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 tolerant to dicamba herbicide (modified *dmo*, *Glycine max* (L.) Merr.) (MON87708, OECD UI : MON-877Ø8-9)
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

Monsanto Company has developed dicamba-tolerant soybean (modified *dmo*, *Glycine max* (L.) Merr.) (MON87708, OECD UI: MON-877Ø8-9) (hereinafter referred to as “this recombinant soybean”) that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide.

In conventional soybean, dicamba is labeled only for pre-seeding (application to weed foliage) and pre-harvest (for the purpose of drying soybeans and weed control for facilitating harvesting operation) applications. The tolerance of this recombinant soybean to dicamba herbicide expands the window of dicamba application, allowing pre-seeding application and application during the growth period up to the early reproductive (R1: beginning of flowering) stage. Table 1 (p. 2) shows the dicamba application system planned for this recombinant soybean after it is commercialized.

Table 1 Application system of dicamba herbicide for this recombinant soybean[1]

	Pre-emergence application	Foliage application		
		Soybean 3-leaf stage	Soybean 6-leaf stage	Soybean early flowering stage
Maximum application system	1.12 kg a.e./ha[2)]	0.56 kg a.e./ha	Not applied	0.56 kg a.e./ha
Recommended application system for normal weed species	0.56 kg a.e./ha	0.28 kg a.e./ha	Not applied	Not applied
Recommended application system for hard-to-control weed species[1)]	0.56 kg a.e./ha	0.28 kg a.e./ha	0.28 kg a.e./ha	Not applied

[1)] Includes weeds of late emergence.
[2)] a.e.; acid equivalent. Herbicide active ingredients are formulated either as the active ingredients themselves or in the form of their salts. In active ingredients formulated as their salts, the acid moieties serve as the active components, and the base moieties vary between formulations. If the dosage of a herbicide is expressed as the amount of the salt of the active ingredient formulated, the amount of the active component cannot be precisely compared between formulations with different base moieties. Thus, acid equivalent was used as the unit for the active component.

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Dicamba herbicide provides control of over 95 annual and biennial weed species, and suppresses growth of over 100 perennial broadleaf and woody species. Dicamba provides more effective weed control than glyphosate herbicide on broadleaf weeds such as lamb's quarters (*Chenopodium album*) and hemp sesbania (*Sesbania exaltata*). Dicamba is also effective in controlling broadleaf weeds that are resistant to herbicides other than dicamba, including glyphosate-resistant weeds such as common ragweed (*Ambrosia artemisiifolia*) and palmer amaranth (*Amaranthus palmeri*).

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of this recombinant soybean are shown in Figure 1 (p. 5) and Table 2 (p. 6–8).

Due to the insertion of a restriction site in the cloning process, the amino acid sequence of dicamba mono-oxygenase (hereinafter referred to as "DMO protein"), which is expressed by the *dmo* gene derived from *Stenotrophomonas maltophilia* DI-6 strain and introduced into this recombinant soybean, has an alanine inserted immediately after the first methionine from the N-terminus (Behrens et al., 2007), compared to the amino acid sequence of the wild-type DMO protein (Herman et al., 2005) derived from *S. maltophilia* DI-6 strain. In addition, tryptophan at b[2] position (c[3] position in the modified DMO protein expressed in this recombinant soybean) in the amino acid sequence of the wild-type DMO protein derived from DI-6 strain is replaced with cysteine. This amino acid replacement was unintentionally induced during PCR amplification of the wild-type *dmo* gene (Behrens et al., 2007). The *dmo* gene introduced into this recombinant soybean is hereinafter referred to as "modified *dmo* gene." As a result of processing of the precursor protein expressed by the modified *dmo* gene expression cassette introduced into this recombinant soybean, two different forms of modified DMO proteins are expressed in this recombinant soybean, which will be discussed later in I.-2-(1)-2)-(b) and Figure 5 (p. 12 and p.17). The deduced amino acid sequences of the two modified DMO protein forms expressed in this recombinant soybean are shown in Annex 1.

Due to the insertion of a restriction site in the cloning process, the amino acid sequence of the CP4 EPSPS protein encoded by the *cp4 epsps* gene introduced into this

[2]Confidential: not disclosed to unauthorized persons
[3]Confidential: not disclosed to unauthorized persons

recombinant soybean has its 2nd residue from the N-terminus changed from serine to leucine, compared to the amino acid sequence of the CP4 EPSPS protein derived from *Agrobacterium* sp. CP4 strain. The *cp4 epsps* gene introduced into this recombinant soybean is hereinafter referred to as "modified *cp4 epsps* gene." However, this recombinant soybean was obtained by applying glyphosate herbicide to the R1 generation at a dose lower than normal and selecting individuals damaged by the herbicide, that is, individuals not containing the modified *cp4 epsps* gene, which were derived from genetic segregation (Figure 9, p. 26).

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

The composition of donor nucleic acid and the origins of component elements used for the development of this recombinant soybean are shown in Figure 1 (p. 5) andTable 2 (p. 6–8).

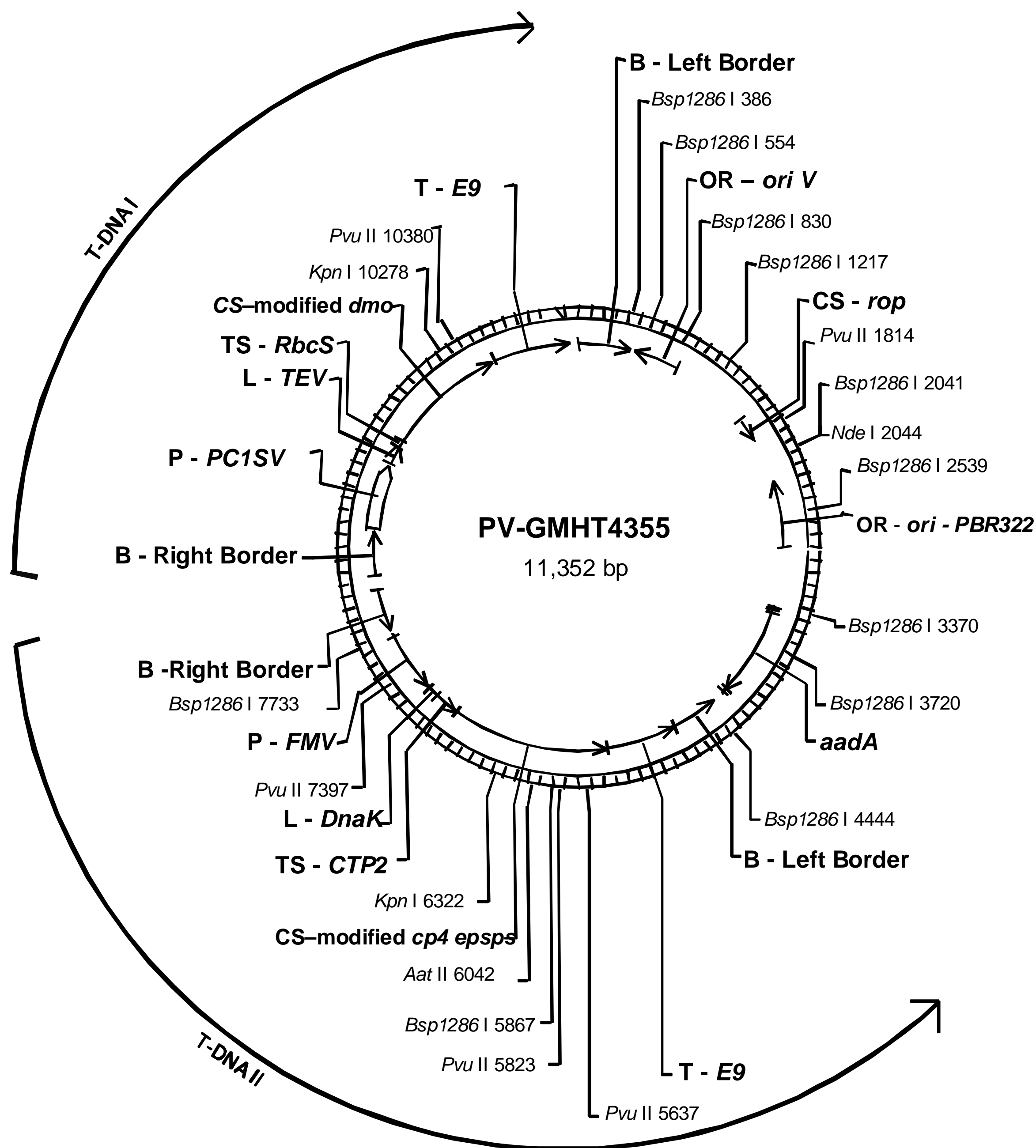

Figure 1 Plasmid map of PV-GMHT4355[4]

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

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Table 2 Component elements of the donor nucleic acids, and their origins and functions[5]

Component elements	Origin and function
T-DNA I	
B[Note 1]-Right Border Region	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982).
Intervening sequence	Sequence used in DNA cloning.
P[Note 2]-*PC1SV*	Promoter inducing transcription of the Full-Length Transcript (FLt; produced by transcription of the whole genome) of peanut chlorotic streak caulimovirus (PC1SV) (Maiti and Shepherd, 1998). It directs constitutive transcription in plant cells.
Intervening sequence	Sequence used in DNA cloning.
L[Note 3]-*TEV*	5′ non-translated region from the Tobacco Etch virus (TEV) (Niepel and Gallie, 1999) that is involved in regulation of gene expression.
Intervening sequence	Sequence used in DNA cloning.
TS[Note 4]-*RbcS*	Sequence encoding the transit peptide and the first 24 amino acids from the N-terminus of the mature protein of the ribulose-1,5-bisphosphate carboxylase small subunit gene (*RbcS*) from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport of the modified DMO precursor protein to the chloroplast.
Intervening Sequence	Sequence used in DNA cloning.
CS[Note 5]-modified *dmo*	Coding sequence for the dicamba mono-oxygenase from *S. maltophilia* (Herman et al., 2005; Wang et al., 1997).
Intervening Sequence	Sequence used in DNA cloning.
T[Note 6]-*E9*	3′ non-translated region from the *RbcS2* gene encoding the ribulose-1,5-bisphosphate carboxylase small subunit of *P. sativum* (pea) , which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984).
Intervening Sequence	Sequence used in DNA cloning.
B-Left Border Region	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983).

