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

Applicant
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Approved Type 1 Use Regulation

Name of the type of Living Modified Organism:	Stearidonic acid producing soybean (modified *Pj.D6D*, modified *Nc.Fad3*, *Glycine max* (L.) Merr.) (MON87769, OECD UI: MON-87769-7)
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 Effects on Biological Diversity

1 Information concerning preparation of living modified organisms

Long-chain omega fatty acid such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are recognized to reduce the risk of cardiovascular diseases (Wang et al., 2006). However, EPA and DHA are more susceptible to oxidation so that their usage in foods is limited. Monsanto Company has developed soybean containing stealidonic acid (SDA), which is a metabolic precursor to those long chain omega-3 fatty acids in the form of oil content. SDA is one of the omega-3-fatty acids and is more stable to oxidation, since SDA has fewer double-bonds than EPA and DHA. It is known that SDA, when ingensted by human and animals, is first converted into EPA in the body, traces of which are additionally transformed to DHA (Hammond *et al.*, 2008; Harris *et al.*, 2007; Harris *et al.*, 2008). SDA soybean oil is used as food ingredient in some products such as margarine, salad dressings, mayonnaise, bread, cakes, bread, cake, cereal bar, cereal, yogurt, smoothie, milk-based dringk, soybean milk, soup, sauce, retort food and prepared meat and fish products for intake of omega-3 fatty acids[1]. In addition, SDA soybean could be used as an alternative to fish oil and other omega-3 fatty acid rich feed components in feed applications such as aquaculture and poultry feeds.

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition and origins of component elements of donor nucleic acids used for developing the stealidonic acid producing soybean (modified *Pj.D6D*, modified *Nc.Fad3*, *Glycine max* (L.) Merr.) (MON87769, OECD UI: MON-87769-7) (hereinafter, referred to as a "present recombinant soybean") are shown in Figure 1 (p5) and Table 1 (p6~9).

[1] Oil used as raw material for margarine includes two types of hydrogenated oil (fat as hydrogenate) and unhydrogenated oil. SDA soybean oil is suitable for utilizing it as unhydrogenated oil.

Length of amino acid sequence of Δ6 desaturase expressed from *Pj.D6D* gene in present recombinant soybean is 16 amino-acids shorter at the N-terminus than that of wild type ofΔ6 desaturase. Accordingly, *Pj.D6D* gene transferred to the present recombinant soybean is referred to as a "modified *Pj.D6D* gene" and the protein as expressed is referred to as a "modified Δ6 desaturase". In addition, amino acid sequence of Δ15 desaturase expressed by *Nc.Fad3* gene in this recombinant soybean has a single amino acid substitution from threonine to alanine in the second amino acid position from the N-terminus to introduce a Kozak consensus sequence, which is important for initiating its translation. Accordingly, the *Nc.Fad3* gene is referred to as a "modified *Nc.Fad3* gene", and the protein expressed is referred to as the "modified Δ15 desaturase".

In addition, 5-enolpyruvylshikimic acid-3-phosphate synthase (CP4 EPSPS) expressed from the *cp4 epsps* gene which is transferred as a selective marker during the process of developing the present recombinant soybean is additionally modified such that the functional activity of CP4 EPSPS is not altered so as to improve its expression in the plant. The CP4 EPSPS is modified in that serine residue at second position from the N-terminal sequence is changed into leucine residue, in comparison with the amino acid sequence derived from *Agrobacterium* sp.CP4 strain. Accordingly, the *cp4 epsps* gene transferred to the present recombinant soybean is referred to as a "modified *cp4 epsps* gene". However, in the development of present recombinant soybean, only those individuals that are free from any modified *cp4 epsps* gene were selected at R1 generation through genetic segregation based on the Invader assay[2] (Figure 3, p18).

Note that deduced amino acid sequences for the modified Δ6 desaturase and the modified Δ15 desaturase are shown in Attachment 1.

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[2] Invader analysis is a technique of signal amplification for detecting the genetic mutation and quantitatively analyzing the gene. Invader analysis is not necessary for the gene amplification by PCR, its detection is made by a cleavage process which is referred to as the Invader® method. In the cleavage process, a gene sequence to be targeted is cleaved by an enzyme, which is referred to as Cleavase®, which can specifically recognize a structure, and fluorescence is detected. Invader® and Cleavase® are registered as trademark of Third Wave Technologies.

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

Function of each component element for the donor nucleic acid used for developing the present recombinant soybean is as shown in Table 1 (p6 to 9).

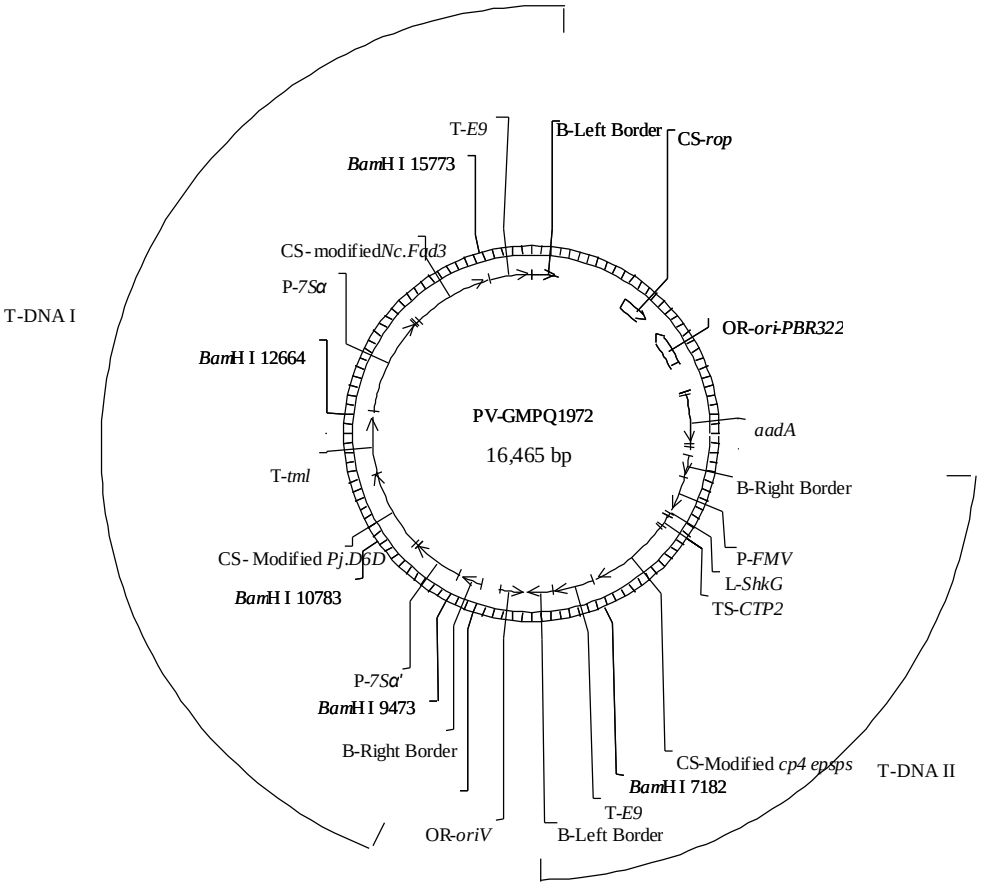

Figure 1 Plasmid map of PV-GMPQ1972[3]

In the development process of the present recombinant soybean, those individuals were selected that contain the T-DNA I region shown above but not contain T-DNA II region.

[3]All the rights pertinent to the information in the present figure and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 1 Component elements of donor nucleic acids, and their origins and functions[4]

Component elements	Origin and function
T-DNA I	
B[*1]-Right Border	Sequence of a DNA region derived from *Agrobacterium tumefaciens*, containing the right border sequence used for transferring T-DNA (Depicker *et al.*, 1982).
Intervening Sequence	Sequence as utilized for the DNA cloning
P[*2]-*7Sα'*	Promoter sequence and leader sequence inducing the transcription of a gene (alpha'-bcsp) encoding β-conglycinin storage protein of *G. max* (Doyle *et al.*, 1986). The sequence induces the transcription of mRNA in embryo-specific manner (Chen *et al.*, 1986).
Intervening Sequence	Sequence as utilized for the DNA cloning
CS[*3]-modified *Pj.D6D*	Coding sequence of Δ6 desaturase of *Primula juliae* (type of primrose) (Ursin *et al.*, 2005).
Intervening Sequence	Sequence as utilized for DNA cloning
T[*4]-*tml*	3'-terminal untranslated region of tml gene of octopine Ti plasmid of *A. tumefaciens* (Kemp *et al.*, 2000). Induces polyadenylation of mRNA.
Intervening Sequence	Sequence as utilized for DNA cloning
P-*7Sα*	Promoter sequence and leader sequence inducing the transcription of a gene (*Sphas2*) encoding β-conglycinin storage protein of *G. max* (Wang *et al.*, 2004). The sequence induces the transcription of mRNA in embryo-specific manner (Chen *et al.*, 1986).
Intervening Sequence	Sequence as utilized for DNA cloning
CS-modified *Nc.Fad3*	Coding sequence ofΔ15 desaturase of *Neurospora crassa* (neurospora) (Ursin *et al.*, 2003).
Intervening Sequence	Sequence as utilized for DNA cloning
T-*E9*	3'-terminal untranslated region derived from *RbcS2* gene, encoding small subunit of ribulose-1,5-bisphosphate carboxylase of *Pisum sativum* (pea). Induces polyadenylation of mRNA (Coruzzi *et al.*, 1984).

