

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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Soybean resistant to <i>Lepidoptera</i> pests and tolerant to glyphosate herbicide (Modified <i>cryIAc</i> , modified <i>cp4 epsps</i> , <i>Glycine max</i> (L.) Merr.) (MON87701 × MON89788, OECD UI: MON-877Ø1-2 × MON-89788-1)
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	The applicant performs the monitoring based on the monitoring plan specified separately.

Outline of the Biological Diversity Risk Assessment Report

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

5 1 Information concerning preparation of living modified organisms

10 Soybean resistant to *Lepidoptera* pests and tolerant to glyphosate herbicide (Modified *cryIAc*, modified *cp4 epsps*, *Glycine max* (L.) Merr.) (MON87701 × MON89788, OECD UI: MON-87701-2 × MON-89788-1) (hereinafter referred to as “this stacked soybean line”) is the stacked line cultivated with the following two modified soybean lines using the conventional crossing.

- 15 a) *Lepidopteran* insect-protected soybean (Modified *cryIAc*, *Glycine max* (L.) Merr.) (MON87701, OECD UI: MON-87701-2) (hereinafter referred to as “MON87701”).
- 15 b) Soybean tolerant to glyphosate herbicide (Modified *cp4 epsps*, *Glycine max* (L.) Merr.) (MON89788, OECD UI: MON-89788-1) (hereinafter referred to as “MON89788”).

20 (1) Information concerning donor nucleic acid

1) Composition and origins of component elements

25 The composition of donor nucleic acids and the origins of component elements used for the development of MON87701 and MON89788 are shown in Tables 1 and 2 (p. 3-7).

2) Function of component elements

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

35 The functions of the component elements of donor nucleic acids used for the development of MON87701 and MON89788 are shown in Tables 1 and 2 (p. 3-7). Of those component elements, the target genes – the modified *cryIAc* and modified *cp4 epsps* genes – are also detailed in Tables 1 and 2 (p. 3-7).

Table 1. Component elements of the donor nucleic acids, and their origins and functions used for the development of MON87701¹

Component elements	Origin and function
T-DNA II (It is not present in MON87701. It is continued from the 15,532 position in the plasmid.)	
Intervening Sequence	Sequence used in DNA cloning.
L¹-ShkG	5'-terminal untranslated region of the <i>ShkG</i> gene of <i>Arabidopsis thaliana</i> (thale cress) coding for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein (Klee et al., 1987; Herrmann, 1995). It is associated with the regulation of gene expression.
TS²-CTP2	Sequence coding for chloroplast transit peptides derived from the <i>ShkG</i> gene coding for the EPSPS protein of <i>A. thaliana</i> (Klee et al., 1987; Herrmann, 1995). It transports the target protein from the cytoplasm to the chloroplasts.
CS³-modified cp4-epsps	Coding sequence of the <i>aroA</i> gene coding for the 5-enolpyruvylshikimate-3-phosphate synthase derived from the <i>Agrobacterium</i> sp. CP4 strain (CP4 EPSP) (Padgett et al., 1996; Barry et al., 2001). In the amino acid sequence of the expressed protein, the serine at the second position from the N-terminal sequence is changed to leucine, compared with the amino acid sequence derived from the <i>Agrobacterium</i> sp. CP4 strain.
Intervening Sequence	Sequence used in DNA cloning.
T⁴-E9	3'-terminal untranslated region of the <i>RbcS2</i> gene coding for the ribulose-1,5-bisphosphate carboxylase small subunit of <i>Pisum sativum</i> (garden pea). It induces polyadenylation of mRNA (Coruzzi et al., 1984).
Intervening Sequence	Sequence used in DNA cloning.
B⁵-Left Border	DNA region derived from <i>Agrobacterium tumefaciens</i> . It contains the left border sequence used for the T-DNA transfer process (Barker et al., 1983).
Vector backbone region (absent in MON87701)	
Intervening Sequence	Sequence used in DNA cloning.
OR⁶-ori V	Origin of replication origin region derived from the broad-host-range plasmid RK2. It allows autonomous replication of vectors in <i>Agrobacterium</i> (Stalker et al., 1981).
Intervening Sequence	Sequence used in DNA cloning.

5 Table 1. Component elements of the donor nucleic acids, and their origins and functions used for

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the development of MON87701 (continued)

Component elements	Origin and function
T-DNA I	
B-Right Border	DNA region derived from <i>A. tumefaciens</i> containing the right border sequence, which is used for the T-DNA transfer process (Zambryski et al., 1982; Depicker et al., 1982).
Intervening Sequence	Sequence used in DNA cloning.
P⁷-RbcS4	Promoter, leader, and 5'-terminal untranslated region of the <i>RbcS4</i> gene coding for the ribulose-1,5-bisphosphate carboxylase small subunit 1A of <i>A. thaliana</i> (Krebbers et al., 1988). It induces expression in the terrestrial part of the plant body.
TS-CTP1	Sequence coding for the transit peptide derived from the <i>RbcS4</i> gene of <i>A. thaliana</i> (Krebbers et al., 1988). It transfers the modified Cry1Ac protein to the chloroplasts.
CS-modified cryIAc	Sequence coding for the modified Cry1Ac protein derived from <i>B. thuringiensis</i> (Fischhoff and Perlak, 1996). Seven amino acids of the modified Cry1Ac protein are different compared with the wild-type Cry1Ac protein generated from <i>B. thuringiensis</i> ssp. <i>kurstaki</i> HD-73 strain.
Intervening Sequence	Sequence used in DNA cloning.
T-7S α'	3'-terminal untranslated region of the <i>Sphas1</i> gene coding for the soybean 7Sα' seed storage protein of <i>G. max</i> . It terminates mRNA transcription and induces polyadenylation (Schuler et al., 1982).
Intervening Sequence	Sequence used in DNA cloning.
B-Left Border	DNA region derived from <i>A. tumefaciens</i> containing the left border sequence, which is used for the T-DNA transfer process (Barker et al., 1983).