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Table 2 (continued) Component elements of the donor nucleic acids, and their origins and functions

Component elements	Origin and function
Plasmid vector backbone (Not present in this recombinant soybean)	
Intervening Sequence	Sequence used in DNA cloning.
OR[Note 7]-*ori V*	Origin of replication from the broad host range plasmid RK2, which confers autonomous replication ability to the vector in *Agrobacterium* (Stalker et al., 1981).
Intervening Sequence	Sequence used in DNA cloning.
CS-*rop*	Coding sequence for repressor of primer protein derived from the ColE1 plasmid, for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).
Intervening Sequence	Sequence used in DNA cloning.
OR-*ori-pBR322*	Origin of replication from pBR322, which confers autonomous replication ability to the vector in *E. coli* (Sutcliffe, 1979).
Intervening Sequence	Sequence used in DNA cloning.
aadA	Bacterial promoter, coding sequence and 3′ non-translated region of an aminoglycoside-modifying enzyme, 3″ (9)-O-nucleotidyltransferase, derived from transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistances.
Intervening Sequence	Sequence used in DNA cloning.
T-DNA II (Not present in this recombinant soybean)	
B-Left Border Region	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983).
Intervening Sequence	Sequence used in DNA cloning.
T-*E9*	3′ non-translated sequence from *RbcS2* gene of *P. sativum* (pea) encoding the ribulose-1,5-bisphosphate carboxylase small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984).
Intervening Sequence	Sequence used in DNA cloning.
CS-modified *cp4 epsps*	Coding sequence of the *aroA (epsps)* gene from *Agrobacterium* CP4 strain encoding 5-enol-pyruvyl-shikimate-3-phosphate synthase (CP4 EPSPS) (Barry et al., 1997; Padgette et al., 1996).

Table 2 (continued) Component elements of the donor nucleic acids, and their origins and functions

Component elements	Origin and function
TS-*CTP2*	Sequence encoding the chloroplast transit peptide from the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene (*ShkG*) of *Arabidopsis thaliana* (Herrmann, 1995; Klee et al., 1987) that directs transport of the modified CP4 EPSPS protein to the chloroplast.
Intervening Sequence	Sequence used in DNA cloning.
L-*DnaK*	5′ non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulation of gene expression.
Intervening Sequence	Sequence used in DNA cloning.
P-*FMV*	Promoter for the 35S RNA from Figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in plant cells.
Intervening Sequence	Sequence used in DNA cloning.
B-Right Border Region	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982).
Plasmid vector backbone (Not present in this recombinant soybean)	
Intervening sequence	Sequence used in DNA cloning.

Note 1 B -border
5 Note 2 P-promoter
Note 3 L-leader
Note 4 TS-targeting sequence
Note 5 CS-coding sequence
Note 6 T-3′ non-translated transcriptional termination sequence and polyadenylation signal sequences
10 Note 7 OR-origin of replication

(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 *dmo* gene]

This recombinant soybean contains the modified *dmo* gene derived from *S. maltophilia*. The modified *dmo* gene expresses the modified DMO protein that confers
10 tolerance to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide to this recombinant soybean. *S. maltophilia* is a gram-negative bacteria commonly present in wet environments, soil, and plants (Denton and Kerr, 1998). The DI-6 strain was isolated from soil (Krueger et al., 1989).

15 The DMO protein is an enzyme that catalyzes demethylation of dicamba to produce non-herbicidal compound DCSA (3,6-dichlorosalicylic acid) and formaldehyde (HCHO), (Chakraborty et al., 2005), thereby conferring dicamba tolerance to plants (Figure 2, p. 10). There is a report that the modified *dmo* gene expression cassette ([P-*PClSV*]-[L-*TEV*]-[TS-*RbcS*]-[CS-modified *dmo*]-[T-*E9*]) that was exactly the same
20 as the one used for developing this recombinant soybean was introduced into soybean, tomato, Arabidopsis, and tobacco and successfully imparted dicamba tolerance to these plants (Behrens et al., 2007).

The DMO protein is a Rieske-type non-heme iron oxygenase that composes a
25 three-component redox system together with a reductase and a ferredoxin. These three proteins work together in a redox system similar to many other oxygenases to transport electrons from nicotinamide adenine dinucleotide (NADH) to oxygen and to catalyze the demethylation of electron-acceptor substrates (in this case, dicamba herbicide) (Behrens et al., 2007), as presented in Figure 2 (p. 10).
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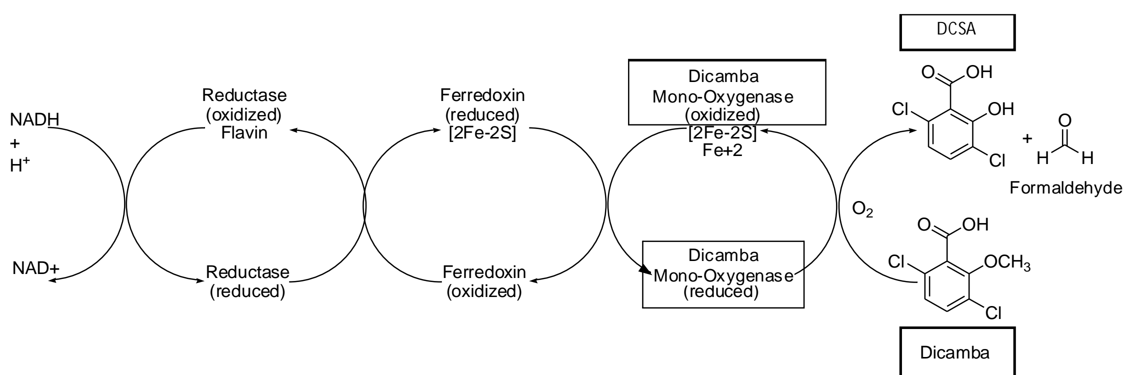

Figure 2 Three-component redox system of the DMO protein[6]

5 The figure presents the electron transport system from NADH to the DMO protein. Demethylation of dicamba produces DCSA.

The crystal structure of the DMO protein has been analyzed using a DMO protein[7]
10 having a histidine tag at the C-terminus (D'Ordine et al., 2009; Dumitru et al., 2009). The crystal of the DMO protein has a trimeric structure comprising three DMO protein monomers (Figure 3, p.11). Each monomer has a Rieske [2Fe-2S] cluster domain containing the Rieske [2Fe-2S] cluster and a non-heme iron center domain containing the non-heme iron center (D'Ordine et al., 2009; Dumitru et al., 2009). These domains,
15 which are commonly found in all Rieske-type mono-oxygenases, are known to be the key domains involved in electron transport (Ferraro et al., 2005).

Electrons transferred from NADH are transferred via a soybean endogenous reductase and ferredoxin to the terminal DMO protein (Figure 2, p.10). The electrons reductively activate oxygen to catalyze the demethylation of dicamba. Since electrons
20 are transported between adjacent monomers, the DMO protein needs to form a trimer to achieve the optimal distance between the monomers and their relative positions (D'Ordine et al., 2009). Within a monomer, due to the large distance between the Rieske [2Fe-2S] cluster domain and the non-heme iron center domain, electron transport does not occur between the two domains (D'Ordine et al., 2009; Dumitru et al.,
25 2009).

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[7] The DMO protein used for crystal structure analysis had the same sequence as the wild-type DMO protein, except for the histidine tag attached to its C-terminus and the alanine inserted at the 2nd position from the N-terminus due to the insertion of a restriction site in the cloning process.

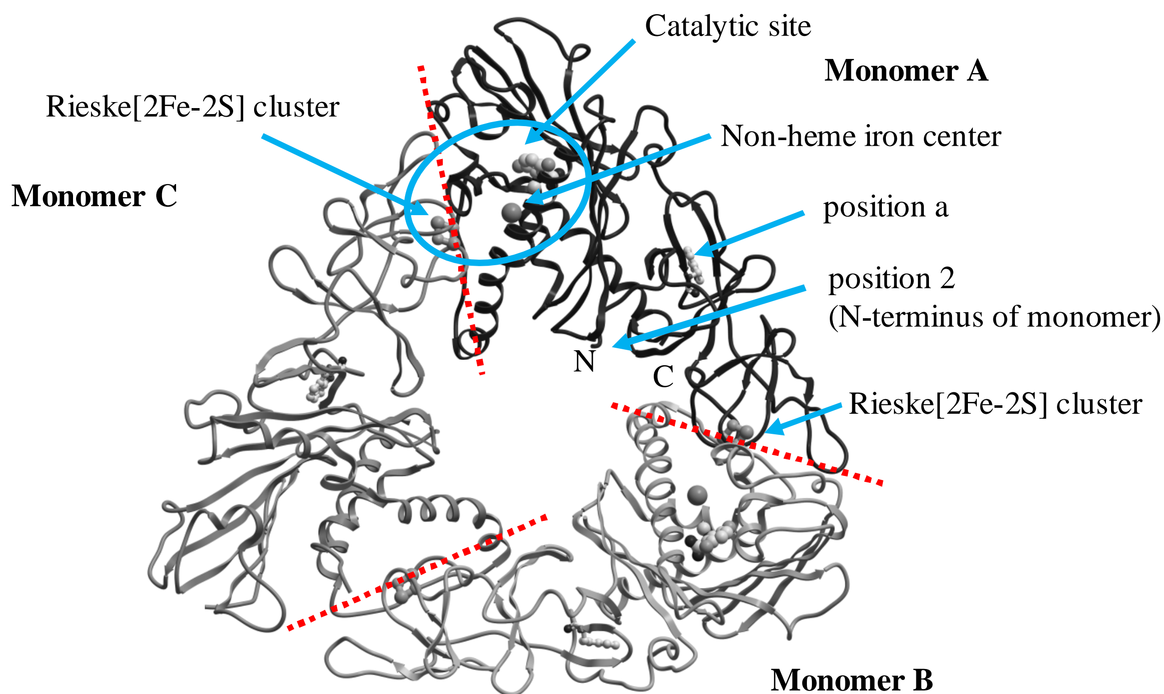

Figure 3 Crystal structure of the DMO protein[8]