[4]All the rights pertinent to the information in the present table and the responsibility for the content rest upon Monsanto Japan Limited.

Table 1 (continued) Component elements of donor nucleic acids, and their origins and functions

Component elements	Origin and function
T-DNA I (continued)	
Intervening Sequence	Sequence as utilized for DNA cloning
B -Left Border	Sequence of a DNA region derived from *A. tumefaciens*, containing the left border sequence used for transferring T-DNA (Barker *et al.*, 1983).
Outside backbone region (not present in the present recombinant soybean)	
Intervening Sequence	Sequence as utilized for DNA cloning
CS -*rop*	Coding sequence of primer repressor protein derived from ColE1 plasmid. Maintains a copy number of the plasmid in *Escherichia coli* (Giza and Huang, 1989).
Intervening Sequence	Sequence as utilized for DNA cloning
OR[*5]-*ori-PBR322*	Initiating region of replication isolated from pBR322, conferring the autonomous replication to vectors in *E.coli* (Sutcliffe, 1978).
Intervening Sequence	Sequence as utilized for DNA cloning
aadA	Bacterial promoter/coding sequence/3'-untranslated region of 3'(9)-O-nucleotidyl transferase (aminoglycoside modified enzyme) of transposon Tn7 (Fling *et al.*,1985). Provides resistances to spectinomycin and streptomycin.
Intervening Sequence	Sequence as utilized for DNA cloning
T-DNA II (not present in this recombinant soybean)	
B-Right Border	DNA region derived from *A. tumefaciens*, containing the right border sequence utilized for transferring T-DNA (Depicker *et al.*, 1982).
Intervening Sequence	Sequence as utilized for the DNA cloning
P-*FMV*	Promoter of 35S RNA of Figwort mosaic virus (FMV) (Rogers, 2000). Induces the transcription within the plant cells.
Intervening Sequence	Sequence as utilized for DNA cloning
L[*6]-*ShkG*	5'-terminal untranslated region of *ShkG* gene encoding 5-enolpyruvylshikimic acid-3-phosphate synthase of *Arabidopsis thaliana* (arabidopsis) (Klee *et al.*, 1987). Responsive for the modulation of the gene expression.

Table 1 (continued) Component elements of donor nucleic acids, and their origins and functions

Component elements	Origin and function
T-DNA II (Not present in the present recombinant soybean) (continued)	
TS[*7]–*CTP2*	Sequence encoding chloroplast transit peptide derived from *ShkG* gene encoding EPSPS of *A. thaliana* (Klee *et al.*, 1987; Herrman, 1995). Transports the modified CP4EPSPS protein into chloroplast.
CS-modified *cp4 epsps*	Coding sequence of *aroA* gene encoding 5-enolpyruvylshikimic acid-3-phosphate synthase (CP4 EPSPS) of *Agrobacterium* CP4 strain (Padgette *et al.*, 1996; Barry *et al.*, 1997).
Intervening Sequence	Sequence as utilized for DNA cloning
T-*E9*	3'-terminal untranslated region derived from *RbcS2* gene encoding small subunit of ribulose-1,5-bisphosphate carboxylase of *Pisum sativum*. Induces the polyadenylation of mRNA (Coruzzi *et al.*, 1984).
Intervening Sequence	Sequence as utilized for DNA cloning
B-Left Border	DNA region derived from *A. tumefaciens*, containing the left border sequence utilized for transferring T-DNA (Barker *et al.*, 1983).
Outside backbone region (not present in the present recombinant soybean)	
Intervening Sequence	Sequence as utilized for DNA cloning
OR-*ori V*	Initiating region of replication derived from the broad-host range plasmid RK2. Permits autonomous replication of vector in *Agrobacterium* (Stalker *et al.*, 1981).
Intervening Sequence	Sequence as utilized for the DNA cloning

[*1] B – Border (border sequence)

[*2] P – Promoter

[*3] CS – Coding Sequence

[*4] T – Transcript Termination Sequence

[*5] OR – Origin of Replication (region at starting replication)

[*6] L – Leader (leader sequence)

[*7] TS – Targeting Sequence

(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 that is known to possess any allergenicity

【Modified *Pj.D6D* gene】

The present recombinant soybean is transferred with the modified *Pj.D6D* gene derived from *P. juliae*. *P. juliae* from which the modified *Pj.D6D* gene is isolated is categorized into a genus of the plant which is generally known as Primrose.

The modified *Pj.D6D* gene encodes the modified Δ6 desaturase which is the front end desaturase (generic name of desaturase which indtoduce double bonds between an existing double bond and carboxyl end). The modified Δ6 desaturase introduces a double bond between the sixth and seventh carbons from the carboxyl end of certain fatty acid. In the plants and animals which are known to produce SDA, SDA is the product of Δ6 desaturation of α-linolenic acid (ALA) and ω-3 desaturation of γ-linolenic acid (GLA) (

Figure 2, p13). However, soybean cannot produce SDA, since it does not have Δ6 desaturase. So, by transferring the modified *Pj.D6D* gene into the present recombinant soybean, SDA, which cannot be originally produced in a soybean, can be produced (

Figure 2, p13). Transfer of the modified Δ6 desaturase results in introduction of double bonds in the oleic acid (18:1) and linoleic acid (18:2) in the present recombinant soybean, causing production of small amounts of isolinoleic acid (ILA, 18:2) and GLA (18:3) (

Figure 2, p13).

In order to investigate whether the modified Δ6 desaturase shares functionally important amino acid sequence with the existence allergen is compared according to FASTA algorithm using the allergen database (AD_2009[5]), no structurally similar sequence with the existence allergen is found.

【Modified *Nc.Fad3* gene】

In addition to the modified *Pj.D6D* gene as mentioned above, the modified *Nc.Fad3* gene derived from *N. crassa* (Japanese name: neurospora) is transferred in the present recombinant soybean. *N. crassa* is a type of fungi categorized in Ascomycotina, and is considered to contain apathogenic and anallergic properties (Perkins and Davis, 2000).

By transferring the modified *Nc.Fad3* gene, the modified Δ15 desaturase is expressed in the present recombinant soybean. The modified Δ15 desaturase introduces a double bond between fifteenth and sixteenth carbons from the carboxyl end of certain fatty acid. Although soybean has endogenous Δ15 desaturase, the activity is known to be low. Expression of the modified Δ15 desaturase from *N. crassa* in the present recombinant soybean accelerates the metabolic pathway from linoleic acid to ALA and GLA to SDA, where GLA is a product from the modified Δ6 desaturase activity on LA (

Figure 2, p13).

As mentioned above, it is indispensable to transfer a gene encoding Δ6 desaturase in order to produce SDA in a soybean. However, it is considered that the level of SDA in the present recombinant soybean would increase efficiently by expressing the modified Δ15 desaturase simultaneously.

In order to investigate whether the modified Δ15 desaturase shares a functionally

[5] Database comprising sequences registered in FARRP (Food Allergy Research and Resource Program) AllergenOnline database (FARRP, 2009)

important amino acid sequence with the existence allergen is compared according to FASTA algorithm using the allergen database (AD_2009), no similar sequence structurally with the existence allergen is found.

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

By nature, soybean lacks Δ6 desaturase and therefore cannot produce SDA. However, the present recombinant soybean is given the capability of producing SDA by expressing the modified Δ6 desaturase and modified Δ15 desaturase. The present recombinant soybean produces approximately 20% to 30% SDA of total fatty acid in soybean oil depending on the environment condition of cultivation (Table 2, p14). In addition, by transferring the modified Δ6 desaturase, a double bond is introduced in linoleic acid (18:2) and as a result, GLA (18:3) is produced.

In fact, as results of analyzing fatty acids in the present recombinant soybean, non-recombinant control soybean and 15 conventional commercial varieties, it was confirmed that SDA (on an average of 26.13% of the total fatty acids) and GLA (on an average of 7.09% of the total fatty acids) are produced in the seed of the present recombinant soybean (Table 2, p14). On the other hand, although the recombinant soybean was expected to produce ILA due to the activity of the modified Δ6 desaturase on oleic acid, ILA was found below limit of quantification as a result of fatty acid composition analysis on the seeds of the present recombinant soybean (Table2, p14). In addition, small amounts of trans-SDA and trans-ALA were detected in the seeds of the present recombinant soybean. However, it has been reported that a trans-isomers of fatty acidss are created in the process of oil extraction and refining (Chardingny *et al.*, 1996). Therefore, it was considered that trans-SDA and trans-ALA might be produced in the oil refining process due to isomerization and are detectable in the oil from the present recombinant soybean in which SDA and ALA levels are relatively high.

In addition, regarding the contents of palmitic acid, oleic acid, linoleic acid, ALA, arachidic acid and behenic acid among eight fatty acids which were detected in both of the present recombinant soybean and the non-recombinant control soybean, a significant difference was confirmed between the present recombinant soybean and the non-recombinant control soybean (Table 2, p14). The levels of oleic acid, linoleic acid and ALA in the present recombinant soybean were found outside the ranges of analytical values of conventional commercial varieties. However, these

fatty acids are all present in the metabolic pathway leading to production of SDA (

Figure 2、p13), and therefore, the content levels were considered to vary according to the expression of Δ6 desaturase and Δ15 desaturase in the present recombinant soybean.

Moreover, the difference in the level of arachidic acid, palmitic acid and behenic acid in the present recombinant soybean and non-recombinant control soybean was found within the range of analytical values of conventional commercial varieties. The arachidic acid, palmitic acid and behenic acid are not contained in the metabolic pathway to production of SDA and then, the difference detected in the levels of fatty acid contents was considered not to result from the direct action of Δ6 desaturase and Δ15 desaturase on the fatty acids.