Table 1. Component elements of the donor nucleic acids, and their origins and functions used for the development of MON87701 (continued)

Component elements	Origin and function
Vector backbone region (absent in MON87701)	
Intervening Sequence	Sequence used in DNA cloning.
CS-rop	Coding sequence of the repressor of primer protein derived from the ColE1 plasmid. It maintains the number of copies of plasmid in <i>E. coli</i> (Giza and Huang, 1989).
Intervening Sequence	Sequence used in DNA cloning.
OR-ori-pBR322	Origin of Replication separated from the pBR32. It allows autonomous replication of vectors in <i>E. coli</i> (Sutcliffe, 1979).
Intervening Sequence	Sequence used in DNA cloning.
CS-aadA	Bacterial promoter, coding sequence, and 3' untranslated region derived from the 3''(9)- <i>O</i> -nucleotidyltransferase, the aminoglycoside modified enzyme of transposon Tn 7 (Fling et al., 1985)(GenBank accession X03043). It confers resistance to spectinomycin and streptomycin.
Intervening Sequence	Sequence used in DNA cloning.
T-DNA II (absent in MON87701. Continued to the head of the Table.)	
B-Right Border	DNA region derived from <i>A. tumefaciens</i> containing the right border sequence, which is used for T-DNA transfer (Zambryski et al., 1982; Depicker et al., 1982).
Intervening Sequence	Sequence used in DNA cloning.
P-FMV	Promoter of the FMV 35S RNA (Rogers, 2000). It induces transcription in plant cells.
Intervening Sequence	Sequence used in DNA cloning.

L¹: Leader (Leader Sequence)

5 **TS**²: Targeting Sequence (Targeting Sequence)

CS³: Coding Sequence (Coding Sequence)

T⁴: Transcription Termination Sequence (Transcription Termination Sequence)

B⁵: Border (Border Sequence)

OR⁶: Origin of Replication (Replication initiation region)

10 **P**⁷: Promoter (Promoter)

Table 2. Component elements of the donor nucleic acids, and their origins and functions used for the development of MON89788³

Component elements	Origin and function
T-DNA Region	
B ¹ -Right Border	DNA region derived from <i>Agrobacterium tumefaciens</i> containing the right border sequence, which is used as the initiation point of T-DNA transfer (Depicker et al., 1982).
P ² -FMV/ <i>Tsf1</i>	Chimera promoter combining the <i>Tsf1</i> promoter of thale cress (Axelos et al., 1989) and the enhancer sequence of Figwort Mosaic Virus (FMV) 35S promoter (Richins et al., 1987). It involves constitutive expression of the target genes in all tissues.
L ³ - <i>Tsf1</i>	Leader sequence of the <i>Tsf1</i> gene coding for the elongation factor of thale cress, EF-1 alpha (exon 1) (Axelos et al., 1989). It is a ribosome binding site in translation.
I ⁴ - <i>Tsf1</i>	Intron sequence of the <i>Tsf1</i> gene coding for the elongation factor of thale cress, EF-1 alpha (Axelos et al., 1989). It enhances expression of the target genes.
TS ⁵ - <i>CTP2</i>	Sequence coding for chloroplast transit peptide derived from the <i>shkG</i> gene of thale cress EPSPS (Klee et al., 1987). It transports the modified CP4 EPSPS protein to plastids, where aromatic amino acids are synthesized.
CS ⁶ -modified <i>cp4 epsps</i>	Coding sequence of the <i>aroA (epsps)</i> gene coding for the 5-enolpyruvylshikimate-3-phosphate synthase derived from the <i>Agrobacterium</i> CP4 strain (CP4 EPSPS) (Padgett et al., 1996; Barry et al., 1997). In order to enhance the level of expression in plants, the nucleotide sequence is modified not to change the functional activity of the CP4 EPSPS protein. As for the amino acid sequence, the serine at the second position from the N-terminal sequence is changed to leucine.
T ⁷ - <i>E9</i>	3'-terminal untranslated region of the ribulose-1,5-bisphosphate carboxylase small subunit (<i>RbcS2</i>) E9 gene of garden pea (<i>Pisum sativum</i>) (Coruzzi et al., 1984). It terminates transcription of mRNA and induces polyadenylation.
B-Left Border	DNA region derived from <i>A. tumefaciens</i> containing the left border sequence, which is used as a termination point of T-DNA. (Barker et al., 1983).