A schematic diagram of the crystal structure of the DMO protein in the active trimer form (D'Ordine et al., 2009). Characteristic structures including the non-heme iron center domain containing the non-heme iron center, the N- and C-termini, and the Rieske[2Fe-2S] cluster domain containing the Rieske [2Fe-2S] cluster are shown in monomer A. The catalytic site formed by monomer A and monomer C is indicated in the blue circle. The Rieske [2Fe-2S] cluster of monomer C is indicated by the arrow. Electron transport takes place between adjacent monomers, so electrons are transported between the Rieske [2Fe-2S] cluster domain of monomer C and the non-heme iron center domain of monomer A. Red dotted lines indicate the interfaces at which electrons are transported between individual subunits of the adjacent non-heme iron center domain and the Rieske [2Fe-2S] cluster domain, as well as the boundaries between monomers. The DMO protein used for the crystal structure analysis was different from the wild-type DMO protein and the modified DMO protein, having a histidine tag attached to its C-terminus and an alanine inserted at the 2nd position from its N-terminus (see Figure 5, p. 17, for modifications in the amino acid sequence) [Confidential: not disclosed to unauthorized persons]

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

Regarding the expression pattern of the modified DMO protein in this recombinant soybean, this recombinant soybean contains a modified *dmo* gene expression cassette, from which a precursor protein (hereinafter referred to as the "modified MON87708 DMO precursor protein") is expressed. The modified MON87708 DMO precursor protein, which is a protein yet to be processed, contains an additional 84 amino acids at the N-terminus of the modified DMO protein. In the peptide consisting of the 84 amino acids at the N-terminus, 57 amino acids are derived from the chloroplast transit peptide (hereinafter referred to as "CTP") from pea ribulose-1,5-bisphosphate carboxylase small subunit, and 24 amino acids are derived from the N-terminal amino acids of the pea ribulose-1,5-bisphosphate carboxylase small subunit (hereinafter referred to as "RbcS") that enhances efficiency of chloroplast transport efficiency (Behrens et al., 2007; Comai et al., 1988). The remaining three amino acids are encoded by an intervening sequence used for cloning purposes (Table 2, p. 6–8).

Analysis of seed extracts of this recombinant soybean by Western blot analysis demonstrated the presence of two immunoreactive bands. Analysis of these two bands revealed the presence of two protein forms (Figure 4, p.13), that is, a full-length protein produced by cleaving off the transit peptide from the modified MON87708 DMO precursor protein and a protein retaining the transit peptide that should have been cleaved off.

Amino acid sequence analysis of the N-terminus revealed that, between these two bands, the lower molecular weight band corresponded to a protein from which CTP, 24 amino acids from RbcS, and 3 amino acids encoded by the intervening sequence had been cleaved off (Figure 4, p. 13). In addition to CTP, RbcS, and the intervening sequence, methionine residue has been cleaved off from the modified DMO protein as a result of N-terminal processing by the methionine aminopeptidase, which is a phenomenon commonly observed in all living organisms (Arfin and Bradshaw, 1988; Bradshaw et al., 1998). This protein had a molecular weight of 39.8 kDa and was a single polypeptide chain of 339 amino acids. Hereinafter, this protein form is referred to as "modified MON87708 DMO protein" (Figure 4, p. 13).

Meanwhile, the higher molecular weight band (approximately 42 kDa) corresponded to the modified MON87708 DMO protein retaining the 27 amino acids derived from RbcS and the intervening sequence at the N-terminus. The N-terminal methionine should be processed in the modified MON87708 DMO protein but was not in this protein form, resulting in a polypeptide of 367 amino acids (Figure 4, p.13). Hereinafter, this protein form is referred to as "modified MON87708 DMO+27 protein".

The amino acid sequence of the modified MON87708 DMO precursor protein was analyzed using an algorithm that has been developed for predicting the CTP cleavage sites (Emanuelsson et al., 1999). The analysis revealed that the precursor protein had two potential CTP cleavage sites; cleavage at one of these sites would result in the modified MON87708 DMO protein from which the 84 amino acids are cleaved off,

while cleavage at the other site would result in the modified MON87708 DMO+27 protein retaining the 27 amino acids. The result suggested that the modified DMO protein in this recombinant soybean has two protein forms.

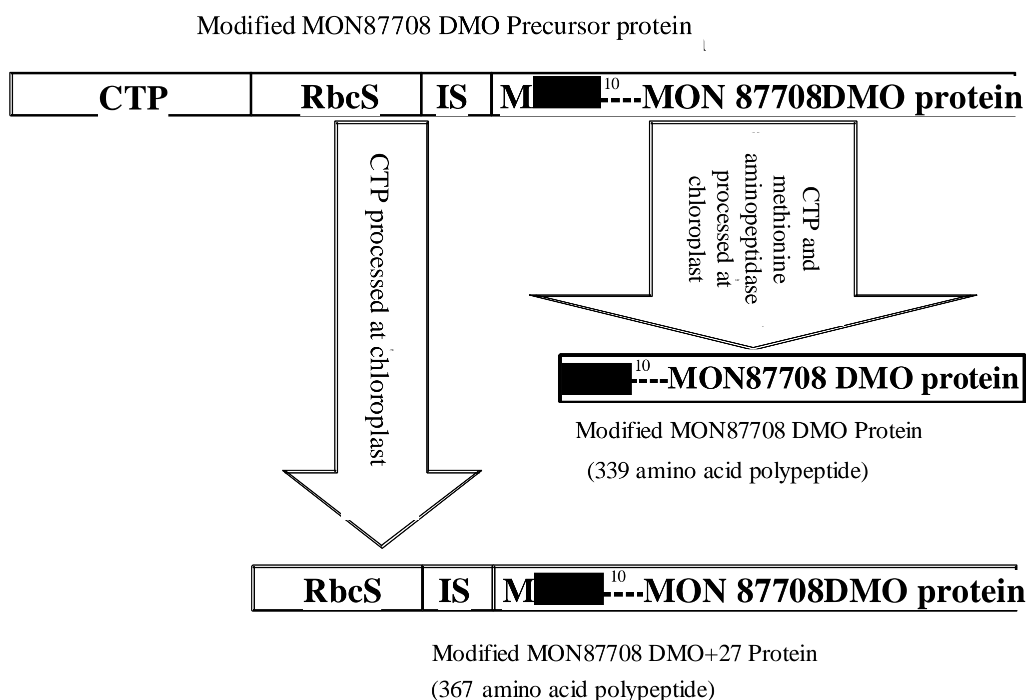

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Figure 4 Processing of the modified MON87708 DMO precursor protein[9]

The modified MON87708 DMO precursor protein produced in this recombinant soybean contains 57 amino acids of the chloroplast transit peptide (CTP), 24 amino acids from the N-terminus of
10 ribulose-1,5-bisphosphate carboxylase small subunit (RbcS), and 3 amino acids encoded by the intervening sequence (IS). M[10] indicates the N-terminal amino acid sequence. Processing of the precursor protein at the chloroplast results in cleavage of CTP, RbcS, IS, and N-terminal methionine (M) and produces the modified MON87708 DMO protein (339 amino acids). Meanwhile, when another mode of processing occurs and only the CTP is cleaved off, the modified MON87708
15 DMO+27 protein consisting of 367 amino acids would be produced, that is, the modified MON87708 DMO protein containing one additional amino acid corresponding to methionine (M) remaining at the N-terminus due to the absence of N-terminal processing by methionine aminopeptidase and additional 27 amino acids from RbcS and IS.

20 The above findings suggest that this recombinant soybean has a DMO protein that lacks the additional 27 amino acids and has the N-terminal methionine cleaved off (modified MON87708 DMO protein) and a DMO protein that has the additional 27 amino acids and retains the N-terminal methionine (modified MON87708 DMO+27

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protein). Thus, hereinafter in this assessment report, the term "modified DMO protein" refers to the modified MON87708 DMO protein and the modified MON87708 DMO+27 protein.

As mentioned earlier (p. 10), in order to confer dicamba tolerance to this recombinant soybean, the modified DMO protein expressed in this recombinant soybean needs to form a trimer in this recombinant soybean. Since this recombinant soybean is tolerant to dicamba herbicide and since the modified DMO protein purified from this recombinant soybean has an enzymatic activity for dicamba demethylation (Figure 2 of Annex 2-C, p. 6), it is assumed that the DMO protein trimer is formed and functioning in this recombinant soybean. Hereinafter, the modified MON87708 DMO protein (monomer), the modified MON87708 DMO+27 protein (monomer), and the trimers comprising either monomer or combination of monomers contained in this recombinant soybean are collectively referred to as "modified MON87708 DMO."

In order to investigate whether the modified MON87708 DMO shares any functionally important amino acid sequences with known allergens, the amino acid sequence of the modified MON87708 DMO+27 protein (Annex 1) was compared with the allergens in the database (AD_2010[11]) using the FASTA algorithm. As a result, it did not share any structurally related sequences with known allergens. Since the amino acid sequence of the modified MON87708 DMO+27 protein contains the amino acid sequence of the modified MON87708 DMO protein, it was also concluded that the modified MON87708 DMO protein did not share any structurally related sequences with known allergens.

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

It was assessed whether the expression of the modified MON87708 DMO causes any change to the metabolic system of the recipient organism.

