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

(3) Crossability

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

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

2) Evaluation of concrete details of adverse effect

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

3) Evaluation of likelihood of adverse effect

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

A. There is no plan of cultivation of this recombinant soybean in the future in Japan. It is considered that any seeds may be spilled during transportation and such spilled seeds germinate and grow, though there is no report so far that soybean becomes self-seeding.

B. Even in the case where this recombinant soybean grows voluntarily near *G. soja* as a rare case:

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

(b) According to existing documents, even when *G. soja* was grown adjacent to *G. max* under such a condition that the *G. max* variety has the same flowering time as *G. soya*, the crossing rate was less than 1%.

(c) As a result of the adjacent cultivation of this recombinant soybean and the non-recombinant control soybean in the Japanese isolated fields, the crossability between them was considered at similar low levels as in the case of conventional soybeans.

In view of the above, it is considered to be very low that this recombinant soybean and *G. soja* could cross with each other and that the transferred gene could diffuse among the population of *G. soja* without remaining at a low level.

4) Judgment of existence of Adverse Effect on Biological Diversity

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant soybean in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

* Cleistogamy means self-pollination within a flower that does not open that occurs in angiosperms. It involves a very low probability of cross-pollination with the pollens of other plants due to the physical obstacles of flower bud (petal/calyx). However, it does not mean any physiological incompatibility, so it may allow cross-pollination with the pollens of other plants transmitted by insects, etc.

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List of Annex for High oleic acid soybean (*GmFad2-1, Glycine max* (L.) Merr.)
(26Ø-Ø5, OECD UI : DD- Ø26Ø5-3)

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

Annex 2 Nucleotide sequences of plasmid pBS43 and plasmid pML102
(Confidential: Not made available or disclosed to unauthorized person)

Annex 3 Identification based on the Northern blotting analysis that the *amp*^{r} gene, *GmFad2-1* gene, *dapA* gene, and *GUS* gene are not expressed
(Confidential: Not made available or disclosed to unauthorized person)

Annex 4 Analysis of seed proteins in the high oleic acid soybean line 260-05
(Confidential: Not made available or disclosed to unauthorized person)

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

Annex 6 Analysis on the oleic acid content
(Confidential: Not made available or disclosed to unauthorized person)

Annex 7 Method for the detection of the high oleic acid soybean line 260-05
(Confidential: Not made available or disclosed to unauthorized person)

Annex 8 Field test results on the high oleic acid soybean line 260-05 in the US and other countries
(Confidential: Not made available or disclosed to unauthorized person)

Annex 9 Isolated field test results on the high oleic acid soybean line 260-05 in Japan
(Confidential: Not made available or disclosed to unauthorized person)

Annex 10 Additional test results on the productivity of harmful substances(the substances secreted from the roots to affect other plants, the substances secreted from the roots to affect microorganisms in soil, and the substances contained in the plant bodies to affect other plants after dying out)
(Confidential: Not made available or disclosed to unauthorized person)