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³ All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited

Table 2. Component elements of the donor nucleic acids, and their origins and functions used for the development of MON89788 (continued)

Component elements	Origin and function
Vector component elements of T-DNA (absent in MON89788)	
OR⁸-ori V	Origin of replication origin region of <i>Agrobacterium</i> derived from the broad-host-range plasmid RK2. It allows autonomous replication of vectors in <i>A. tumefaciens</i> (Stalker et al., 1981).
CS-rop	Coding sequence of the repressor of primer protein. It maintains the number of copies of plasmid in <i>Escherichia coli</i> (Giza and Huang, 1989).
OR-ori-PBR322	Origin of Replication separated from the pBR32. It allows autonomous replication of vectors in <i>E. coli</i> (Sutcliffe, 1978).
aadA	Bacterial promoter and coding sequence of the 3'-(9)- <i>O</i> -nucleotidyltransferase, the aminoglycoside modified enzyme derived from transposon Tn 7 (Fling et al., 1985). It confers resistance to spectinomycin and streptomycin.

5 ¹B - Border (Border Sequence)

²P - Promoter (Promoter)

³L - Leader (Leader Sequence)

⁴I - Intron (Intron)

⁵TS - Targeting Sequence (Targeting Sequence)

10 ⁶CS - Coding Sequence (Coding Sequence)

⁷T - Transcription Termination Sequence (Transcription Termination Sequence)

⁸OR - Origin of Replication (Replication initiation region)

(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

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【Modified Cry1Ac protein】

10 In MON87701, resistance to certain *Lepidoptera* pests is conferred by expression of the modified Cry1Ac protein coding for the modified *cry1Ac* derived from *Bacillus thuringiensis* ssp. *Kurstaki*.

15 MON87701 has been cultivated in order to reduce or eliminate the use of pesticides currently used for *Lepidoptera* pest control mainly in some regions of South America classified as tropical and subtropic regions, which suffer serious damage by *Lepidoptera* pests. In fact, this recombinant soybean has been observed to exhibit insecticidal activity against major *Lepidoptera* pests in soybean cultivation in South America, such as velvetbean caterpillar (Biroud mame kemushi) (*Anticarsia gemmatalis*), soybean looper (*Pseudoplusia includes*), soybean axil borer (*Epinotia aporema*), and sunflower looper (*Rachiplusia nu*) (Monsanto Company, 2008b; MacRae and Kabuye, 2002; MacRae, 2011b; MacRae, 2011a). In addition, it was identified from the literature review that the Cry1Ac protein, despite variants and origins, does not have insecticidal activity against insect species other than *Lepidoptera* insects.

25

Based on the above, it was confirmed that the modified Cry1Ac protein exhibits selective insecticidal activity against only *Lepidoptera* pests, and does not against other insects.

30 In the modified Cry1Ac protein, seven amino acids are replaced, compared with the wild-type Cry1Ac protein. In addition, four amino acids derived from CTP1 are added to the N-terminal region (Monsanto Japan Limited, 2008).

【Modified CP4 EPSPS protein】

35 The plants treated with glyphosate herbicide wither and die because

5-enolpyruvylshikimate 3-phosphate synthetase (enzyme number: E.C.2.5.1.19, hereinafter referred to as “EPSPS protein”) is inhibited and then the aromatic amino acids essential to protein synthesis cannot be synthesized. The modified *cp4 epsps* gene, the target gene of MON89788, expresses the modified CP4 EPSPS protein with high tolerance to glyphosate herbicide. The activity of the modified CP4 EPSPS protein is not inhibited even under the presence of glyphosate herbicide, and therefore, the recombinant plants expressing this protein can grow by the normal synthesis of shikimic acid.

10 Moreover, for the modified *cp4 epsps* gene, the nucleotide sequence of the wild-type *cp4 epsps* gene is modified in order not to change the functional activity of the wild-type CP4 EPSPS protein to improve the level of expression in plants. As for the amino acid sequence of the expressed protein, only serine at the second position from the N-terminal is substituted by leucine.

15

It was examined whether or not the modified Cry1Ac and modified CP4 EPSPS proteins expressed in parent lines shared similar amino acid sequences to those of known allergens by a FASTA-type algorithm, using AD_2010²; the results showed no similarity of sequences with those of known allergens.

20

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

【Modified Cry1Ac protein】

25 The modified Cry1Ac protein is a crystal insecticidal protein (Bt protein) derived from *B. thuringiensis*. A number of studies have been performed on the mechanism of the insecticidal activity of these Bt proteins (OECD, 2007), and so far it has not been reported that the Bt proteins have other functions. Therefore, it is not concluded that these Bt proteins have enzyme activities and change the metabolic system of recipient organisms.

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² AD_2010: This is the database developed based on the sequences obtained from Food Allergy Research and Resource Program Database (FARRP) (<http://www.allergenonline.com>), and contains 1,471 sequences.