Substrate specificy of Δ6 desaturase and Δ15 desaturase has been investigated in an *in vitro* yeast expression system (Attachment 2). As a result, it was confirmed that desaturation in the modified Δ6 desaturase from *P. juliae* is specific and it only performs Δ6 desaturation of certain unsaturated fatty acids such as oleic acid, linoleic acid and ALA. Similarly, it was confirmed that also the modified Δ15 desaturase from in *N. crassa*, is specific to ω-3 desaturation of fatty acid substrates such as linoleic acid, GLA, dihomo γ-linoleic acid (DGLA) and arachidonic acid.

Therefore, it was concluded unlikely that there would be any change in the metabolic system of host organism other than above-mentioned changes in fatty acid composition due to the expression of the modified Δ6 and Δ15 desaturases in the present recombinant soybean.

In addition, SDA, a fatty acid produced newly in the present recombinant soybean, is a normal component of fish oil and is a metabolic precursor to EPA and DHA as described in section 2 (p2) of chapter I. GLA is part of the human diet, found in human breast milk, organ meat and plant seed oils, and ILA is also present in human diets, such as various fish oils and dietary supplements and in fish.

In the seed of the present recombinant soybean, SDA and GLA, which soybean cannot produced naturally, are produced. Fatty acidsin soybean seeds are

accumulated and stored as a part of triacylglycerol (TAG) in seed oil contents. TAG is known to be a major storage lipid within the soybean seed, and is known to be utilized as an energy source during the germination period (Liu and Brown, 1996; Taiz and Zeiger, 1998). In the process of utilizing TAG as an energy source, TAG is hydrolyzed by lipases and to glycerol and fatty acids which are further metabolized via β-oxidation to acyl-CoA. It has been reported to date that lipase in sunflower, poppies and flaxseeds does not have substrate specificity (Fernandez-Moya et al., 2000, Prokofev and Novitskaya, 1958), and that isomerase and reductase required for β-oxidation of polyunsaturated fatty acids are present in all plants and animals (Stryer, 1995). Therefore, it is considered that β-oxidation of SDA and GLA in soybean take place in the similar manner as in the case of β-oxidation of linoleic acid and ALA. In fact, as results of fatty acid composition analysis on the seed and the cotyledons in the germination period of the present recombinant soybean, SDA and GLA contents were found decreasing with germination, suggesting that the fatty acids are utilized for energy metabolism, similar to soybean endogenous fatty acids such as linoleic acid and ALA (Attachment 3).

Based on above, it is considered that SDA and GLA produced in the present recombinant soybean play similar biological role as other fatty acids present in soybean seeds.

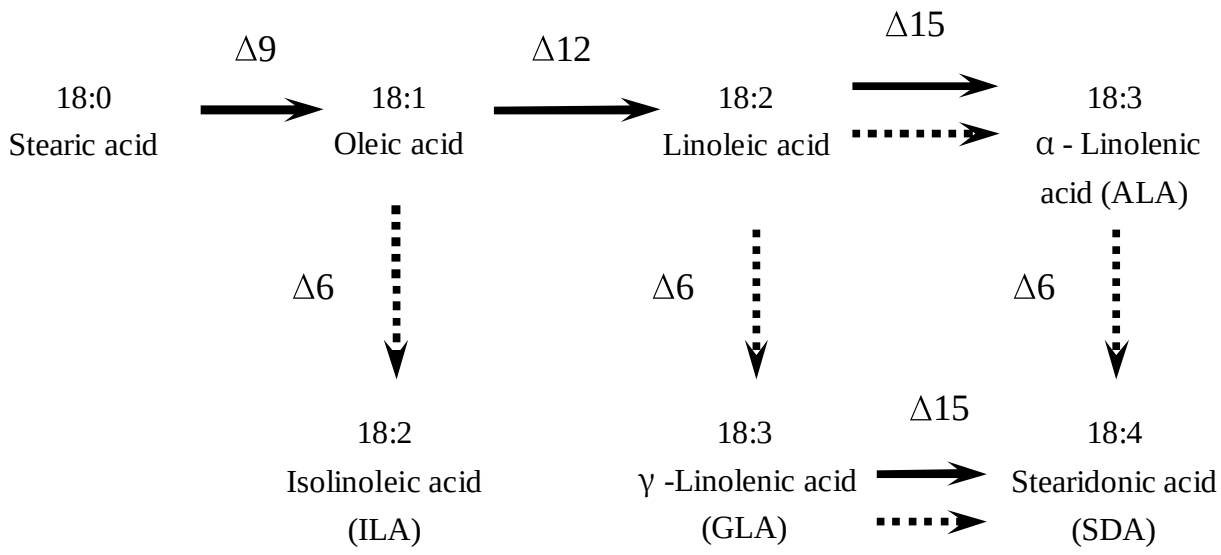

Figure 2 Production of SDA in the present recombinant soybean in which the modified Δ6 desaturase from *P. juliae* and the modified Δ15 desaturase derived from *N. crassa* are transferred [6]

Fatty acid synthesis pathway where soybean endogenous desaturase work or may work

Fatty acid synthesis pathway where the transferred modified Δ6 desaturase and modified Δ15 desaturase work

[6]All the rights pertinent to the information in the present figure and the responsibility for the content rest upon Monsanto Japan Limited.

Table 2 Fatty acid composition in soybean oils of the present recombinant soybean, the non-recombinant control soybean and conventional commercial varieties, and fat content in seeds [1] [7]

	Present recombinant soybean Average value (%) [range (%)]	Non-recombinant control soybean Average value (%) [range (%)]	p value	Conventional commercial product (range (%)) [99% Tol. Int.[2] (%)]
Fatty acid composition (content per total fatty acids)				
16:0 Palmitic (Palmitic acid)	12.06 [11.53 - 12.54]	11.77 [11.14 - 12.08]	<0.001	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic (Stearic acid)	4.19 [3.73 - 4.53]	4.15 [3.85 - 4.44]	0.245	(3.68 - 4.89) [2.87, 5.85]
18:1 Oleic (Oleic acid)	15.18 [12.66 - 18.80]	19.19 [17.24 - 21.17]	0.001	(16.70 - 23.16) [12.56, 27.98]
18:2 Linoleic (Linoleic acid)	22.78 [16.46 - 30.81]	54.93 [54.05 - 56.04]	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:2 Isolinolenic (ILA) (Isolinoleic acid)	- [3]	-	NA[4]	-
18:3 Linolenic (ALA) (α-Linoleic acid)	11.18 [10.20 - 11.80]	9.20 [7.42 - 10.66]	0.016	(6.95 - 10.58) [3.72, 13.46]
18:3 trans-ALA (trans α-linolenic acid)	0.44 [0.38 – 0.48]	-	NA	-
18:3 γ-linolenic (GLA) (γ-linolenic acid)	7.09 [6.07 - 8.03]	-	NA	-
18:4 Stearidonic (SDA) (Stearidonic acid)	26.13 [16.83 – 33.92]	-	NA	-
18:4 trans-SDA (trans stearidonic acid)	0.18 [0.058 – 0.26]	-	NA	-
20:0 Arachidic (Arachidic acid)	0.34 [0.31 - 0.37]	0.31 [0.28 - 0.34]	<0.001	(0.27 - 0.36) [0.20, 0.45]
20:1 Eicosanoic (Eicosenoic acid)	0.14 [0.075 - 0.20]	0.13 [0.069 - 0.19]	0.282	(0.071 - 0.19) [0, 0.31]
22:0 Behenic (Behenic acid)	0.29 [0.26 - 0.31]	0.32 [0.28 - 0.37]	0.023	(0.30 - 0.41) [0.22, 0.49]
Fat content in dried seed				
Total Fat (Fat)	15.91 [12.95 - 19.03]	15.94 [12.73 - 18.80]	0.955	(13.99 - 20.56) [11.04, 25.03]

[1] Samples obtained from 5 farm fields in the US were analyzed, and results were statistically analyzed with ANOVA (n=5).
[2] Tolerance interval includes 99% of the expressed value in population of the conventional commercial product with 95% reliability. The limit of the lowest value is set as 0
[3] - ; less than the lower limit for quantification (the limit for quantification is 0.13% of total fatty acids)
[4] NA; not applied, since the analyzed value in the non-recombinant control soybean, or the both of analyzed values in the present recombinant soybean and the non-recombinant control soybean are less than the lower limit for quantification.

(2) Information concerning vectors

1) Name and origin

The vectors used for the production of the present recombinant soybean were assembled from plasmids including the vector pBR322 from *E. coli*.

2) Properties

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

The total number of base pairs in PV-GMPQ1972 used for the production of the present recombinant soybean is 16,465 bp. The entire nucleotide sequences of the present plasmid vector is shown in Attachment 4.

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

The *aadA* gene derived from transposon Tn7 conferring spectinomycin and streptomycin as the selective marker of the construction vector in *E. coli* is present outside of T-DNA region.

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

The present vector is not known to be infectious.

(3) Method of preparing living modified organisms

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

The component elements of the present plasmid vector transferred to the recipient organism are described in Table 1 (p6 to 9). In addition, the location of the component element of the donor nucleic acid within the vector and the section to be cleaved by restriction enzymes are shown in Figure 1 (p5).

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

The plasmid vector PV-GMPQ1972 was transferred into the embryo cell derived from the non-recombinant soybean variety A3525 by *Agrobacterium* method.