The substrate specificity of an enzyme is generally determined by the presence or absence of a structure required for the enzymatic catalytic reaction. The specificity of the DMO protein to dicamba owes to the specific interactions that occur in the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). Crystal structure analysis of the DMO protein demonstrated that, when dicamba is metabolized, a carboxyl group and chlorine

[11] AD_2010: A database developed based on the sequences obtained from Food Allergy Research and Resource Program Database (FARRP) (http://www.allergenonline. com). It consists of 1,471 sequences.

atoms in dicamba interact with the amino acids located in the catalytic site of the DMO protein (Dumitru et al., 2009). In the catalytic site of the DMO protein, the carboxyl group forms six hydrogen bonds with the amino acids. These hydrogen bonds play an important role in binding the enzyme to the substrate. Meanwhile, the chloro group in dicamba has a role of stabilizing the substrate in the proper position. In the crystallographic study of the DMO protein, these interactions were observed when dicamba was present at the catalytic site of the DMO protein. These results suggest that, in addition to the benzene ring in dicamba, these chemical groups also play very important roles in proper positioning of the substrate, which is required for the catalytic action (D'Ordine et al., 2009; Dumitru et al., 2009). The DMO protein used for the analyses conducted by D'Ordine et al. (2009) and Dumitru et al. (2009) was the wild-type DMO protein having an alanine inserted at the 2nd position from the N-terminus and a histidine tag attached to its C-terminus (hereinafter referred to as "C-terminal his-DMO protein") (Figure 5, p. 17).

The results of the above studies on the catalytic site of the DMO protein suggest that compounds that are structurally similar to dicamba (phenylcarboxylic acids containing methoxy groups) may potentially react with the DMO protein as substrates. In order to confirm the substrate specificity of the modified MON87708 DMO, the following studies were conducted in the U.S.: (1) studies on substrate reactivity of the DMO protein with various herbicides (studies A-C) and (2) a study on substrate reactivity of the DMO protein with endogenous compounds in soybean (study D) (Table 3, p.16).

Since these studies were conducted using different materials, information regarding the study materials is provided in the following section I. Section II summarizes the study results, while section III discusses the substrate specificity of the modified MON87708 DMO and whether the expression of the modified MON87708 DMO causes any change to the metabolic system of the recipient organism.

Table 3 Study on substrate specificity of the modified MON87708 DMO[12]

Purpose	Study	Description	Study materials	Reference figures and tables
Study on substrate reactivity of the DMO protein with various herbicides	A	Application test of various herbicides (*in vivo*)	This recombinant soybean and non-recombinant control soybean A3525	Table 4 (p.22) of this assessment report Figure 6 (p.18) of this assessment report Table 5 (p. 23) of this assessment report Table 3 (p. 12) of Annex 2-A
	B	Metabolism test of 2,4-D by DMO protein (*in vitro*)	N-terminal his-wild-type DMO protein (produced in and purified from *E. coli*)	Figure 3 (p. 16) of Annex 2-A
	C	Study on binding affinity of 2,4-D to DMO protein crystal (*in vitro*)	C-terminal his-DMO protein (produced in and purified from *E. coli*)	Figure 7 (p. 20) of this assessment report
Study on substrate reactivity of the DMO protein with endogenous compounds in soybean	D	Metabolism test of endogenous compounds in soybean by DMO protein (*in vitro*)	N-terminal his-wild-type DMO protein (produced in and purified from *E. coli*) Modified MON87708 DMO (extracted and purified from seeds of this recombinant soybean)	Figure 8 (p. 21) of this assessment report Figure 1 (p. 4) of Annex 2-B Figures 3–5 (p. 10–12) of Annex 2-B Figure 3 (p. 7) of Annex 2-C

I. Study materials

5 Study A (*in vivo*) was conducted using this recombinant soybean and the non-recombinant control soybean A3525. Studies B–D (*in vitro*) were conducted using three types of DMO proteins; studies B and D were conducted using a wild-type DMO protein derived from *S. maltophilia* DI-6 strain with a histidine tag attached to its N terminus (hereinafter referred to as "N-terminal his-wild-type DMO protein") (Figure 5,
10 p. 17), while study C was conducted using the C-terminal his-DMO protein, which was a wild-type DMO protein having an alanine inserted at the 2nd position from the N-terminus and a histidine tag attached to its C-terminus (Figure 5, p. 17). Thus, the only differences of the two DMO proteins used in the *in vitro* studies from the modified DMO protein expressed in this recombinant soybean were the presence or absence of

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the histidine tag, the presence or absence of the alanine at the 2nd position from the N-terminus in the amino acid sequence, and whether the amino acid residue at position c[13] is tryptophan or cysteine (Figure 5, p. 17). Since the positions of the amino acids at the 2nd and c[14] positions and the histidine tag are sterically distant from the catalytic site of the DMO protein (Figure 3, p. 11) and since it is generally believed that histidine tags do not affect protein structures (Carson et al., 2007), it was concluded that these differences in the amino acid sequence would not affect the substrate specificity of the DMO protein or results of *in vitro* studies. In study D, the modified MON87708 DMO extracted from the seeds of this recombinant soybean was also used to examine whether its substrate specificity was equivalent to that of the DMO protein used for the substrate specificity analysis.

[Confidential: not disclosed to unauthorized persons]

Figure 5 Amino acid sequence comparison between the DMO proteins used in studies A–D and the wild-type DMO protein, the modified MON87708 DMO protein, and the modified MON87708 DMO+27 protein

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[13]Confidential: not disclosed to unauthorized persons
[14]Confidential: not disclosed to unauthorized persons

II. Study results

II-1. Study on substrate reactivity of the DMO protein with various herbicides (studies A–D)

A. Application test of various herbicides (*in vivo*)

An application test of 20 herbicides representing eight families with distinct modes of action was conducted using this recombinant soybean and the non-recombinant control soybean variety A3525 (Table 4, p. 22). The soybean lines were cultivated in a U.S. greenhouse, and each herbicide was applied at two different doses to 10 plants each of the soybean lines prior to germination or at 2- or 3-leaf stages. The level of herbicide injury was examined 20–21 days after application to evaluate the tolerance to herbicides.

As a result, this recombinant soybean exhibited high tolerance to dicamba among the 20 herbicides tested. It was also slightly tolerant to synthetic auxin herbicides: 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2,4-dichlorophenoxybutyric acid (2,4-DB) (Figure 6, p.18) (Table 3 of Annex 2-A, p. 12; Table 5, p. 23). Regarding other herbicides, there was no difference in the level of herbicide injury between this recombinant soybean and the non-recombinant control soybean.

Based on these results, it has been confirmed that the modified MON87708 DMO metabolizes dicamba. It is also suggested that the protein potentially metabolizes 2,4-D, MCPA and 2,4-DB.

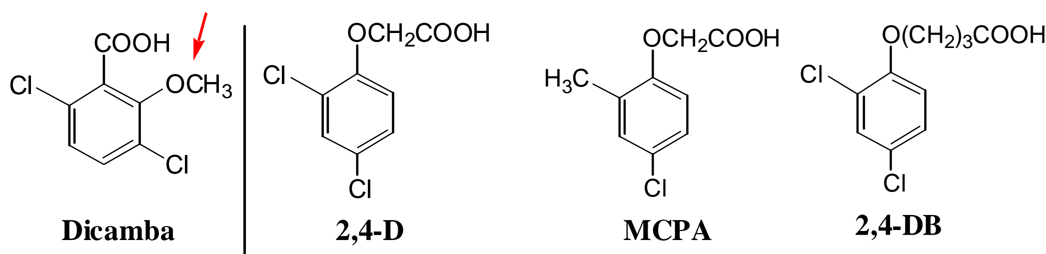

Figure 6 Dicamba and the auxin herbicides to which this recombinant soybean showed low tolerance[15]

Red arrow indicates the methyl group removed by the DMO protein.

B. Metabolism test of 2,4-D by DMO protein (*in vitro*)

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As 2,4-D was most structurally similar to dicamba among the three synthetic auxin herbicides to which this recombinant soybean exhibited low tolerance in study A, it was selected as a representative to study whether herbicides other than dicamba can be metabolized by the DMO protein. Since the DMO protein catalyzes the oxidative *O*-demethylation reaction of dicamba, if the DMO protein is active against 2,4-D, it would catalyze the oxidative *O*-demethylation reaction of 2,4-D and produce 2,4-dichlorophenol (2,4-DCP). To confirm this, 2,4-D was reacted with the N-terminal his-wild-type DMO protein, and formation of 2,4-DCP or any other compound was monitored using LC/UV and LC/MS analyses.

As a result, neither decrease in 2,4-D nor formation of 2,4-DCP or any other compound was detected in either analysis (Figure 3 of Annex 2-A, p. 16).

Therefore, it was concluded that the N-terminal his-wild-type DMO protein cannot metabolize 2,4-D, although this recombinant soybean exhibited slight tolerance to 2,4-D in study A. A possible explanation for this is that 2,4-D binds to the catalytic site of the DMO protein, thereby reducing the amount of herbicidal ingredient in 2,4-D acting on the plants. To confirm this, study C was conducted.

C. Study on binding affinity of 2,4-D to DMO protein crystal (*in vitro*)

In order to assess whether the DMO protein can bind to 2,4-D, crystals of C-terminal his-DMO protein were soaked in solutions of dicamba and 2,4-D and their structures were analyzed.

The result demonstrated that 2,4-D can bind to the C-terminal his-DMO protein with low affinity, although its position in the catalytic site was different from that of dicamba (Figure 7, p. 20). As mentioned earlier, both the carboxyl group and the chlorine atoms (chloro group) in dicamba interact with the amino acids in the catalytic site of the DMO protein to position the substrate (dicamba) in the catalytic site of the DMO protein (D'Ordine et al., 2009; Dumitru et al., 2009). Since 2,4-D also has a chloro group, it can bind to the C-terminal his-DMO protein. However, because the position of the chloro group is different from that in dicamba, it is unlikely that 2,4-D can properly bind to the active site, which explains the failure of the C-terminal his-DMO protein to metabolize 2,4-D. As mentioned above, 2,4-D was selected for this study because it was most structurally similar to dicamba among the three synthetic auxin herbicides (2,4-D, 2,4-DB, and MCPA) to which this recombinant soybean exhibited low tolerance (Figure 6, p. 18). Although 2,4-DB and MCPA were not subjected to metabolism studies and binding affinity studies with the DMO protein, their high structural similarity with 2,4-D suggests that they would possibly bind to the DMO protein but unlikely to be metabolized by the DMO protein.

Therefore, it was concluded that this recombinant soybean exhibited low tolerance to 2,4-D, MCPA, and 2,4-DB, not because the modified MON87708 DMO metabolized

these herbicides but because the modified MON87708 DMO bound to these herbicides to reduce the amount of herbicide active ingredients acting on the plants.