【 Modified CP4 EPSPS protein】

The EPSPS protein, functionally identical to the modified CP4 EPSPS protein, is an enzyme protein, which catalyzes the shikimic acid pathway for biosynthesis of aromatic amino acids, but is not a rate-limiting enzyme, and therefore it has been considered that the levels of the aromatic amino acids, the end products of this pathway, do not increase even with the increased activities of the EPSPS proteins. In addition, it has been identified that the EPSPS protein specifically reacts with the substrates, phosphoenolpyruvate and shikimate-3-phosphate (hereinafter referred to as “S3P”) (Gruys *et al.*, 1992). Other than those substrates, only shikimic acid, an analog of S3P, is known to react with the EPSPS protein. However, the comparison of the reaction of the EPSPS proteins with shikimic acid and S3P by the specificity constant (k_{cat}/K_m), which represents the degree of occurrence of reaction, showed that the reaction specificity between the EPSPS protein and shikimic acid is one-two millionth of that between the EPSPS and S3P (Gruys *et al.*, 1992), and shikimic acid is unlikely to react as a substrate of the EPSPS protein. Therefore, it is not thought that the modified CP4 EPSPS protein changes the metabolic system of recipient organisms.

20 (2) Information concerning vectors

3) Name and origin

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

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MON87701: PV-GMIR9 constructed from the plasmid pBR322 derived from *E. coli* and others

MON89788: PV-GMGOX20 constructed from the vector pBR322 derived from *E. coli* and others

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4) Properties

(d) The number of base pairs and nucleotide sequence of vectors

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The numbers of base pairs in the plasmid vectors used for the development of

parent lines are as follows:

MON87701: PV-GMIR9; 15,532 bp

MON89788: PV-GMGOX20; 9,664 bp

- 5 (e) Presence or absence of nucleotide sequence having specific functions, if present, and the functions

10 The antibiotic resistance gene, which is used as a selective marker of the constructed vector in *E. coli* used for the development of MON87701 and MON89788, is the *aadA* gene conferring resistance to spectinomycin and streptomycin. This antibiotic resistance gene has not been transferred to any recipient organisms.

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

The infectivity of PV-GMIR9 or PV-GMGOX20 is not known.

- 20 (3) Method of preparing living modified organisms

- 5) Structure of the entire nucleic acid transferred to the recipient organism

25 The positions of the component elements and sites cleaved by restriction enzymes of the donor nucleic acid transferred to the recipient organisms, MON87701 and MON89788, are shown in Figures 1 and 2, respectively (p. 11-12).

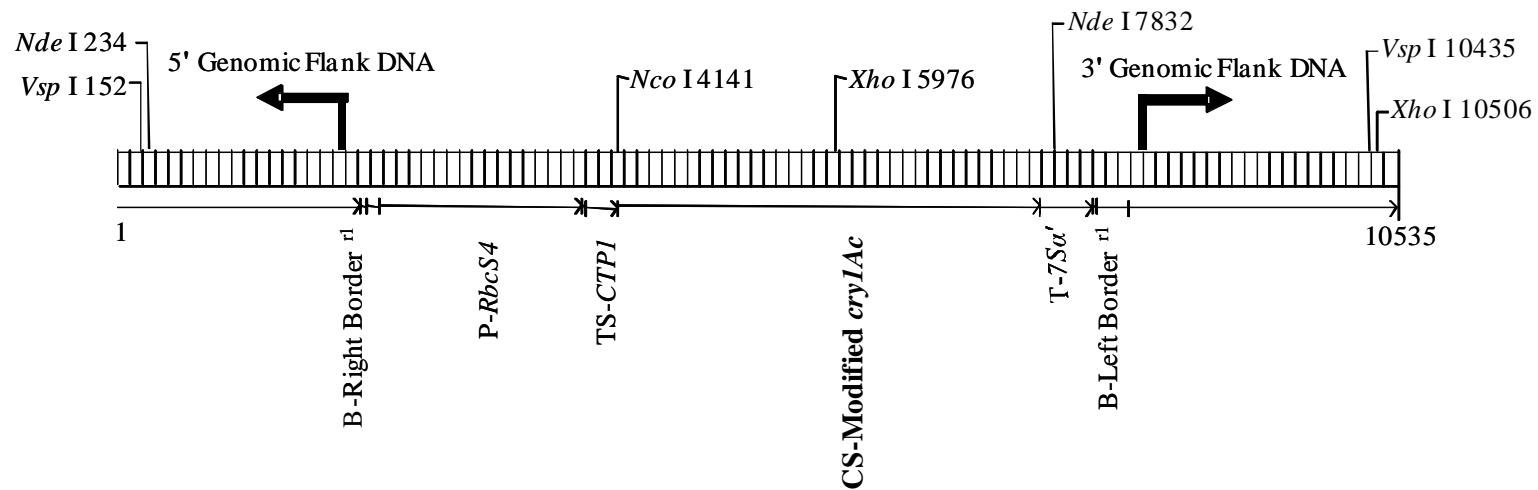


Figure 1. Map of the transferred genes of MON87701⁵

5 The arrows in the figure are the 5'- and 3'-terminals and the subsequent adjacent endogenous sequences of soybean.

The numbers in the figure show the positions in the soybean genome, and therefore, do not correspond to the figures in the plasmids shown in Table 1 (p. 3-5).