3) Process of growing living modified organisms

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

Meristem tissues were excised from the embryos of conventional soybean variety A3525. After co-culturing with the *A. tumefaciencs* ABI strain carrying the plasmid vector PV-GMPQ1972, the meristems were placed on the tissue culture medium containing glyphosate, carbenicillin and claforan for selection of cells. At the time, plant cells untransformed by the glyphosate were removed.

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

The Agrobacterium bacterium which is used for transformation is removed by the tissue culture medium supplemented with carbenicillin and claforan. Further, as a sult of PCR analysis targeting outside backbone region of the plasmid vector PV-GMPQ1972 used for the transformation in R4 generation of the present recombinant soybean, it was found that the outside backbone region of the plasmid vector is not present in the present recombinant soybean (Attachment 5). Consequently, the absence of *Agrobacterium* used for the transformation was confirmed in the present recombinant soybean.

(c) Process 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 Effects on Biological Diversity

The re-differentiated individual (R0), as transformed, was inbred to select an individual having T-DNA I region (a region having an expressing cassette of the modified *Pj.D6D* gene and an expressing cassette of the modified *Nc.Fad3* gene) as homo and having T-DNA II region (a region having an expression cassette of the

modified *cp4 epsps* gene) as separated, in progeny R1 generation. It has been confirmed by the Invader analysis in R2 generation that this individual does not have T-DNA II region. The progeny of the selected individual was to be subjected for analyzing the transferred gene and investigating morphological properties. As a result, the MON87769 line was finally selected as the line to be commercialized.

The process of growing the present recombinant soybean was shown in Figure 3 (p18). Meanwhile, the scope of approval for Type 1 Use Regulation includes R4 generation of the present recombinant soybean and the progeny hybrids delived from the R4 generation of the present recombinant soybean.

【Confidential: not disclosed to unauthorized persons】

Figure 3 Process of growing of the present recombinant soybean

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

(a) Place where the replication product of transferred nucleic acid exists

In order to investigate whether or not the transferred gene of the present recombinant soybean is present on the chromosome (R4 generation) containing the transferred genes as homo, and the soybean without any recombination were bred to produce a F1 individual and the genotype of the transferred gene was investigated using the Invader analysis for F2 generation obtained from inbreeding the one individual at F1 generation to detect a segregation ratio (Attachment 6). As a result, the segregation ratio of the transferred gene complied with the segregation ratio of 1:2:1 which is expected in accordance with the Mendel law (Table 3, p.22). Further, F3 generation obtained from inbreeding a single individual of heterozygote at F2 generation, and F4 generation obtained from inbreeding a single individual of heterozygote at F3 generation complied with the segregation ratio as expected (Table 3, p.22). Therefore, it is considered that the transferred gene of the present recombinant soybean is present on the chromosome.

Table 3 Segregation ratio of the transferred gene in the progeny of the present recombinant soybean[8]

		Observed values			Expected values containing a segregation ratio of 1:2:1 [3]				
Generation[1]	Number of Individuals to be provided[2]	Positive/homo Number of Individuals	Positive/hetero Number of individuals	Negative Number of individuals	Positive/homo Number of Individuals	Positive/homo Number of Individuals	Negative Number of Individuals	χ^2	p value
F2	47	11	23	13	11.75	23.5	11.75	0.2	0.9087
F3	174	48	81	45	43.5	87	43.5	0.9	0.6278
F4	222	60	102	60	55.5	111	55.5	1.5	0.482

[1] The F1 generation was obtained by breeding the present recombinant soybean and the soybean without any recombination which does not have the transferred gene of the present recombinant soybean. In addition, F3 generation and F4 generation were obtained from inbreeding F2

[8] All rights pertinent to the information in the present table above and the responsibility for the content rest upon Monsanto Japan Limited.

generation and F3 generation, each of which are heterozygotes.

[2] Whether or not there is T-*tml*, which is a portion of the transferred genes, was assayed using the Invader analysis to confirm the genotype.

[3] In order to evaluate whether or not the observed value complies with the expected value in accordance with the Mendel law in each generation to be investigated, the value of χ^2 was tested.

(b) The number of copies of replication products of transferred nucleic acid and stability of its inheritance across multiple generations

As a result of analyzing the transferred gene using Southern blotting analysis, it was confirmed that 1 copy of T-DNA I region is incorporated in one location in the genome of the present recombinant soybean (Fig. 5, p.43 of Attachment 7). In addition, it was confirmed that the outside backbone region and T-DNA II region were not transferred (Fig. 6 to 7, p.44 to 45 and Fig. 15 to 16, p.53 to 54 of Attachment 7), and that all component elements of the expression cassette of the modified *Pj.D6D* gene and the expression cassette of the modified *Nc.Fad3* gene within T-DNA I region are incorporated (Fig. 8 to 13, p.46 to 51 of Attachment 7). Further, it has been shown that the transferred gene is stably inherited to the progeny using the Southern blot analysis in several generations (generations R3 to R6) (Fig. 14, p.52 of Attachment 7).

The schematic figure of the transferred gene in the present recombinant soybean is shown in p.24 as Figure 4.

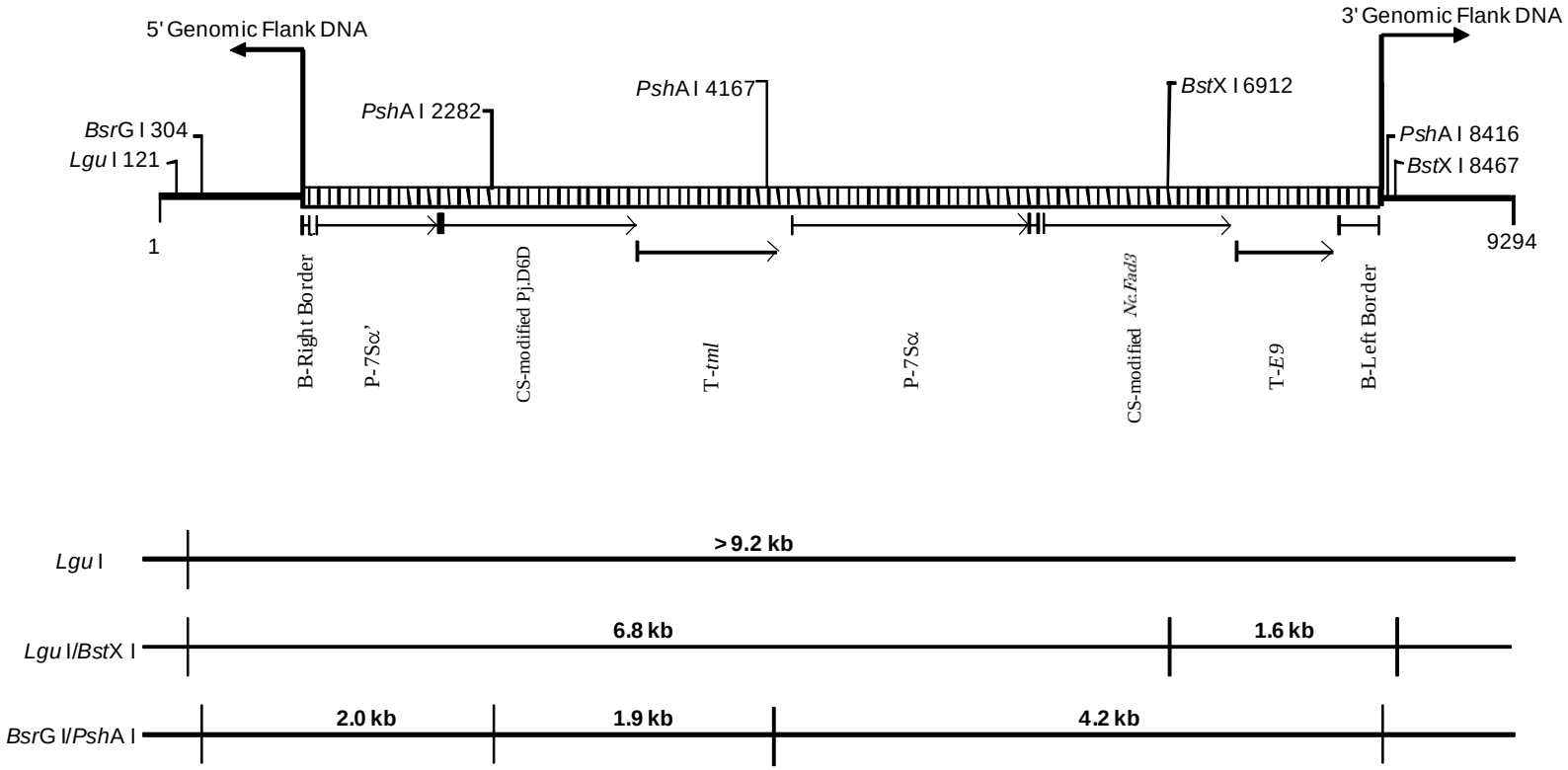

Figure 4 Transferred gene map of the present recombinant soybean[9]

[9]All the rights pertinent to the information in the present table above and the responsibility for the content rest upon Monsanto Japan Limited.

(c) In the case of multiple copies existing in a chromosome, whether they are neighbored or spaced from each other

This item is not applicable, since there is one copy for all genes (Fig. 5, p.43 of Attachment 7).

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

Analysis using the Western blotting analysis was made for the expressed amount of the modified Δ6 desaturase and the modified Δ15 desaturase in leaf, immature seed and mature seed of the present recombinant soybean which was cultivated by the randomized block design for 3 repeats in 3 farm field in US (Nebraska, Arkansas and Wisconsin) (Attachment 8).