Based on these findings, it has been confirmed that the DMO protein has high specificity to its substrate dicamba and has no potential to metabolize any other structurally related herbicides to produce any new metabolites.

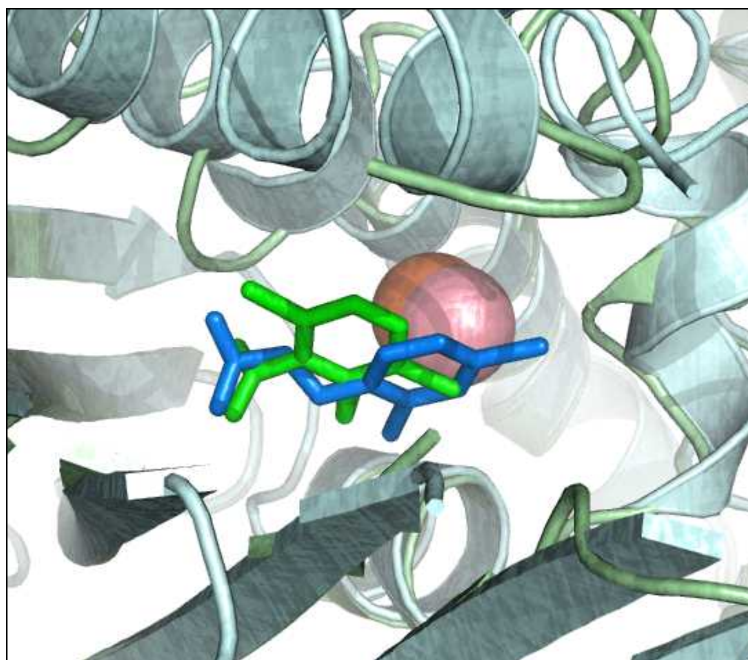

Figure 7 Positions of dicamba and 2,4-D in the catalytic site of the C-terminal his-DMO protein[16]

Dicamba molecule is shown in green, and 2,4-D molecule is shown in blue. Dicamba and 2,4-D cannot bind to the protein at the same time.

II-2. Study on substrate reactivity of the DMO protein with endogenous compounds in soybean (study D)

D. Metabolism test of endogenous compounds in soybean by DMO protein (*in vitro*)

As mentioned earlier (p. 14–22), it is assumed that potential substrates of the DMO protein are limited to compounds that are structurally similar to dicamba (phenylcarboxylic acids containing methoxy groups). Moreover, the result of study C suggests that the chloro group plays a key role in the substrate. Soybean contains many phenolic compounds (Janas et al., 2000; Kim et al., 2006) but is not known to contain any chlorinated aromatic compound. In fact, chlorinated aromatic compounds have a

[16]All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited

limited distribution among plants or other eukaryotes (Gribble, 1998; 2004; Wishart, 2010; Wishart et al., 2009). Among the endogenous compounds in soybean, five compounds (*o*-anisic acid, vanillic acid, syringic acid, ferulic acid, and sinapic acid) that were characterized as those structurally related to dicamba and having methoxy and phenylcarboxylic moieties were subjected to a metabolism study by the DMO protein (Figure 1 of Annex 2-B, p. 4; Figure 8, p. 21).

The five endogenous compounds were added to reaction solutions with or without the N-terminal his-wild-type DMO protein, and the reaction solutions were incubated before they were analyzed by LC/UV and LC/MS to detect any decrease in the added compounds or formation of any other compounds by the DMO protein. As a result, dicamba decreased in both analyses, but neither decrease in the five endogenous compounds nor formation of any other compound was detected in either analysis (Figures 3–5 of Annex 2-B, p. 10–12).

Furthermore, the modified MON87708 DMO was extracted from seeds of this recombinant soybean and reacted with *o*-anisic acid, which was the most structurally similar endogenous compound to dicamba. As a result, *o*-anisic acid was not metabolized by the modified MON87708 DMO (Figure 3 of Annex 2-C, p. 7), which demonstrated that the chloro group is also required for the substrate to be properly positioned in the catalytic site of the modified MON87708 DMO. Moreover, the slight modification in the amino acid sequence of the DMO protein used in this study was not considered to affect the substrate specificity, as the site of modification was distant from the catalytic site of the DMO protein (Figure 3, p. 11).

Based on these findings, it was concluded that the modified MON87708 DMO cannot metabolize any endogenous compounds in soybean to produce any new metabolites.

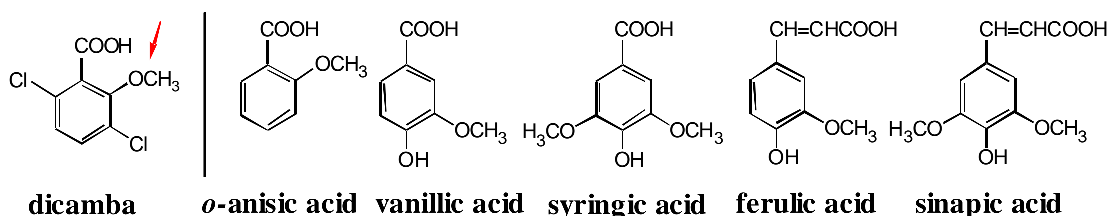

Figure 8 Dicamba and potential endogenous substrates used in the *in vitro* study using the DMO protein[17]

Red arrow indicates the methyl group removed by the DMO protein.

[17]All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited

III. Discussion regarding substrate specificity of the modified MON87708 DMO and effect of the modified MON87708 DMO on the metabolic system of the recipient organism

Based on the results of the above-mentioned studies on substrate reactivity of the DMO protein and the modified MON87708 DMO with various herbicides (studies A-C) and studies on substrate reactivity of the DMO protein and the modified MON87708 DMO with endogenous compounds in soybean (study D), it is very unlikely that the modified MON87708 DMO metabolizes compounds other than dicamba herbicide and affects the metabolic pathway of the recipient organism soybean.

Table 4 Herbicides tested in the application study of various herbicides on this recombinant soybean[18]

Herbicide active ingredient	Herbicide family (mode of action)[1]
Dicamba	Phenoxycarboxylic acid (synthetic auxin)
2,4-D	Phenoxycarboxylic acid (synthetic auxin)
2,4-DB	Phenoxycarboxylic acid (synthetic auxin)
MCPA	Phenoxycarboxylic acid (synthetic auxin)
Triclopyr	Phenoxycarboxylic acid (synthetic auxin)
Clopyralid	Phenoxycarboxylic acid (synthetic auxin)
Picloram	Phenoxycarboxylic acid (synthetic auxin)
Alachlor	Chloroacetamide (inhibition of very long chain fatty acid synthesis)
Acetochlor	Chloroacetamide (inhibition of very long chain fatty acid synthesis)
Atrazine	Triazine (inhibition of photosystem II)
Linuron	Urea (inhibition of photosystem II)
Oxyfluorfen	Diphenylether (inhibition of protoporphyrinogen oxidase)
Lactofen	Diphenylether (inhibition of protoporphyrinogen oxidase)
Chlorimuron	Sulfonylurea (inhibition of acetolactate synthase)
Chlorsulfuron	Sulfonylurea (inhibition of acetolactate synthase)
Halosulfuron	Sulfonylurea (inhibition of acetolactate synthase)
Imazapyr	Imidazolinone (inhibition of acetolactate synthase)
Trifluralin	Dinitroaniline (spindle microtubule assembly inhibition)
Paraquat	Bipyridinium (photosystem I electron diversion)
Glyphosate	Glycine (inhibition of 5-enolpyruvylshikimate-3-phosphate synthase)

[1] (HRAC, 2009)

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

Table 5 Results of the application study of dicamba, 2,4-D, MCPA, and 2,4-DB

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10 [Confidential: not disclosed to unauthorized persons]

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(2) Information concerning vectors

20 1) Name and origin

The vector PV-GMHT4355 used for the development of this recombinant soybean was constructed from several plasmid vectors including plasmid pBR322 derived from *E. coli* (Sutcliffe, 1979).

25

2) Properties

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

30 The total number of base pairs in the plasmid PV-GMHT4355 used for the development of this recombinant soybean is 11,352 bp.

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

35

As a marker gene for selecting the constructed vector in *E. coli*, the *aadA* gene derived from the transposon Tn7 conferring resistance to spectinomycin and streptomycin is present outside the T-DNA region.

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

The infectivity of this vector is not known.

5

(3) Method of preparing living modified organisms

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

10 The component elements of the plasmid vector transferred to the recipient organism are listed in Table 2 (p. 6–8). The positions of the component elements of the donor nucleic acid and sites cleaved by restriction enzymes in the vector are shown in Figure 1 (p. 5).

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

The Agrobacterium method was used to transfer the plasmid vector PV-GMHT4355 into meristems isolated from embryos of the non-recombinant soybean variety A3525.

20 3) Process of rearing of living modified organisms

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

Meristems isolated from embryos of the non-recombinant soybean variety A3525
25 were co-cultivated with *A. tumefaciens* ABI strain containing the plasmid vector PV-GMHT4355, which were subsequently incubated on the tissue culture medium containing glyphosate to select transformed cells.

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

Carbenicillin, Cefotaxime, and ticarcillin-clavulanic acid were added to the tissue culture medium to remove any residual *Agrobacterium* used for transformation. Then, at the R3 generation of this recombinant soybean, PCR analysis was conducted for the
35 backbone region of the plasmid vector PV-GMHT4355 used for transformation. As a result, the plasmid vector backbone region was not detected from this recombinant

soybean (Annex 3), and thus, it was considered that there was no residual *Agrobacterium* used for transformation in this 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 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. Plants damaged by glyphosate were selected as individuals not containing the T-DNA II region (region containing the modified *cp4 epsps* gene expression cassette). Using the selected R1 plants which did not containing the T-DNA II region, further selection was carried out based on the Invader assay[19] and Southern blot analysis to select individuals containing a single copy of the T-DNA I region (region containing the modified *dmo* gene expression cassette) in a homozygous state. The progenies of the selected plants were subjected to the analysis of the inserted genes and morphological assessments, and based on the results,this recombinant soybean was selected as the final commercial line.