10

⁵ All the rights pertinent to the information in this figure and the responsibility for the content remain with Monsanto Japan Limited.

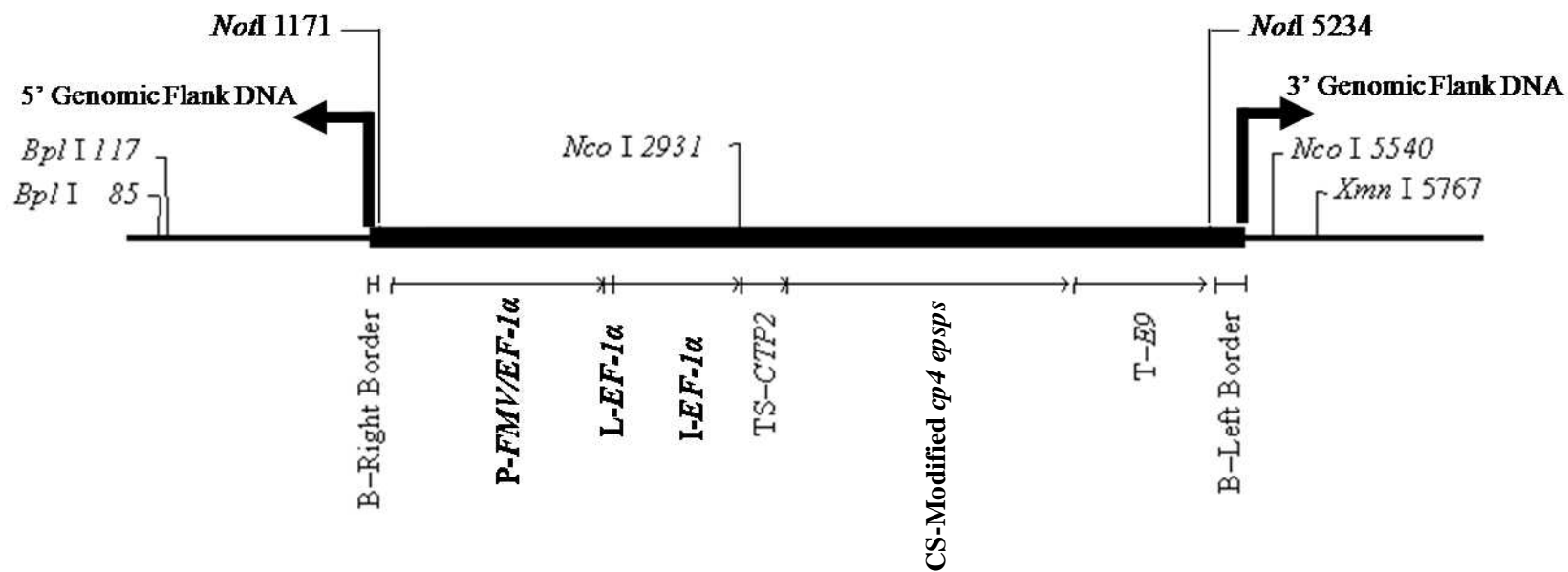


Figure 2. Map of the transferred genes of MON89788⁶

5 The arrows in the figure are the 5'- and 3'-terminals and the subsequent adjacent endogenous sequences of soybean.

The numbers in the figure show the positions in the soybean genome, and therefore, do not correspond to the figures in the plasmids shown in Table 2 (p. 6-7).

⁶ All the rights pertinent to the information in this figure and the responsibility for the content remain with Monsanto Japan Limited.

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

The transferring of nucleic acids to the recipient organism was performed using the following method:

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MON87701: The agrobacterium method was used to transfer the T-DNA I and II regions of the plasmid vector PV-GMIR9. Then the transformed regenerated plants (R0) were self-pollinated, and the subsequent R1 plants were screened for the presence of the modified *cp4 epsps* gene by applying glyphosate herbicide at a dose lower than normal. The plants damaged by glyphosate were selected as individuals not containing the T-DNA II region (region containing the modified *cp4 epsps* gene expression cassette).

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MON89788: The agrobacterium method was used to transfer the T-DNA region of the plasmid vector PV-GMGOX20.

7) Process of rearing of living modified organisms

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

The transformed cells were selected in the media added with MON87701, MON89788, and glyphosate.

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

30

Agrobacterium in MON87701 and MON89788 was removed by adding carbenicillin, cefotaxime and ticarcillin-clavulanic acid, and carbenicillin and cefotaxime to the medium, respectively.

35

Then, in MON87701 and MON89788, PCR analysis was conducted for the vector backbone regions of the plasmid vectors, PV-GMIR9 and PV-GMGOX20, used for transformation. As a result, the vector backbone regions of the plasmid vectors, PV-GMIR9 and PV-GMGOX20 were not detected, and thus, it was confirmed that there was no residual *Agrobacterium* used for transformation in MON87701 and

MON89788 (Monsanto Company, 2008a; Urquhart and Paul, 2011).

5 (i) 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

10 This stacked soybean line was cultivated by crossing of MON87701 with MON89788. Figure 3 (p. 15) shows an example of the rearing process of this stacked line. The status of application and approval of MON87701, MON89788, and this stacked soybean line in Japan is described below (Table 3, p. 16).