As a result, the expressed amount of the modified Δ6 desaturase was an average of 20 μg/g fwt and a range of 15 to 25 μg/g fwt in the immature seed, and was an average of 0.99 μg/g fwt and a range of 0.43 to 1.9 μg/g fwt in the mature seed (Table 1, p.17 of Attachment 8). On the other hand, the expressed amount of the modified Δ6 desaturase in the leaf was less than the lower limit for quantification (LOQ=0.625 μg/g).

The expressed amount of the modified Δ15 desaturase was an average of 48 μg/g fwt and a range of 26 to 62 μg/g fwt in the immature seed, and was an average of 21 μg/g fwt and a range of 5.3 to 52 μg/g fwt in the mature seed (Table 1, p.17 of Attachment 8). On the other hand, the expressed amount of the modified Δ15 desaturase was less than lower limit for quantification (LOQ=0.625 μg/g fwt).

In addition, in order to investigate the inter-generational stability of the modified Δ6 desaturase and the modified Δ15 desaturase in the present recombinant soybean, proteins were extracted from samples of immature seed, at autonomous progeny four generations (R3, R4, R5, R6) of the present recombinant soybean to analyze the expression of the modified Δ6 desaturase and the modified Δ15 desaturase using the Western blotting analysis. As a result, bands which are identical to the modified Δ6 desaturase and the modified Δ15 desaturase were detected for all generations to be provided, and it was confirmed that traits to be purposed were stably expressed (Fig. 1, p.15 and Fig. 2, p.16 of Attachment 9).

According to the results, it was indicated that the modified *Pj.D6D* gene and the modified *Nc.Fad3* gene transferred to the present recombinant soybean are stably inherited to several progenies, and that the modified Δ6 desaturase and the modified Δ15 desaturase are expressed at these progenies.

(e) 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 possibility that the transferred nucleic acid is transmitted to wild animals and wild plants and the like under natural conditions, since regarding the plasmid vector PV-GMPQ1972, the region of recipient organism, which allows autonomous replication, is limited to gram-negative bacteria such as *E. coli* and *A. tumefaciens*.

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

Detection is available using the PCR method (Attachment 10). This method has sufficient sensitivity to evaluate each one seed. It is recommended that the concentration of DNA used for evaluation 5 to 10 ng per one reaction of PCR. A confirmation test was performed for the reproductive precision of this method using 180 seeds of the present recombinant soybean and 90 seeds of the soybean without any recombination.

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

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

In the present recombinant soybean, the modified Δ6 desaturase and the modified Δ15 desaturase are expressed by transferring the modified *Pj.D6D* gene and the modified *Nc.Fad3* gene, respectively, to produce SDA and GLA as a result. Since the modified *Pj.D6D* gene and the modified *Nc.Fad3* gene are controlled by the embryo-specific *7Sα'* promoter and the *7Sα* promoter the modified Δ6 desaturase and

the modified Δ15 desaturase was confirmed to express only in seed. In addition, expression level of both proteins in immature seeds is higher than that of mature seeds, and the expression level is confirmed to decrease as the seeds reach maturity (Table 1, p.17 of Attachment 8).

(b) With respect to the physiological or ecological characteristics listed below, presence or absence of differences between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present[10]

In an isolated field of Kawauchi research farm of Japan Monsanto Limited, an isolated field test of the present recombinant soybean was performed in 2008. The R6 generation of the present recombinant soybean was provided for the test (Figure 3, p18). As the non-recombinant control soybean, A3525, which is the parent line to be transferred for the present recombinant soybean, was used. In addition to the field test described above, a test for condition of low temperature stress and an antioxidation test were performed in a greenhouse at Monsanto Company (US).

a Morphological and growth characteristics

In order to compare the morphology and the growth characteristics, differences of the morphological characteristics and the growth between the present recombinant soybean and the non-recombinant control soybean were investigated for 20 items (starting of germination, during germination, uniformity of germination, number of germinated plants, germination rate, shape of little leaf, number of trichomes, time of flower initiation, time of flower completion, growing type, maturation period, main stem length, number of culm nodes, tiller number, height of lowest stem node, plant type, weight of above-ground parts at the harvest time, shape of harvested seed (grain color, grain uniformity and grain shape)) according to the seedling characteristic categorized list for the seedling registration. As a result, no significant difference between the present recombinant soybean and the non-recombinant control soybean was found for the items which were statistically analyzed (number of individuals to be germinated, main stem length, number of culm nodes, tiller number, height of lowest

[10]All the rights pertinent to the information described in the following a to h in the present item and the responsibility for the content rest upon Monsanto Japan Limited.

stem node, weight of above-ground parts at the harvest time) (Table 2, p.9 of Attachment 11). In addition, it was perceived that the starting of germination in the present recombinant soybean and the non-recombinant control soybean were different among the items which were not statistically analyzed (starting of germination, during germination, uniformity of germination, germination rate, shape of little leaf, number of trichomes, time of flower initiation, time of flower completion, growing type, maturation period, plant type, shape of harvested seed (grain color, grain uniformity and grain shape)) (Table 2, p.8, and Figures 3 to 5, p.9 and p.10 of Attachment 11). The starting of germination was July 30 for the present recombinant soybean, and August 1 for the non-recombinant control soybean (Table 2, p.8 of Attachment 11).

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

Low temperature resistance test at early stage of growth was conducted on an environmently controlled room of the Monsanto Company (US).

Low temperature stress experiment 1 (Attachment 12, 2006)

The present recombinant soybean, the non-recombinant control soybean A3525 and 4 different conventional commercial varieties were grown at 29°C in daytime/21°C at nighttime in a greenhouse until two-leaf stage were transferred to the environmently controlled room, and cultivated for 20 days at 15°C in daytime/8°C at nighttime to investigate dry weight, fresh weight, growth stage, main stem length and plant vigour[11](Attachment 12). As a result, no statistically significant difference was observed between the present recombinant soybean and the non-recombinant control soybean for dry weight, fresh weight, main stem length and plant vigour, and no difference was observed between the present recombinant soybean and the non-recombinant control soybean were found for growth stage, which were not statistically analyzed (Table 2, p.5 of Attachment 12).

In the seed of the present recombinant soybean, SDA and GLA, which are fatty acids not originally produced in a soybean, are produced due to the expression of the transferred gene. Since the transferred gene is controlled by the embryo-specific promoter, it is considered that SDA and GLA cannot be produced in tissues other

[11]Plant vigour is evaluated as stages 1 to 9, and the lower the number indicates the worse the growth.

than the seed. However, during application of the isolated field test, reviewer pointed out that the soybean plants could have increased cold tolerance in case if SDA and GLA can be produced in the biological membrane, resulting in increased level of unsaturated fatty acid. So, in order to investigate in detail that resistance to low temperatures is not increased in the present recombinant soybean, the growth of the present recombinant soybean at 3 different low temperature conditions were evaluated (Attachment 13).

Low temperature stress experiment 2 (Attachment 13, 2009)

The present recombinant soybean and the non-recombinant control soybean were seeded in a greenhouse, grown at 30°C in daytime/20°C at nighttime until first-leaf stage. Then they were cultivated in the environmently controlled room set at the optimal temperatures[12] (29°C in daytime/24°C at nighttime) and 3 different low temperature conditions (20°C /15°C, 15°C /8°C and 7°C /2°C in daytime/nighttime) to investigate dry weight, fresh weight, growth stage, main stem length and plant vigour[13] after 22 days transferred to the environmently controlled room (after 9 days at 7°C /2°C) (Attachment 13).

As a result, statistically significant differences between the present recombinant soybean and the non-recombinant control soybean were found in the dry weight for 20°C /15°C and 7°C /2°C and the main stem length for 20°C /15°C (Table 4, p30 and Table 3, p. 21 of Attachment 13). Regarding the growth stage which was not statistically analyzed, no differences between the present recombinant soybean and the non-recombinant control soybean were found.

The dry weight was 3.0 g for the present recombinant soybean and 2.6 g for the non-recombinant control soybean at 20°C /15°C, and 0.50 g for the present recombinant soybean and 0.46 g for the non-recombinant control soybean at 7°C /2°C, the present recombinant soybean was heavier (Table 4, p30 and Table 3, p21 of Attachment 13).

The main stem length was 17.5 cm for the present recombinant soybean and 16.2 cm for the non-recombinant control soybean at 20°C /15°C, and the present recombinant soybean was higher (Table 4, p30 and Table 3, p21 of Attachment 13).

[12]Present recombinant soybean at the optimal temperature (29/24 °C) was not evaluated.

[13]Plant vigour is evaluated as stages 1 to 9, and the bigger the number indicates the worse the growth.

In the above low temperature stress test 2, while the difference found in the dry weight and the main stem length was small, it was not able to fully deny the possibility of increased cold tolerence in present recombinant soybean cannot be denied. Therefore, follow-up study was conducted as a low temperature stress test 3.

Low temperature stress test 3 (Attachment 14, 2010)

The present recombinant soybean, the non-recombinant control soybean and 4 conventional commercial varieties were seeded in the greenhouse to grow them at 30°C in daytime/20°C in nighttime until a primary leaf is developed. Then, they were cultivated in an environmently controlled room under optimal conditions (29°C in daytime/24°C at nighttime) and under 3 different low temperature conditions (20°C /15°C, 15°C /8°C and 7°C /2°C in daytime/nighttime) to investigate dry weight, growing step, main stem length and plant vigour[14], followed by statistically analyzing the dry weight, main stem length and plant vigour (Attachment 14). The statistical analysis for plant vigour was subject to the difference in plant vigour before and after the treatment with low temperature.