The process of rearing of this recombinant soybean is illustrated in Figure 9 (p. 26). The scope of approval for Type 1 Use Regulation covers the R3 generation of this recombinant soybean and any cross progeny derived from the R3 generation.

[19]Invader analysis is a signal amplification technology for detection of genetic mutations or quantitative detection of genes. Invader analysis can detect genes based on cleavage events called the Invader® method and requires no PCR amplification of the genes. In these cleavage events, the target nucleotide sequences are cleaved by an enzyme called Cleavase® recognizing specific structures. Cleavage of the target nucleotide sequence generates fluorescence signals, which can be detected. Invader® and Cleavase® are registered trademarks of Third Wave Technologies, Inc.

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[Confidential: not disclosed to unauthorized persons]

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Figure 9 Process of rearing of this recombinant soybean

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[Confidential: not disclosed to unauthorized persons]

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(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

5

In order to confirm whether the transferred gene exists on the chromosome of this recombinant soybean, homozygous R4 plants of this recombinant soybean were crossed via traditional crossbreeding method with a soybean variety not containing the modified *dmo* gene expression cassette to produce heterozygous F1 plants. The F1 plants were subsequently self-pollinated to produce the F2 generation. The F2 plants were tested for the presence of the modified *dmo* gene expression cassette by Invader analysis, and heterozygous F2 plants were selected and self- pollinated to produce the F3 generation. Then, heterozygous F3 plants were selected and self-pollinated to produce the F4 generation. The resulting F3 and F4 generations were subjected to Invader analysis, and the segregation ratio of the modified *dmo* gene expression cassette in this recombinant soybean was determined based on zygosity (Table 6, p. 28; Annex 4). In this case, the segregation ratio of the modified *dmo* gene expression cassette was expected to be 1:2:1 (homozygote + +: heterozygote + -: homozygote - -) according to Mendel's law.

10

15

As a result, regarding the F3 and F4 generations, no statistically significant difference was found between the observed and expected segregation ratios based on the Chi-square test. Consequently, it was concluded that the transferred gene resides on the chromosome of this recombinant soybean and is inherited by offspring according to Mendel's law.

20

Table 6 Segregation of the modified *dmo* gene during the rearing process of this recombinant soybean[20]

Generation[1]	Number of plants tested[2]	Observed value			Expected value based on 1:2:1 segregation ratio				
		Number of Positive homozygotes	Number of positive heterozygotes	Number of negative homozygotes	Number of positive homozygotes	Number of positive heterozygotes	Number of negative homozygotes	χ^2	p value[3]
F3	118	29	52	37	29.5	59	29.5	2.7	0.253
F4	343	83	171	89	85.75	171.5	85.75	0.2	0.899

[1] F3 and F4 generations were produced by self-pollinating the respective parental generations (F2 and F3 generations) that are heterozygous for the modified
5 *dmo* gene expression cassette. Plants of the F3 and F4 generations were produced from two plants of the respective parental generations.
[2] The presence or absence of the modified *dmo* gene expression cassette was tested by Invader analysis.
[3] The segregation ratios obtained for the F3 and F4 generations were analyzed by Chi-square test ($p \leq 0.05$).

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

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

As a result of Southern blot analysis for existence of the transferred gene, it was confirmed that a single copy of the T-DNA I region was transferred at a single site in the genome of this recombinant soybean (Figures 3–5 of Annex 5, p. 36–38) and was stably inherited in offspring across multiple generations (R2-R6 generations) (Figure 15 of Annex 5, p. 50). In addition, it was confirmed that the backbone region and the T-DNA II region were not transferred to this recombinant soybean (Figures 6–9 of Annex 5, p. 39–42).

The map of the transferred gene in this recombinant soybean is shown in Figure 10 (p. 30).

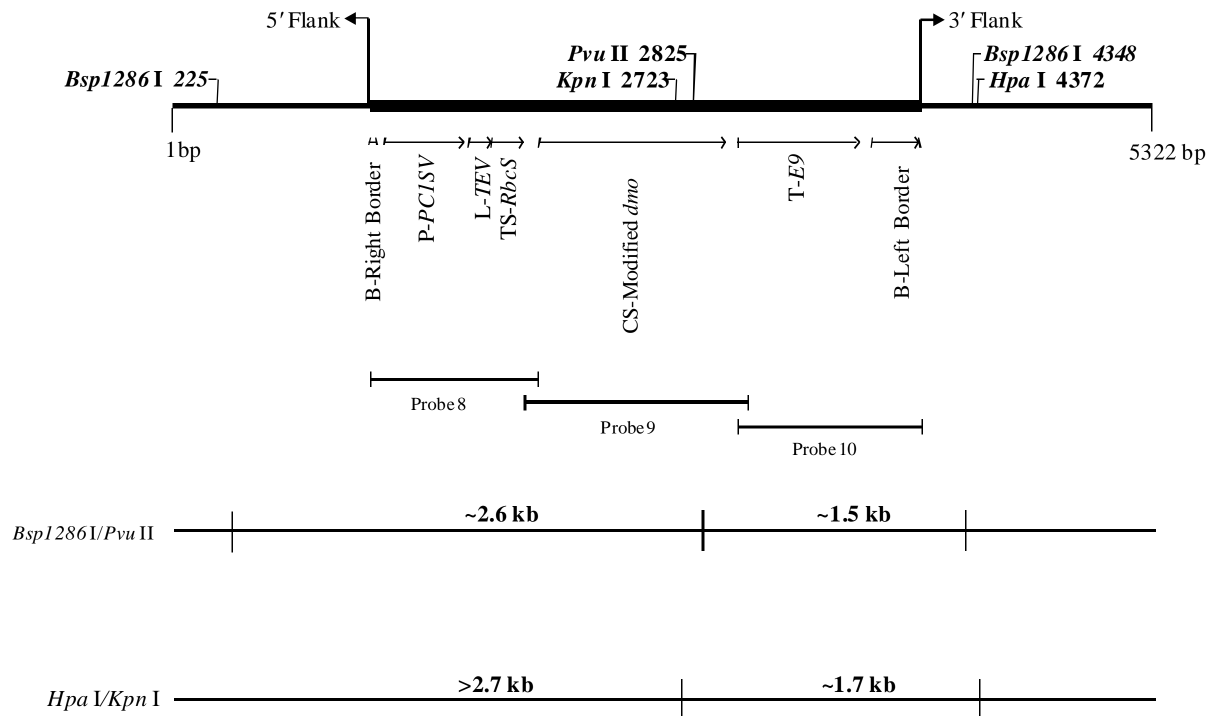

Figure 10 Map of the transferred gene in this recombinant soybean[21]

The top diagram represents the map of the transferred gene and the flanking sequences in this recombinant soybean. The map shows the relative positions of the component elements in the transferred gene and the restriction sites used in Southern blot analysis. The middle diagram shows the relative sizes and positions of the T-DNA I probes shown in Figure 1 of Annex 5 (p. 34). The bottom two diagrams show the expected sizes of the DNA fragments produced after cleavage by the respective restriction enzymes. The arrows (⟶) indicate the 5′ and 3′ ends of the transferred gene and the beginning of the flanking genomic DNA sequences at both ends. The arrows (→) indicate the sequence orientation of the component elements in this recombinant soybean.

[21] All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon Monsanto Japan Limited

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

This item is not applicable because there is only one copy (Figures 3–5 of Annex 5, p. 36–38).

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

Based on Western blot analysis, it has been confirmed that the modified MON87708 DMO is stably expressed across multiple generations (R2-R6 generations) of this recombinant soybean (Figure 2 of Annex 6, p. 17).

Moreover, in 2008, this recombinant soybean was cultivated in three replicated plots in five field sites in the U.S. (one site in Iowa, two sites in Illinois, one site in Indiana, and one site in Pennsylvania), and the expression levels of the modified MON87708 DMO in the over-season leaf (OSL 1~4), roots, forage, and seeds were determined by ELISA (Table 1 of Annex 7, p. 16). Over-season leaf (OSL 1~4), roots, forage, and seeds were sampled from each replicated plot at all field sites, and the leaves were sampled four times at different growth stages (OSL-1: 3- to 4-leaf stage, OSL-2: 5- to 8-leaf stage, OSL-3: full bloom to 12-leaf stage, and OSL-4: beginning seed to 16-leaf stage).

The ELISA used in this study detected all forms of the modified MON87708 DMO, that is, the modified MON87708 DMO protein, the modified MON87708 DMO+27 protein, and all trimers comprising either form or combination of both, and therefore, the expression levels represent the total of the modified MON87708 DMO.

The results obtained from the ELISA demonstrated the expression of the modified MON87708 DMO in the leaves, roots, forage, and seeds of this recombinant soybean (Table 7, p. 32).

Based on the above results, it was found that the modified *dmo* gene transferred to this recombinant soybean is stably inherited across multiple generations and that the modified MON87708 DMO is expressed in the subsequent generations.