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【 Confidential: not disclosed to unauthorized persons 】

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Figure 3. Example of rearing process of this stacked line

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Table 3. The status of application and approval of MON87701, MON89788, and this stacked soybean line in Japan⁷

As of November 2012

Safety assessment	MON87701	MON89788	This stacked soybean line
Food ⁸	March 2011 Confirmed safety	November 2007 Confirmed safety	June 2011 Confirmed safety
Feed ³	September 2011 Confirmed safety	October 2007 Confirmed safety	September 2011 Confirmed safety
Environment ⁴	July 2012 Application for Type I Use Regulation	January 2008 Approved for Type I Use Regulation	September 2012 Application for Type I Use Regulation

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

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

10 It was confirmed that the transferred genes in MON87701 and MON89788 existed on the chromosome (Phillips et al., 2008; Monsanto Company, 2006).

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

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[MON87701]

20 As a result of Southern blot analysis for existence of the transferred gene, it has been confirmed that a single copy of the T-DNA I region was transferred at a single site in the genome of MON87701. It was confirmed that the T-DNA II region other than the T-DNA I region and the vector backbone region were not transferred and that all component elements of the modified *cryI*Ac gene expression cassettes in the T-DNA I region were transferred. In addition, it was confirmed by Southern blot analysis on multiple generations that the transferred genes were stably inherited to subsequent

⁷ All the rights pertinent to the information in this table and the responsibility for the contents rest upon Monsanto Japan Limited.

⁸ Based on the Food Sanitation Act

³ Based on the Act on Safety Assurance and Quality Improvement of Feeds

⁴ Based on the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms

generations (Arackal et al., 2009).

[MON89788]

5 As a result of Southern blot analysis for existence of the transferred gene, it has been confirmed that a single copy of the T-DNA region was transferred at a single site in the genome of MON89788. It was confirmed that the vector backbone region other than the T-DNA region was not transferred and that all component elements of the modified *cp4 epsps* gene expression cassettes in the T-DNA region were transferred. In addition, it was confirmed by Southernblot analysis on multiple generations that the transferred
10 genes were stably inherited to subsequent generations (Dickinson et al., 2006).

(l) The position relationship in the case of multiple copies existing in a chromosome

15 This item is not applicable because there is only one copy for both MON87701 and MON89788 (Arackal et al., 2009; Dickinson et al., 2006).

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

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The expression stability was confirmed as follows:

MON87701: Confirming the expression of the modified Cry1Ac protein by Western blot analysis (Zhao and Silvanovich, 2008)

25 MON89788: Confirming the expression of the modified CP4 EPSPS protein by Western blot analysis (Mozaffar and Silvanovich, 2006)

(n) 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

30

The sequences of the nucleic acids transferred to MON87701 and MON89788 do not have the function allowing transmission. Therefore, it is presumed that the transferred nucleic acids are unlikely to transmit through virus infection and/or other routes to any other wild animals and wild plants.

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

40 The PCR, in which the DNA sequences of the transferred genes and the nearby plant

genomes are used as primers, allows to specifically detect MON87701 and MON89788 (Cole, 2008; Dickinson and Masucci, 2006). In order to detect and identify this stacked soybean line, the above-mentioned methods must be applied to each sample derived from an individual.

5

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

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

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This stacked soybean line contains the following traits derived from individual parent lines.

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MON87701: Resistance to the *Lepidoptera* pests due to the modified Cry1Ac protein derived from the transferred genes.

MON89788: Tolerance to glyphosate herbicide due to the modified CP4 EPSPS protein derived from the transferred genes.

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As for those proteins, a. functional interaction between them, and b. ecological effects induced by obtaining traits derived from the parent line were examined.

a. Functional interaction among proteins

25

The modified Cry1Ac protein expressed in MON87701 is a crystal insecticidal protein (Bt protein) derived from *B. thuringiensis*. A number of studies have been performed on the mechanism of the insecticidal activity of these Bt proteins (OECD, 2007), and so far it has not been reported that the Bt proteins have other functions; in addition, it is not believed that these Bt proteins have enzyme activities. Therefore, these Bt proteins including the modified Cry1Ac protein are not considered to affect metabolic pathways in plants.

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On the other hand, the EPSPS protein, functionally identical to the modified CP4 EPSPS protein expressed in MON89788, is an enzyme, which catalyzes the shikimic acid pathway for biosynthesis of aromatic amino acids. Since the EPSPS protein with the high substrate specificity is not a rate-limiting enzyme of the synthetic pathway of shikimic acid, the levels of the aromatic amino acids, the end products of this pathway, do not increase even with the increased activities of the EPSPS proteins by the expression of the modified CP4 EPSPS protein.