As a result, it was confirmed under optimal conditions to have a statistically significant difference between the present recombinant soybean and the non-recombinant control soybean in the main stem length and plant vigour (Table 5, p31 and Table 2, p.9 of Attachment 14). The average main stem length under optimal conditions was 35.8 cm for the present recombinant soybean and 30.3 cm for the non-recombinant control soybean, and the present recombinant soybean was higher. However, the average value for the present recombinant soybean was within the range for conventional commercial varieties (31.1 to 104.5 cm). Plant vigour was 1.1 for the present recombinant soybean and 1.0 for the non-recombinant control soybean before the treatment, and 1.0 for both the present recombinant soybean and the non-recombinant control soybean after the treatment.

Under 3 different low temperature conditions, a statistically significant difference was confirmed between the present recombinant soybean and the non-recombinant control soybean in the dry weight at 15°C /8°C and 7°C /2°C and plant vigour at 20°C /15°C and 7°C /2°C among the items which were statistically analyzed (dry weight, main stem length and plant vigour) (Table 5, p.31 and Table 2, p.9 of Attachment 14). In addition, regarding the growth stages which were not

[14] The plant vigour is evaluated as stages 1 to 9, and the bigger the number indicates the worse the growth.

statistically analyzed, no difference between the present recombinant soybean and the non-recombinant control soybean could be confirmed.

The dry weight was 0.68 g for the present recombinant soybean and 0.82 g for the non-recombinant control soybean at 15°C/8°C, and 0.13 g for the present recombinant soybean and 0.15 g for the non-recombinant control soybean at 7°C/2°C, the present recombinant soybean is lower in both tests. However, the average values for the present recombinant soybean under these temperature conditions were within the range for the conventional commercial varieties (0.46 to 1.00 g at 15°C/8°C and 0.12 to 0.17 g at 7°C/2°C) (Table 5, p.31 and Table 2, p.9 of Attachment 14).

Plant vigour was 1.3 for the present recombinant soybean and 1.0 for the non-recombinant control soybean before the treatment at 20°C/15°C, and 3.9 for the present recombinant soybean and 4.1 for the non-recombinant control soybean after the treatment. Plant vigour was 1.2 for the present recombinant soybean and 1.0 for the non-recombinant control soybean before treatment at 7°C/2°C and 9.0 (discharge) for both the present recombinant soybean and the non-recombinant control soybean after the treatment (Table 5, p31 and Table 2, p.9 of Attachment 14). Distribution of the plant vigour in the individual to be provided under each temperature condition was shown in Table 6 (p.36 and Table 3, p.10 of Attachment 14).

Table 4 Growth characteristics of the present recombinant soybean and the non-recombinant control soybean in the low temperature stress test 2 (Results before the treatment and day 22 after the treatment)[15]

Temperature condition	Growth characteristics[2,3]		Average value[1]	
			Present recombinant soybean	Non-recombinant control soybean
29°C/24°C [4] (Optimal temperature conditions)	Dry weight (g)	After treatment	N.A.	4.45
	Fresh weight (g)	After treatment	N.A.	15.42
	Growth stage	After treatment	N.A.	R2- R3
	Main stem length (cm)	After treatment	N.A.	36.8
	Plant vigour	Before treatment	N.A.	1.0
		After treatment	N.A.	2.0
20/15 °C	Dry weight (g)	After treatment	3.0*	2.6
	Fresh weight (g)	After treatment	9.7	8.9
	Growth stage	After treatment	V3-V4	V3-V4
	Main stem length (cm)	Before treatment	9.4	9.4
		After treatment	17.5*	16.2
	Plant vigour	Before treatment	1.0	1.0
		After treatment	3.4	3.6
15/8 °C	Dry weight (g)	After treatment	2.0	2.0
	Fresh weight (g)	After treatment	7.3	6.9
	Growth stage	After treatment	V2-V3	V2-V3
	Main stem length (cm)	Before treatment	9.4	9.4
		After treatment	12.2	11.9
	Plant vigour	Before treatment	1.0	1.0
		After treatment	5.0	5.0
7/2 °C [5]	Dry weight (g)	After treatment [6]	0.5*	0.5
	Fresh weight (g)	After treatment	1.9	1.7
	Growth stage	After treatment	V1 (V1)	V1 (V1)
	Main stem length (cm)	Before treatment	9.4	9.4
		After treatment	9.6 (9.4)	9.3 (9.3)
	Plant vigour	Before treatment	1.0	1.0
		After treatment	8.9 (6.4)	8.9 (6.6)

Note) The main stem length and the plant vigour before the treatment are not compared by statistically analyzing those obtained from the present recombinant soybean and the non-recombinant control soybean. The main stem length after the treatment was statistically analyzed using ANOVA with the main stem length before the treatment as a covariate in the statistical analysis. The dry weight and fresh weight after the treatment was analyzed using ANOVA without using the covariant for the statistical analysis, since they do not have any correlation with the main stem length before the treatment. The plant vigour was statistically analyzed using ANOVA for a change of values before and after the treatment.

*Statistically significant differences those from the present recombinant soybean and the non-recombinant control soybean were confirmed ($p<0.05$).

[1] n=20

[2] Growing step: Vn=main leaf n leaves stage, R2=time of flower initiation, R3= early stage of node growing

[3] Plant vigour was evaluated as stages 1 to 9. 1 indicates that the growth is good, the higher the number the worse the growth, and 9 indicates discharge.

[4] The present recombinant soybean under the optimal condition (29/24 °C) was not evaluated.

[5] Regarding the growth stage, main stem length and plant vigour at 7°C /2°C, values in parentheses ares data at 9 days after the treatment.

[6] While the average value of the dry weight at 7°C /2°C was 0.50 g for the present recombinant soybean and 0.46 g for the non-recombinant control soybean, both values are shown as 0.5 g in the Table.

[15]All the rights pertinent to the information in the present table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 5 Growth characteristics of the present recombinant soybean, the non-recombinant control soybean and the conventional commercial product in the low temperature stress test 3 (Results before the treatment and day 21 after the treatment) [16]

Temperature condition	Growth characteristics [3,4]		Average value (SE)[1]		Range of the conventional commercial product [2]	
			Present recombinant soybean	Non-recombinant control soybean	Minimum value	Maximum value
29/24 °C (Optimal temperature condition)	Dry weight (g)	After treatment	4.26 (0.17)	4.31 (0.13)	3.66	5.08
	Growing step	After treatment	R1	R1	V7	R3
	Main stem length (cm)	Before treatment	7.9 (0.13)	7.5 (0.15)	7.0	7.6
		After treatment	35.8 (0.50)*	30.3 (0.93)	31.1	104.5
	Plant vigour *	Before treatment	1.1 (0.07)	1.0 (0.00)	1.0	1.0
		After treatment	1.0 (0.00)	1.0 (0.00)	1.0	1.0
20/15 °C	Dry weight (g)	After treatment	2.21 (0.12)	2.45 (0.11)	2.25	2.85
	Growing step	After treatment	V3-V5	V3-V5	V3	V5
	Main stem length (cm)	Before treatment	7.7 (0.17)	7.6 (0.14)	7.0	7.3
		After treatment	16.3 (0.33)	15.6 (0.37)	13.9	29.4
	Plant vigour *	Before treatment	1.3 (0.10)	1.0 (0.00)	1.0	1.0
		After treatment	3.9 (0.08)	4.1 (0.09)	3.2	5.0
15/8 °C	Dry weight (g)	After treatment	0.68 (0.03)*	0.82 (0.04)	0.46	1.00
	Growing step	After treatment	V1	V1	V1	V1
	Main stem length (cm)	Before treatment	7.8 (0.14)	7.5 (0.15)	6.2	8.0
		After treatment	10.1(0.14)	10.0(0.15)	8.5	11.0
	Plant vigour	Before treatment	1.3 (0.12)	1.0 (0.00)	1.0	1.0
		After treatment	6.0(0.05)	6.1(0.09)	5.2	7.5
7/2 °C	Dry weight (g)	After treatment	0.13 (0.01)*	0.15 (0.00)	0.12	0.17
	Growing step	After treatment	VC	VC	VC	VC
	Main stem length (cm)	Before treatment	7.7 (0.16)	7.4 (0.13)	6.8	7.9
		After treatment	7.9 (0.19)	7.9 (0.20)	6.1	7.2
	Plant vigour *	Before treatment	1.2 (0.08)	1.0 (0.00)	1.0	1.0
		After treatment	9.0 (dead)	9.0 (dead)	9.0 (dead)	9.0 (dead)

Note) The main stem length and the plant vigour before the treatment are not compared for those of the present recombinant soybean and the non-recombinant control soybean by the statistical analysis. The dry weight at 15°C /8°C and 7°C /2°C after the treatment and the main stem length after the treatment were statistically analyzed using ANOVA, with the main stem length before the treatment as covariants in the statistical analysis. The dry weight at 29°C /24°C and 20°C /15°C after the treatment was statistically analyzed using ANOVA without using any covariants in the statistical analysis, since the dry weight does not have any correlation with the main stem length before the treatment. The plant vigour was statistically analyzed for a change in values before the treatment and after the treatment using ANOVA.
*Statistically significant difference between the present recombinant soybean and the non-recombinant control soybean was confirmed ($p<0.05$).
[1] n=20, SE: Standard error
[2] Minimum value and maximum value of average value for 4 Conventional commercial varieties (AG0604, AG00501, ANAND and Schillinger 235.T)
[3] Growth stage: VC=time of developing Primary leaf, Vn=main leaf n leaves stage, R1=time of flower initiation, R3=early stage of node growing
[4] Plant vigour was evaluated as stages 1 to 9. 1 indicates that the growth is good, the higher the number the worse the growth, and 9 indicates discharge.