Table 7 Expression levels of the modified MON87708 DMO in leaves, roots, forage, and seeds of this recombinant soybean (2008, U.S.)[22]

Tissue type[8]	Modified MON87708 DMO[1] Mean (SD)[2] (µg/g fresh weight)[3]	Range[4] (µg/g fresh weight)	Modified MON87708 DMO Mean (SD) (µg/g dry weight)[5]	Range (µg/g dry weight)	LOQ/LOD (µg/g fresh weight)[6,7]
OSL-1	3.1 (1.9)	0.87 – 6.8	17 (7.7)	6.2 – 29	0.63/0.20
OSL-2	5.2 (2.6)	1.4 – 9.8	31 (13)	12 – 54	0.63/0.20
OSL-3	6.0 (2.2)	3.5 – 11	44 (14)	25 – 71	0.63/0.20
OSL-4	16 (12)	4.6 – 43	69 (46)	23 – 180	0.63/0.20
Root	1.9 (0.73)	1.2 – 3.6	6.1 (2.1)	3.9 – 11	0.031/0.015
Forage	12 (2.5)	7.0 – 17	53 (18)	25 – 84	0.63/0.10
Seed	43 (7.7)	31 – 55	47 (8.7)	34 – 59	1.3/0.21

[1]Represents total of the modified MON87708 DMO (i.e., the two processed protein forms and all
5 trimers comprising either form or combination of both).
[2]The mean and standard deviation (SD) were calculated (n = 15) (1 sample/replicate, 3 replicates/field site, 5 sites in total).
[3]Protein expression levels are expressed as protein weight (µg) per tissue weight (g) on fresh weight basis.
10 [4]Minimum and maximum values were determined for each tissue type.
[5]Protein expression levels are expressed as µg/g dry weight. The dry weight values were calculated by dividing the µg/g fresh weight by the dry weight conversion factors obtained from moisture analysis data.
[6]The limit of quantitation (LOQ) was calculated based on the lowest concentration of the standard
15 DMO protein produced by *E. coli*. The "ng/ml" value was converted to "µg/g fresh weight" using the respective dilution factor and tissue-to-buffer ratio.
[7]The limit of detection (LOD) was calculated as the mean value plus three times the standard deviation determined using the non-recombinant control soybean for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fresh weight" using the respective dilution factor and
20 tissue-to-buffer ratio.
[8]Samples of the individual tissue types were collected at the following growth stages:
OSL-1: V3-V4 (3- to 4-leaf stage) OSL-2: V5-V8 (5- to 8-leaf stage)
OSL-3: R2-V12 (full bloom to 12-leaf stage) OSL-4: R5-V16 (beginning seed to 16-leaf stage)
Root: R6 (full seed) Forage: R6 (full seed)
25 Seed: R8 (full maturity)

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

(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

The transferred nucleic acid does not contain any sequence allowing transmission. Therefore, it is considered unlikely that it could be transmitted through virus infection and/or other routes to any other wild animals and wild plants.

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

This recombinant soybean can be specifically detected and identified by End-Point TaqMan PCR using a primer set specifically binding to this recombinant soybean (Annex 8). The recommended DNA concentration for this assay is 5–10 ng per PCR reaction, so the assay can be performed using a single seed.

The reproducibility and reliability of this method was verified using 46 seeds from this recombinant soybean and 92 seeds from the non-recombinant soybean (Annex 8).

(6) Difference from the recipient organism or the 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

The modified *dmo* gene transferred into this recombinant soybean expresses the modified MON87708 DMO to impart tolerance to dicamba herbicide.

(b) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present[23]

In 2010 and 2011, isolated field tests were carried out in Kawachi Research Farm, Monsanto Japan Limited, using this recombinant soybean. The tests were conducted

[23] All the rights pertinent to the information in a. through g. in this section and the responsibility for the contents rest upon Monsanto Japan Limited

using the R6 generation of this recombinant soybean (Figure 9, p. 26). As the non-recombinant control soybean, A3525, the host plant of this recombinant soybean for gene transfer was used.

a. Morphological and growth characteristics

The differences in morphological and growth characteristics were investigated for 19 items, based on the designated items for classification of seeds and seedling characteristics for registration of seeds and seedlings (initiation of germination, date of germination, uniformity of germination, number of germinated plants, germination rate, shape of leaflet, trichome quantity, time of flower initiation, time of flower completion, elongation type, maturation period, main stem length, number of main stem nodes, number of branches, the lowest main stem node position of podding, weight of forage at harvest time, and appearance of harvested seed (seed hull color, uniformity of seeds, and seed shape)). Statistical analyses were conducted on the data of germination rate, main stem length, number of main stem nodes, number of branches, the lowest main stem node position of podding, and weight of forage at harvest time, but not on the data of initiation of germination, date of germination, uniformity of germination, number of germinated plants, shape of leaflet, trichome quantity, time of flower initiation, time of flower completion, elongation type, maturation period, and appearance of harvested seed (seed hull color, uniformity of seeds, and seed shape). As a result, a statistically significant difference was observed in the germination rate between this recombinant soybean and the non-recombinant control soybean. A difference was also observed in the uniformity of germination between this recombinant soybean and the non-recombinant control soybean (Table 3 of Annex 9, p. 12).

The germination rates of this recombinant soybean and the non-recombinant control soybean were 99.2% and 100%, respectively. There was a one-day difference in the uniformity of germination between this recombinant soybean and the non-recombinant control soybean, which were observed on June 22 and 23, respectively (Table 3 of Annex 9, p. 12).

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

Cold tolerance at the early stage of growth was evaluated in 2008 in the U.S. This recombinant soybean, the non-recombinant control soybean A3525, and six conventional commercial cultivars were grown in a greenhouse, and 21 days after

sowing, the seedlings were transferred to and grown in a climate chamber at 15°C (day)/8°C (night) for 23 days to examine and compare plant vigor, main stem length, growth stage, fresh weight, and dry weight. As a result, for the items subjected to statistical analyses (plant vigor, main stem length, fresh weight, and dry weight), no statistically significant difference was observed between this recombinant soybean and the non-recombinant control soybean, and for the item not subjected to statistical analysis (growth stage), no difference was observed between this recombinant soybean and the non-recombinant control soybean (Table 4 of Annex 10, p. 21).

c. Wintering ability and summer survival of the mature plant

This recombinant soybean and the non-recombinant control soybean raised in an isolated field were left to grow after the maturation period to observe the growth conditions in winter in Japan. As a result of observation made on January 5, 2011, in the plot for investigation of wintering ability, this recombinant soybean and the non-recombinant control soybean were both found dead (Figure 7 of Annex 9, p. 15).

d. Fertility and size of the pollen

Pollens were sampled from this recombinant soybean and the non-recombinant control soybean grown in an isolated field, and the samples were stained with iodine potassium iodide solution to observe their fertility and size. As a result, no significant difference was observed in pollen fertility between this recombinant soybean and the non-recombinant control soybean. Furthermore, no difference was observed in shape or size of pollen (Figure 8 of Annex 9, p. 16).

In addition, in 2008, pollens were sampled from this recombinant soybean and the non-recombinant control soybean grown in a field in Illinois, U.S., to examine their fertility and size. As a result, no statistically significant difference was observed in pollen fertility or size (Table 2 and Figure 1 of Annex 11, p. 15 and p. 16).

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

Items related to seed production (number of ripe pods per plant, approximate grain weight per plant, precise grain weight per plant, and 100-seed weight) were examined in this recombinant soybean and the non-recombinant control soybean grown in an isolated field, and the obtained data were subjected to statistical analyses. As a result,

statistically significant differences were observed in approximate grain weight per plant and 100-seed weight between this recombinant soybean and the non-recombinant control soybean (Table 4 of Annex 9, p. 18). The approximate grain weight per plant was larger in this recombinant soybean (54.7 g) than in the non-recombinant control soybean (51.0 g), and the 100-seed weight was larger in this recombinant soybean (20.6 g) than in the non-recombinant control soybean (19.6 g) (Table 4 of Annex 9, p. 18).

Regarding the shattering habit, this recombinant soybean and the non-recombinant control soybean grown in an isolated field were harvested during the maturation period, and the harvested plants were left to air-dry in a vinyl house before examining the degree of pod shattering. As a result, this recombinant soybean and the non-recombinant control soybean were both found to be shattering-resistant and showed no difference in the pod shattering habit (Table 4 of Annex 9, p. 18).

Regarding dormancy and germination rate, seeds were collected immediately after harvesting this recombinant soybean and the non-recombinant control soybean grown in an isolated field, and the seeds were incubated on a Petri dish at 25ºC to examine the number of germinated plants over time. As a result, the germination rates of this recombinant soybean and the non-recombinant control soybean were both high (98.5% and 98.0%, respectively) and showed no statistically significant difference. Dormancy was not observed in this recombinant soybean or the non-recombinant control soybean (Table 4 of Annex 9, p. 18).

f. Crossability

This recombinant soybean was used as the pollen parent to examine the crossability between this recombinant soybean and the non-recombinant control soybean. The frequency of occurrence of hybrids in the harvested seeds of the non-recombinant control soybean was identified to examine the crossability. Identification of the hybrid was based on whether the progeny exhibits dicamba tolerance as this recombinant soybean used as the pollen parent.

In the plot for investigation of morphological and growth characteristics in an isolated field, seeds were harvested from non-recombinant control soybean plants that were cultivated in rows (except for three plants at each end) neighboring this recombinant soybean. The non-recombinant control soybean plants were neighbored by this recombinant soybean on its southeast and northwest borders at a distance of 1.65 m (Figure 3 of Annex 9, p. 6). The plot was not covered with an insect net during the

flowering period. Five hundred seeds were randomly selected from the harvested seeds and sown in pots in a greenhouse. As soon as they reached 2- to 3-leaf stages, dicamba herbicide was sprayed at a concentration of 0.56 kg a.e./ha[24]. On the 22nd day after herbicide application, the number of surviving plants was counted.

5 Among the 500 seeds sown in this study, none survived till the 22nd day after dicamba herbicide application. Thus, it was concluded that no crossing was observed between this recombinant soybean and the non-recombinant control soybean in this study (Annex 9, p. 19).

10 g. Productivity of harmful substances

To confirm whether or not this recombinant soybean produces any substances affecting soil microbes and other plants, a soil microflora test, a plow-in test and a succeeding crop test were conducted. As a result, no statistically significant difference
15 was observed between this recombinant soybean and the non-recombinant control soybean regarding the number soil microbes and the germination rate and dry weight of radish (Tables 6–8 of Annex 9, p. 20).

In addition, to examine whether this recombinant soybean produces any harmful substances under dicamba application, this recombinant soybean was sprayed with
20 dicamba herbicide at a concentration of 0.56 kg a.e./ha at the 3-leaf stage and was subsequently subjected to a soil microflora tests, a plow-in test, and a succeeding crop test. As a result, no statistically significant difference was observed between the plots of this recombinant soybean to which dicamba herbicide was applied and not applied, regarding the number soil microbes and the germination rate and dry weight of radish
25 (Tables 9–11 of Annex 9, p. 27).

[24]The dicamba concentration sprayed was 0.56 kg a.e./ha, which corresponded to the maximum dose per foliage application among the application system of dicamba herbicide planned for this recombinant soybean after it is commercialized.