Based on the above, it is believed that the modified Cry1Ac and the modified CP4 EPSPS proteins expressed in this stacked soybean line have a respective distinct mechanism of action and act independently. Also, it is unlikely that the expressed proteins derived from respective parent lines interact with one another in this stacked soybean line.

b. Ecological effects induced by obtaining traits derived from the parent lines

This stacked soybean line has tolerance to glyphosate herbicide derived from MON89788 as well as resistance to *Lepidoptera* pests derived from MON87701. As for the ecological effects induced by obtaining traits derived from the parent lines, on roadsides of the highway, where this stacked soybean line is thought to grow, a transport pathway of imported soybeans, the following was examined: 1) potential application of glyphosate herbicide, and 2) whether or not the degree of the occurrence of crossing with *Glycine soja* and the survival rate of the hybrid are both higher for this stacked soybean line, compared with those for the soybean resistant to *Lepidoptera* pests, MON87701, by the presence or absence of application of glyphosate herbicide.

At first, the potential application of glyphosate herbicide on roadsides of the highway, where this stacked soybean line is thought to grow, a transport pathway of imported soybeans was examined.

It has been reported that physical weeding, such as brush cutting, is mainly applied for weeding in roadsides of the highway, a transport pathway of imported soybeans (Editorial committee of Public works management journal, 2000). Since glyphosate herbicide is nonselective and an absorption- and translocation-type herbicide, it has to be used carefully when there is vegetation, such as street trees, to be maintained near the location the herbicide is applied. In addition there are many options of herbicides available for roadsides, and then some other herbicides, which this stacked soybean line does not exhibit resistance to, are likely to be used even for roadside herbicide application. Therefore, it was determined that glyphosate herbicide was unlikely to be used regularly on highway roadsides.

Next, the degree of the occurrence of crossing between this stacked soybean line and *Glycine soja* and the survival rate of the hybrids based on the presence or absence of the application of glyphosate herbicide to this stacked soybean line were examined.

a) When glyphosate herbicide is not usually applied

When glyphosate herbicide was not usually applied, the tolerance to glyphosate herbicide does not increase competitiveness, and therefore, crossing with *Glycine soja* was unlikely to occur, as with the parent line, MON87701.

5

b) When glyphosate herbicide is annually applied

When glyphosate herbicide was annually applied, this stacked soybean line did not die, but survived; however, *Glycine soja* growing next to this stacked soybean line was killed. Therefore, crossing between this stacked soybean line and *Glycine soja* was extremely unlikely to occur.

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c) When glyphosate herbicide is irregularly applied

When glyphosate herbicide is irregularly applied, as described above, crossing between this stacked soybean line and *Glycine soja* adjacently growing was extremely unlikely to occur in a year when glyphosate herbicide was used. However, in a year without application of glyphosate herbicide, as with the parent line, MON87701, crossing between this stacked soybean line and *Glycine soja* adjacently growing was possible but low. In this case, the hybrid seeds germinate and grow the following year or the year after the next and glyphosate herbicide is applied to the habitat, and then hybrid individuals are likely to survive with no deaths. However, the sites, to which glyphosate herbicide is applied, are considered to be managed for weed control by people. Then the hybrid individuals survived after application of glyphosate herbicide are likely to be removed as a target weed to be controlled by brush cutting or use of any other herbicides other than glyphosate by an administrator. Therefore, it was concluded that the probability to survive for the hybrid between this stacked soybean line and *Glycine soja* was not higher than that for the hybrid of soybean resistant to *Lepidoptera* pests, MON87701.

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Based on the above, it was concluded that the degree of the occurrence of crossing with *Glycine soja* and the survival rate of the hybrid for this stacked soybean line are not higher than those for the parent line, MON87701, even when this stacked soybean line has resistance to *Lepidoptera* pests and tolerance to glyphosate herbicide conferred by the expressed modified Cry1Ac and modified CP4 EPSPS proteins, respectively.

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Therefore, it was concluded that the expressed proteins derived from respective parent lines are unlikely to interact with one another and change their own respective characteristics in this stacked soybean line. It was also concluded that the ecological

effects of this stacked line were not higher than the range of those of the parent line by obtaining traits derived from the parent lines. Thus, differences in the physiological and ecological characteristics between this stacked soybean line and the soybeans, the species of the taxonomy to which the recipient organism belongs, were evaluated based on the results of the individual examination of the parent lines, MON87701 and MON89788.

(p) With respect to the physiological or ecological characteristics listed below, the presence or absence of a difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present⁵

As described in the previous section, it is believed that the expressed proteins derived from the respective parent lines are unlikely to newly affect the physiological and ecological characteristics in this stacked soybean line. Therefore, differences in the physiological and ecological characteristics between this stacked soybean line and the soybeans, the species of the taxonomy to which the recipient organism belongs, can be evaluated based on the results of the individual examinations of a and c-g for the parent line MON87701 in Japan, and the results of the individual examinations of a-g for the parent line MON89788 in Japan. As a result, it has been confirmed that there are no differences between the parent lines and their controls, non-recombinant soybeans (Monsanto Japan Limited, 2010; Monsanto Japan Limited, 2007).

Moreover, no difference between the parent lines and their controls, and non-recombinant soybeans was confirmed by the evaluation based on the results of the evaluation of b for the parent line MON87701 in the artificial climate chamber in the US (Baltazar and Kendrik, 2008).

As for the information on the physiological or ecological characteristics, see the website of the Japan Biosafety Clearing House¹¹.