[16] All the rights pertinent to the information in the present table above and the responsibility for the content rest upon Monsanto Japan Limited.

Table 6 The plant vigour of each individual to be provided of the present recombinant soybean and the non-recombinant control soybean in the low temperature stress test 3 (Result before the treatment and day 21 after the treatment)[17]

Temperature condition	Soybean to be provided (Time for evaluation)		Plant vigour (number of individuals at evaluation items)[1]								
			1	2	3	4	5	6	7	8	9
29°C /24°C (Optimal temperature condition)	Present recombinant soybean	Before treatment	18	2	—	—	—	—	—	—	—
	Non-recombinant control soybean	After treatment	20	—	—	—	—	—	—	—	—
	Present recombinant soybean	Before treatment	20	—	—	—	—	—	—	—	—
	Non-recombinant control soybean	After treatment	20	—	—	—	—	—	—	—	—
20°C /15°C	Present recombinant soybean	Before treatment	15	5	—	—	—	—	—	—	—
	Non-recombinant control soybean	After treatment	20	—	—	—	—	—	—	—	—
	Present recombinant soybean	Before treatment	—	—	3	17	—	—	—	—	—
	Non-recombinant control soybean	After treatment	—	—	1	17	2	—	—	—	—
15°C /8°C	Present recombinant soybean	Before treatment	16	3	1	—	—	—	—	—	—
	Non-recombinant control soybean	After treatment	20	—	—	—	—	—	—	—	—
	Present recombinant soybean	Before treatment	—	—	—	—	1	19	—	—	—
	Non-recombinant control soybean	After treatment	—	—	—	—	1	17	2	—	—
7°C /2°C	Present recombinant soybean	Before treatment	17	3	—	—	—	—	—	—	—
	Non-recombinant control soybean	After treatment	20	—	—	—	—	—	—	—	—
	Present recombinant soybean	Before treatment	—	—	—	—	—	—	—	—	20
	Non-recombinant control soybean	After treatment	—	—	—	—	—	—	—	—	20

[1] Plant vigour was evaluated as stages 1 to 9. 1 indicates that the growth is good, the higher the number the worse the growth, and 9 indicates discharge. The values described in columns in each evaluation item indicate number of individuals to be provided corresponding to the evaluation item.

[17]All the rights pertinent to the information in the present table above and the responsibility for the content rest upon Monsanto Japan Limited.

c Wintering ability and summer survival of the mature plant

The present recombinant soybean and the non-recombinant control soybean which were grown in the isolated farm field were continuously grown after the maturation period to observe the growing status in the winder season in Japan. While the growing status in the winter season is confirmed at January 20, both of the present recombinant soybean and the non-recombinant control soybean were fired (Figure 6, p.11 of Annex 11.

d Fertility and pollen size

Pollen collected from the present recombinant soybean and the non-recombinant control soybean were stained with potassium iodide solution to compare fertility and pollen size. As a result, both the present recombinant soybean and the non-recombinant control soybean indicated high fertility of pollen, and a great difference in fertility could not be confirmed. In addition, a difference in the morphology and size of the pollen could not be confirmed (Figure 7, p.12 of Attachment 11).

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

Regarding the present recombinant soybean and the non-recombinant control soybean which were cultivated under the same conditions, items relating to the produced amount of seed (number of ripened pods, weight of crude seed per single stock, weight of precise seed per single stock, and weight of 100 seeds) were investigated. As a result in which these items were statistically analyzed, a significant difference in the weight of 100 seeds was confirmed (Table 3, p.15 of Attachment 11). The average value of the weight of 100 seeds was 20.33 g for the present recombinant soybean and 21.58 g for the non-recombinant control soybean, and those of the present recombinant soybean were lower.

Regarding the shedding habit, the present recombinant soybean and the non-recombinant control soybean were harvested at the mature stage to observe the degree of dehiscence after the plant was naturally dried in a vinyl house. As a result, both the present recombinant soybean and the non-recombinant control soybean had difficulty in pod bursting, and a difference in the pod bursting of the seed was not seen (Table 3, p.15 of Attachment 11).

Regarding the dormancy and germination rate, the seed just after harvesting was

layered on a petri dish, and incubated at 25°C to investigate number of germinated seeds in time dependent manner. As a result, while some variations of germination vigour were observed, high germination rate was indicated in both the present recombinant soybean and the non-recombinant control soybean, and a statistically significant difference was not confirmed in the final number of germinated individuals (Table 3, p.15 of Attachment 11).

f Crossability

In order to investigate the crossability of the present recombinant soybean, frequency of hybrid plants in the harvested seed of the non-recombinant control soybean with the present recombinant soybean as a pollen parent was investigated. Regarding the determination of the hybrid plant, the transferred gene of the present recombinant soybean which is the pollen parent was used as an index.

Seeds were bulk harvested from the non-recombinant control soybean which was cultivated at a site for investigating morphology and growing characteristics. The individual from which the seed was harvested was cultivated at 2 inner furrows in the plot with a clearance of 1 m provided from the plot for this recombinant soybean adjacent to the non-recombinant soybean plot (Figure 2, p.5 of Attachment 11). PCR determining whether or not the transferred gene is present was conducted in each seed for 499 seeds which were randomly selected from the harvested seeds. Taqman-PCR method, which can detect the transferred gene and an endogenous gene region corresponding to a portion to be transferred, was applied for the above-mentioned PCR.

491 seeds were analyzed among the 499 seeds subjected to PCR. 8 seeds could not be analyzed, as a sufficient amount of DNA could not be extracted from the 8 seeds. For the rest 491 seeds available to analysis, no transferred genes were detected (Table 5, p.16 of Attachment 11) and therefore, it was considered that crossability could not be observed in this investigation. Therefore, it was considered that the crossing rate in this investigation did not go beyond the previously reported natural crossing rate between the soybean products (0.03 to 6.32%) (Woodworth, 1922; Garber and Odland, 1926; Cutler, 1934; Weber and Hanson, 1961; Caviness, 1966; Beard and Knowles, 1971; Ahrent and Caviness, 1994; Abud et al., 2003; Ray et al., 2003).

g Productivity of harmful substances

Soil microflora test, plow-in test and succeeding crop test were conducted in order

to confirm that substances which provide an effect on the soil microflora or other plant are not produced from the present recombinant soybean. As a result, no statistical significant difference was confirmed in the number of soil microflora, number of germinated line of radioxenon and dry weight of the present recombinant soybean and the non-recombinant control soybean, and no difference was found in the germination rate which was not statistically analyzed (Tables 6 to 8, p.18 of Attachment 11).

h Antioxidative property

In the seed of the present recombinant soybean, SDA and GLA, fatty acids which cannot be produced by nature by soybean, are produced due to the transferred genes of the modified Δ6 desaturase and the modified Δ15 desaturase. Since these transferred genes are controlled by the embryo-specific promoter, it is considered that SDA and GLA are not produced in tissues other than the seed. However, during application of the isolated field test, reviewer pointed out that the soybean plants could have increased cold tolerance in case if SDA and GLA can be produced in the biological membrane, resulting in increased level of unsaturated fatty acid. So, in order to confirm whether or not the antioxidative properties of the present recombinant soybean is increased, 3 separated concentrations (30, 70 and 200 g active ingredient (a.i.)/ha) of herbicide paraquat, which has been known to induce oxidative stress, was sprayed on an embryo plant in an environmently controlled room in Monsanto Company (US) (Attachment 15). As a result, although damage to the leaf was observed at day 3 and day 7 after spraying for each group, no statistical significant difference between the present recombinant soybean and the non-recombinant control soybean was found in any sprayed concentration (Table 2, p.17 of Attachment 15).

In this test, the present recombinant soybean and the non-recombinant control soybean were seeded in the greenhouse, the seeds were grown at 30°C in daytime/20°C at nighttime until 3 leaf-stage or 4 leaf-stage, then sprayed with paraquat

II. Results of the review by persons with specialized knowledge and experience concerning Adverse Effects 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) Results of the assessment of Adverse Effects on Biological Diversity

The present recombinant soybean is produced by transferring PV-GMPQ1972 based on the plasmid pBR322 derived from E. coli, and the like using the *Agrobacterium* method.

The present recombinant soybean is introduced with one copy on the chromosome with T-DNA I region comprising the modified *Pj.D6D* gene coding for the Δ6 desaturase derived from primrose and the modified *Nc.Fad3* gene coding for the Δ15 desaturase derived from neurospora, and it is confirmed by separation mode of genes and using the Southern blotting analysis that these genes are stably transmitted over several generations. In addition, it is confirmed using the Western blotting analysis that these genes stably expressed over several generations.

a. Competitiveness

It has not been reported that soybean grows naturally while the soybean, which is a biological species of which the recipient organism belongs, is cultivated over a long-term in Japan.

In the isolated farm field in Japan and the greenhouse in US, characteristics associated with competitiveness were compared between the non-recombinant control soybean. As a result, the statistical significant difference between the present recombinant soybean and the non-recombinant control soybean was obaserved for the weight of 100 seeds in the producion of the seed and for the dry weight, main stem length and plant vigour in the low temperature stress test. In addition, regarding the items not subjected to statistical analysis, the difference was observed between the present recombinant soybean and the non-recombinant control soybean for the beginning of germination.