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 Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms. Results of the review are listed below.

(1) Item-by-item assessment of Adverse Effects on Biological Diversity

This recombinant soybean was developed by transferring the T-DNA region of the plasmid PV-GMHT4355, constructed based on the plasmid pBR322, etc., derived from *E. coli*, by the *Agrobacterium* method.

Based on the segregation ratio of the transferred gene and the Southern blot analysis, it has been confirmed that a single copy of the T-DNA region, which contains the modified *dmo* gene encoding the modified DMO protein (dicamba mono-oxygenase) derived from *Stenotrophomonas maltophilia*, resides on the chromosome of this recombinant soybean and is stably inherited across multiple generations. In addition, it has been confirmed by Western blot analysis and ELISA that the target gene is stably expressed across multiple generations.

1) Competitiveness

Soybean, the taxonomical species to which the recipient organism belongs, has been cultivated for a long time in Japan, but there is no report that it grows voluntarily in the natural environment in Japan.

Studies on various characteristics related to competitiveness of this recombinant soybean were carried out in 2008 in U.S. fields and in 2010 in isolated fields in Japan. Significant differences were observed between this recombinant soybean and the non-recombinant control soybean regarding germination rate, approximate grain weight per plant, and 100-seed weight in the isolated field test in Japan. Regarding the items not subjected to statistical analyses in the isolated field test in Japan, a difference was observed in uniformity of germination between this recombinant soybean and the non-recombinant control soybean.

The germination rates of this recombinant soybean and the non-recombinant control soybean were 99.2% and 100%, respectively, which were both high. Therefore, it is considered unlikely that the difference in germination rate observed in this study could

enhance the competitiveness of this recombinant soybean.

The uniformity of germination of this recombinant soybean and the non-recombinant control soybean was observed on June 22 and 23, respectively, and was 1 day earlier in this recombinant soybean. However, this was a slight difference, and there was no difference in initiation of germination. Therefore, it is considered unlikely that the observed difference in uniformity of germination enhances the competitiveness of this recombinant soybean.

The mean value of the approximate grain weight per plant was larger in this recombinant soybean (54.7 g) than in the non-recombinant control soybean (51.0 g). However, the mean value of the approximate grain weight per plant of this recombinant soybean falls within the range of the approximate grain weight of conventional soybean observed in isolated field tests conducted in 2006 through 2009. Regarding the 100-seed weight, the mean value was larger in this recombinant soybean (20.6 g) than in the non-recombinant control soybean (19.6 g). However, the mean value of the 100-seed weight of this recombinant soybean falls within the range of the 100-seed weight of conventional soybean observed in isolated field tests conducted in 2006 through 2009 and falls within the range of the 100-seed weight of conventional soybean reported to date. Consequently, it is considered unlikely that the differences observed in the approximate grain weight per plant and 100-seed weight in this study enhance the competitiveness of this recombinant soybean.

This recombinant soybean is given the trait to be tolerant to dicamba herbicide due to the expression of the modified DMO protein. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of dicamba herbicide, the tolerance to dicamba would increase the competitiveness of this recombinant soybean.

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

2) Productivity of harmful substances

Regarding the plant species of soybean to which the recipient organism belongs, there is no report that it produces any substance harmful to wild animals and wild plants.

This recombinant soybean expresses the modified DMO protein, though there is no report that this protein would be a harmful substance, and it has been confirmed to have no amino acid sequence homology with any known allergens. In addition, the modified DMO protein has substrate specificity to dicamba and cannot use any endogenous

substance in soybean structurally related to dicamba as a substrate. Consequently, it is considered very unlikely that this protein would affect the metabolic pathway of the recipient organism and produce any harmful substances.

In addition, as a result of soil microflora tests, plow-in tests and succeeding crop tests carried out in the isolated field in Japan to examine the production of harmful substances by this recombinant soybean (the substances secreted from the roots, which can affect other plants and microorganisms in soil; the substances existing in the plant body, which can affect other plants after dying), no difference was observed between this recombinant soybean and the non-recombinant control soybean.

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

3) Crossability

Since *Glycine soja* Sieb. et Zucc., which is known as a relative of soybean, has the same chromosome number (2n = 40) as soybean and can be crossed with soybean, it was specified and assessed as a potentially affected wild plant.

There is no obstacle to the growth of the hybrid obtained from artificial crossing between soybean and *G. soja*. Thus, in the case where this recombinant soybean and *G. soja* crossed with each other in the natural environment in Japan, there is a possibility that the hybrid would grow and that the gene transferred into this recombinant soybean could diffuse among the population of *G. soja* through backcrossing of the hybrid to *G. soja*. Moreover, since *G. soja* grows voluntarily and widely throughout Japan on riversides, banks, around fields, in orchards, etc., in the case where this recombinant soybean grows near *G. soja*, they could cross with each other.

However,

(a) there is a report regarding hybridization between soybean and *G. soja* and gene penetrance to the progenies that, in a follow-up study conducted on *G. soja* population located near soybean fields throughout Japan for several years, the occurrence of crossing between *G. soja* and soybean was checked using genetic markers, etc., but none of the obtained results demonstrated continuous existence of hybrid progenies;

(b) it is known that the flowering times of soybean and *G. soja* rarely overlap with each other, and there is a report that, even when a soybean variety whose flowering time overlaps that of *G. soja* and *G. soja* were alternately grown at distances of 50 cm, the crossing rate was 0.73%; and

(c) there is a report that, in a crossing study conducted by sowing seeds of glyphosate-tolerant recombinant soybean and *G. soja* at different timings and growing the two species with the *G. soja* vines wrapped around the soybean plants, among the seeds harvested from *G. soja*, one seed was found to be a hybrid with soybean in a plot (11,860 seeds) in which the blooming times of the two species were the closest to each other.

In addition, in an isolated field test conducted in Japan in 2010, this recombinant soybean and the non-recombinant control soybean were cultivated in neighboring plots to examine the occurrence of natural crossing of this recombinant soybean to the non-recombinant soybean, but no crossing was observed. Moreover, when various characteristics related to reproduction were compared between this recombinant soybean and the non-recombinant control soybean, statistically significant differences were observed in the approximate grain weight per plant and the 100-seed weight. However, the mean values of the approximate grain weight per plant and the 100-seed weight, in which statistically significant differences were observed, fell within the range of the values of conventional soybean experimentally cultivated in isolated fields to date. Based on these findings, it was concluded that the reproductive characteristics of this recombinant soybean are within the range of variations in conventional soybean characteristics and that there is no difference in the reproductive characteristics of this recombinant soybean from those of the non-recombinant control, which would affect its crossability with *G. soja*. Consequently, it is likely that the crossability of this recombinant soybean with *G. soja* is extremely low, as in the case of conventional soybean.

Moreover, if this recombinant soybean and *G. soja* crosses with each other, the resulting hybrid would exhibit dicamba tolerance due to the presence of the modified *dmo* gene. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of herbicides, this trait would increase the competitiveness of the hybrid. Therefore, even if such hybrids with dicamba tolerance are produced, they are unlikely to become dominant in *G. soja* populations.

Based on the above understanding, it was judged that the conclusion made by the applicant that the use of this recombinant soybean poses no risk of Adverse Effects on Biological Diversity attributable to competitiveness is reasonable.

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

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant soybean, in accordance

with 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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20 22-23.

List of Annexes for Soybean tolerant to dicamba herbicide (modified *dmo*, *Glycine max* (L.) Merr.) (MON87708, OECD UI: MON-877Ø8-9)

Annex 1	Amino Acid Sequence of the Modified MON87708 DMO+27 Deduced from the Modified *dmo* Gene Used for Developing this Recombinant Soybean (Confidential)
Annex 2-A	Specificity of Dicamba Mono-Oxygenase (DMO) When Different Herbicides are Applied to MON 87708 and Conventional Soybean (RAR-09-500) (Confidential)
Annex 2-B	Specificity of Dicamba Mono-Oxygenase for Potential Endogenous Substrates (RPN-10-365)(Confidential)
Annex 2-C	Specificity of Dicamba Mono-Oxygenase (DMO) Enzyme from MON 87708 Using *o*-Anisic Acid as a Substrate (RPN-10-499)(Confidential)
Annex 3	Summary of PCR Analysis to Confirm the Absence of Agrobacterium Used To Produce Dicamba-Tolerant Soybean MON 87708 (Confidential)
Annex 4	Revised Summary: Heritability and Stability of the *dmo* Expression Cassette in Dicamba-Tolerant Soybean MON 87708 Across Multiple Generations (RPN-08-505)(Confidential)
Annex 5	Amended Report for MSL0022670: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708 (MSL0023278)(Confidential)
Annex 6	Western Blot Analysis of DMO Protein in Dicamba-Tolerant Soybean MON 87708 Leaf Across Multiple Generations Produced in the Greenhouse During 2007 and 2008 (MSL0021459)(Confidential)
Annex 7	Assessment of Total DMO Protein Levels in Soybean Tissues Collected from MON 87708 Produced in United States Field Trials During 2008 (MSL0022510)(Confidential)
Annex 8	A) Soybean GM_A92205 EndPoint TaqMan PCR with *PUB* Internal Control for Single Seed (BQ-QC-10711-01)(Confidential)

B) Supplemental File for BQ-QC-10711-01 Soybean GM_A92205 EndPoint TaqMan PCR with *PUB* Internal Control for Single Seed (Confidential)

5 Annex 9 Biological Diversity Risk Assessment Report of Soybean Tolerant to Dicamba Herbicide (Modified *dmo*, *Glycine max* (L.) Merr.) (MON87708, OECD UI: MON-877Ø8-9) in Isolated Field (Confidential)

10 Annex 10 An Assessment of the Effect of Cold Stress on the Growth of Dicamba-Tolerant Soybean MON 87708 under Growth Chamber Conditions (MSL0021852)(Confidential)

Annex 11 Viability and Morphology Evaluation of Pollen from
15 Dicamba-Tolerant Soybean MON 87708 Produced in a U.S. Field Trial During 2008 (MSL0021880)(Confidential)

Annex 12 Report on Monitoring Results (Confidential)