¹¹ Visit the website of the Japan Biosafety Clearing House and search as follows:

[MON87701]

1. In the website, http://www.bch.biodic.go.jp/bch_3_2.html, select “persons with specialized knowledge and experience concerning Adverse Effects on Biological Diversity.”
2. Select “General review meeting (open)” on September 7, 2012
3. Select “Outline of application form, etc. PDF”

[MON89788]

1. In the website, https://ch.biodic.go.jp/bch/OpenSearch.do_imput “Soybean” in the box of “Name of Organism” and then select “Search/View.”
2. Select “Name of Organism-Soybean” of the appropriate line.

- a. Morphological and growth characteristics
- b. Cold-resistance and heat-resistance at the early stage of growth
- c. Wintering ability and summer survival of the mature plant
- 5 d. Fertility and size of the pollen
- e. Production, shedding habit, dormancy, and germination rate of the seed
- f. Crossability
- g. Productivity of harmful substances

-
- 3. Select "Attached documents."
 - 4. Select "Document 1."

II. 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 Effects on Biological Diversity (called Experts) for possible Adverse Effects 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 Effects on Biological Diversity

Soybean resistant to *Lepidoptera* pests and tolerant to glyphosate herbicide (hereinafter referred to as “this stacked line”) was developed with the following lines by crossing.

(a) Soybean resistant to *Lepidoptera* pests, to which the modified *cry1Ac* gene coding for the modified Cry1Ac protein was transferred (hereinafter referred to as “MON87701”), and

(b) Soybean tolerant to glyphosate herbicide, to which the modified *cp4 epsps* gene coding for the modified CP4 EPSPS protein (5-enolpyruvylshikimate 3-phosphate synthetase) was transferred (hereinafter referred to as “MON89788”).

The modified Cry1Ac and modified CP4 EPSPS proteins expressed by the genes transferred to this stacked line have a respective distinct mechanism of action and act independently. Therefore, it was concluded that these proteins did not interact to change the metabolic system of the recipient organism to produce an unexpected metabolite in this stacked line.

In the examination conducted on roadsides of the highway, where this stacked line falls during transport and grows, for differences in degree of the occurrence of crossing between this stacked soybean line, which falls and grows naturally, and *Glycine soja*, and the survival rate of the hybrids (ecological effects) based on the presence or absence of application of glyphosate herbicide, it was concluded that the degree of the occurrence of crossing with *Glycine soja* and the survival rate of the hybrids (ecological effects) for this stacked soybean line, which has a resistance to *Lepidoptera* pests and tolerance to glyphosate herbicide, are not higher than those for the parent line, MON87701, based on the following results:

(a) In the location without application of glyphosate herbicide, this stacked line is not thought to have higher competitiveness, compared with the parent line MON87701, and

therefore, the possibility of crossing with *Glycine soja* is not thought to increase,

(b) in the location where glyphosate herbicide is applied annually, *Glycine soja* is killed by glyphosate herbicide, and

(c) in the location with unscheduled application of glyphosate herbicide, the hybrids
5 between this stacked line and *Glycine soja* are likely to be produced. However, the location is considered to be properly managed for weed control by an administrator, and therefore, the hybrids are more likely to be removed by brush cutting or use of herbicides other than glyphosate.

10 Based on the above, it was unlikely that these proteins derived from respective parent lines functionally interact with one another in the plant body of this stacked soybean line, and the ecological effects were not higher than the range of those of the parents, MON87701 and MON89788. Therefore, it was concluded that there
15 were no trait changes to be evaluated, except having the traits that the parent line had.

The examination of the respective evaluation items in the general review meeting has already been completed*. Based on the results of the examination, the conclusion
20 described in the Biological Diversity Risk Assessment Report that the use of the respective parent lines in accordance with the Type I Use Regulation causes no Adverse Effect on Biological Diversity in Japan has been judged to be reasonable.

a. Competitiveness

b. Productivity of harmful substances

25 **c. Crossability**

In addition, in order to understand the changes of the situations used as premises for those evaluations and the growth situation of this stacked line, monitoring by the applicant based on the monitoring plan is included in the Type 1 Use Regulation for
30 the Type 1 Use of this recombinant soybean and monitoring will be performed.

* The results of the evaluation of the respective parent lines are available as described below.

35 **[MON87701]**

1. In the website, http://www.bch.biodic.go.jp/bch_3_1.html, select

“Agriculture, Forestry and Fisheries-2012.”

2. Select “Results of the review in the Sub- committee of agricultural crops-PDF” of the 56th appropriate line.

5 **[MON89788]**

1. In the website, <https://ch.biodic.go.jp/bch/OpenSearch.do>, input “Soybean” in the box of “Name of Organism” and then select “Search/View.”

2. Select “Name of Organism-Soybean” of the appropriate line.

3. Select “Attached documents.”

10 4. Select “Document 2.”

(2) Conclusion based on the Biological Diversity Risk Assessment Report

15 Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stacked line, in accordance with the Type 1 Use Regulation, causes Adverse Effects on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is reasonable.

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- 10 Document 2: Results from the review meeting of the Biological Diversity Risk Assessment, “Soybean tolerant to glyphosate herbicide (modified *cp4 epsps*, *Glycine max* (L.) Merr.) (MON89788, OECD UI: MON-89788-1)”
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