The weight of 100 seeds was 20.33 g for the present recombinant soybean, and 21.58 g for the non-recombinant control soybean, showing smaller weight of 100 seeds in the present recombinant soybean. However, the average value of the weight of 100 seeds for the present recombinant soybean was within the previously published range of weight of 100 seeds for conventional soybean varieties.

While a significant difference in a portion of the items was confirmed in the low temperature stress test, these differences in the relevant items were not inconsistent.

The beginning of germination was July 30 for the present recombinant soybean, and August 1 for the non-recombinant control soybean, showing only a slight differences between the both plants. In addition, no significant difference between the present recombinant soybean and the non-recombinant control soybean was confirmed for the number of germinated individuals and the number of germinated individuals of the harvested seed.

Based on the study result described above,, it was considered that the observed differences would not increase the competitivess of the present recombinant soybean.

In the present recombinant soybean, Stealidonic acid (SDA) and γ-linoleic acid (GLA) which cannot be produced by nature by a soybean, are produced due to expression of transferred modified *Pj.D6D* gene and the modified *Nc.Fad3* gene driven by the embryo-specific promoter, and the fatty acids are accumulated in the seed.

Typically it is known that fatty acids produced in soybean seeds are stored as an energy source in a soybean seed, and it is mainly utilized for germination, it was considered that SDA and GLA in the present recombinant soybean has the same role. SDA and GLA contents stored in the seed of the present recombinant soybean were actually investigated overtime. As a result, it was suggested that the levels of SDA and GLA contents decrease with germination and the fatty acids are utilized for energy metabolism, similar to those of endogenous fatty acids in a soybean. In addition, from the result of isolated field tests, it was concluded that thre is no difference between the present recombinant soybean and the non-recombinant control soybean for germination characteristics.

Consequently, it was determined that SDA and GLA produced in the seed of the present recombinant soybean would play similar biological role as the endogenous fatty acids. Therefore, it was concluded that SDA and GLA produced in the present recombinant soybean would not increase the competitiveness of the present recombinant soybean.

As mentioned above, it was determined that the conclusion made by the applicant that wild animals, wild plants and the like, on which there are possible effects, cannot be specified and possible effects of biological diversity are not produced due to superiority in the competition, is reasonable.

b. Productivity of harmful substances

It has not been reported that a soybean, of the biological species to which the recipient belongs, produce harmful substances against wild animals and wild plants.

While the present recombinant soybean expresses the modified Δ6 desaturase and the modified Δ15 desaturase, it has not been reported that these proteins are harmful

substances, and it has been confirmed that the proteins do not have a structurally similar sequence with known allergens. In addition, since the modified Δ6 desaturase and the modified Δ15 desaturase have high substrate specificity, the possibility was considered to be extremely low that these desaturases affect any other metabolic systems of the recipient organism and produce any new harmful substances. Furthermore, there has been no report to date that fatty acid like SDA and GLA which are new to soybean are harmful substances.

It was considered likely that SDA and GLA contained in the seed of the present recombinant soybean might affect wild biological organisms if the present recombinant soybean were fed by wildlife that damages soybean seeds by feeding. However, when the present recombinant soybean is fed by the wildlife, SDA and GLA contained in the seed of the present recombinant soybean are considered to be metabolized in the wildlife. Therefore it is considered unlikely that fatty acids like SDA and GLA contained in the seeds of this recombinant soybean could affet on wildlife.

In the isolated fields in Japan, the present recombinant soybean was investigated for productivity of harmful substances (which secrete from the roots to affect other plants and soil microflora, and which are contained in plant bodies and affect other plants after discharge) based on the soil microflora test, plow-in test and succeeding crop test in the isolated farm field in Japan, no difference between the present recombinant soybean and the non-recombinant control soybean were found.

Based on above, it was determined that the conclusion made by the applicant that wild animals and wild plants which are possibly affected are not specified and that no possibility of affecting on the biological diversity because of whether or not any harmful substances can be produced is found, is reasonable.

c. Crossability

It is known that Glycine soja is a related species, and both organisms have 2n=40 in chromosome number and can be crossed. So, the following items are examined upon specifying Glycine soja as a wild plant which is possibly affected by the present recombinant soybean.

Since there is no particular failure for growing a crossbreed of which the soybean is artificially crossed with Glycine soja, when the present recombinant soybean is crossed with Glycine soja in the natural environment in Japan, there is a possibility that its crossbreed is grown and the crossbreed is back crossed to Glycine soja, then the transferred genes to the present recombinant soybean is spread to a population of Glycine soja, not only with a low ratio, but also with a large ratio. In addition, since Glycine soja is distributed throughout Japan and grows naturally on riversides, banks, surrounding farms, orchards and the like, there is a possibility to crossbreed when the present recombinant soybean is grown in proximity thereto.

However, in addition to:

① regarding the formation of crossbreed between the soybean and Glycine soja and the gene penetration, the population of Glycine soja is subjected to the follow-up investigation around soybean fields in Japan to analyze whether or not any crossbreeds are produced using gene markers and the like. As a result, there is a report suggesting the presence of crossbreed progenies was not obtained;

② it is generally known that the soybean and Glycine soja hardly overlap in time of flower initiation, and that the crossing rate is only 0.73% even when both of the times of flower initiation are artificially arranged to be coincident with each other and closely cultivated alternatively with a distance of 50 cm between both lines; and

③ in the crossing test during each time of flower initiation for the recombinant soybean resistant to the herbicide glyphosate, 40-3-2 line and Glycine soja was arranged to be coincident, and both were closely cultivated to grow so as that Glycine soja was wound with the soybean, one seed among 32,502 seeds of Glycine soja crossed with the soybean,

when the present recombinant soybean and the non-recombinant control soybean were cultivated at neighboring test sites of the isolated farm field in Japan to investigate the natural crossing with the soybean without any recombination, no crossing was observed. In addition, while the traits relating to the reproduction (fertility of pollen, morphology of pollen, producibility of seed) were investigated, the characteristics of the present recombinant soybean did not go beyond the range of those with the species, and it was speculated that the crossability between the present recombinant soybean and the non-recombinant control soybean is extremely low, similar to the relationship between conventional soybean products and Glycine soja.

Further, since it is considered, in the present recombinant soybean, that there are no effects by the transferred gene changes the metabolic system of which the recipient organism has to differ from the recipient organism for the physiological or ecological characteristics relating to the crossability, it is considered that the crossing rate between the present recombinant soybean and Glycine soja is low, similar to the crossing rate between conventional soybean and Glycine soja.

As mentioned above, it was decided that the conclusion made by the applicant that there is no possibility of Adverse Effects on Biological Diversity that is attributable to crossability, is reasonable.

(2) Conclusion based on the Biological Diversity Risk Assessment

Based on the above understanding, the conclusion described in the Biological Diversity Risk Assessment Report that use of the present recombinant soybean in

accordance with the type 1 Use Regulation causes no Adverse Effects on Biological Diversity in Japan has been judged to be reasonable.

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List of Attachments for Stearidonic acid producing soybean (modified *Pj.D6D*, modified *Nc.Fad3*, *Glycine max* (L.) Merr.)
(MON87769, OECD UI: MON-87769-7)

Attachment 1 Amino acid sequences of modified Δ6 desaturase and modified Δ15 desaturase deduced from modified *Pj.D6D* gene and modified *Nc.Fad3* gene used for producing present recombinant soybean (Confidential)

Attachment 2 Functional Characterization of NcD15 and PjD6 Desaturases (PAR-07-309)(Confidential)

Attachment 3 Fatty Acid Analyses of Germinating Cotyledons of Stearidonic Acid-Containing Soybean MON 87769 (MSL0021821) (Confidential)

Attachment 4 Sequence of the Genetic Elements in Plasmid Vector PV-GMPQ1972(Confidential)

Attachment 5 Summary of PCR analysis to confirm the absence of *Agrobacterium* containing PV-GMPQ1972(Confidential)

Attachment 6 Heritability and Stability of Genes Present in MON 87769 from an F_2 to F_4 Generation(RPN-08-177) (Confidential)

Attachment 7 Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769 (MSL0021926) (Confidential)

Attachment 8 Assessment of Delta 6 and Delta 15 Desaturase Protein Levels in Tissues from MON 87769 Soybeans in Support of a Japan Stage III Application (MSL 0020845) (Confidential)

Attachment 9 Western Blot Analysis of PjD6D and NcD15D Proteins in Immature Seed of Soybean MON 87769 across Multiple Generations(MSL0021711) (Confidential)

Attachment 10 SDA Soybean GM_A38136 Zygosity EndPoint TaqMan® PCR for Single Seeds(Confidential)

Attachment 11 Report of Adverse Effect of Biological Diversity test in isolated farm field of stearidonic acid producing soybean (modified *Pj.D6D*, modified *Nc.Fad3*, *Glycine max* (L.) Merr.)(MON87769, OECD UI: MON-87769-7) (Confidential)

Attachment 12 Assessment of the Effect of Sub-Optimal Temperature on SDA Soybean MON 87769 under Growth Chamber Conditions in 2006(Study # 06-01-83-21) (Confidential)

Attachment 13 Assessment of the Effect of Cold Stress of SDA Soybean MON 87769 Under Growth Chamber Conditions(MSL0023334) (Confidential)

Attachment 14 Assessment of Cold Temperature Stress on Growth of SDA Soybean MON 87769 Under Growth Chamber Conditions in 2010 (PLC-2010-0639) (Confidential)

Attachment 15 Evaluation of the Photo-Oxidative Stress Tolerance of Soybean MON 87769 when Treated with Varying Concentrations of a Commercial Formulation of Paraquat Herbicide(MSL0021835)(Confidential)