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

Name: Monsanto Japan Limited
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Approved Type 1 Use Regulation

Name of the type of Living Modified Organism:	Cotton tolerant to dicamba and glufosinate herbicides (modified *dmo*, *bar*, *Gossypium hirsutum* L.) (MON88701, OECD UI : MON-887Ø1-3)
Content of the Type 1 Use of Living Modified Organism:	Provision as food, provision as feed, processing, storage, transportation, disposal, and acts incidental to them
Method of the Type 1 Use of Living Modified Organism:	—

Outline of the Biological Diversity Risk Assessment Report

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

1 Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

Monsanto company has produced a cotton tolerant to herbicides dicamba and glufosinate, provided with tolerant to herbicides dicamba (3,6-dichloro-2-methoxybenzoic acid) and glufosinate (2-amino-4-(hydroxymethylphosphinyl) butanoic acid) (modified *dmo*, *bar*, *Gossypium hirsutum* L.) (MON88701, OECD UI : MON-887Ø1-3) (hereinafter, referred to as "present recombinant cotton").

1) Composition and origins of component element

Composition of donor nucleic acid used for producing the present recombinant cotton and origins of component elements are shown in Figure 1 (p.3) and Table 1 (p.4 to 6).

In the present recombinant cotton, the *dmo* gene derived from *Stenotrophomonas maltophilia* DI-6 strain and the *bar* gene derived from *Streptomyces hygroscopicus* are transferred.

Amino acid sequence of dicamba mono-oxygenase (hereinafter, referred to as "DMO protein") expressed from the *dmo* gene transferred to the present recombinant cotton is in that leucine is inserted just next to methionine at position 1 from N-terminal end on the purpose of facilitate the cleavage of chloroplast transit peptide (hereinafter, referred to as "CTP2"). Hence, the *dmo* gene transferred to the present recombinant cotton is referred to as "modified *dmo* gene", and its expressed protein is referred to as "modified MON88701 DMO protein". 9 amino acids derived from CTP2 are bound in the modified MON88701 DMO protein at its N-terminal end.

Amino acid sequence of phosphinothricin N-acetyl transferase (PAT) expressed from the *bar* gene transferred to the present recombinant cotton is the same as the wild type of PAT protein (hereinafter, referred to as "PAT protein") derived from *S. hygroscopicus*.

Deduced amino acid sequences of the modified MON88701 DMO protein and the PAT protein which are expressed in the present recombinant cotton are indicated in Attachment 1.

2) Function of component elements

1. Functions of individual component elements of donor nucleic acid including target gene, expression regulatory region, localization signal, and selective marker

The function of the donor nucleic acid used for producing the present recombinant cotton is shown in Table 1 (p.4 to 6).

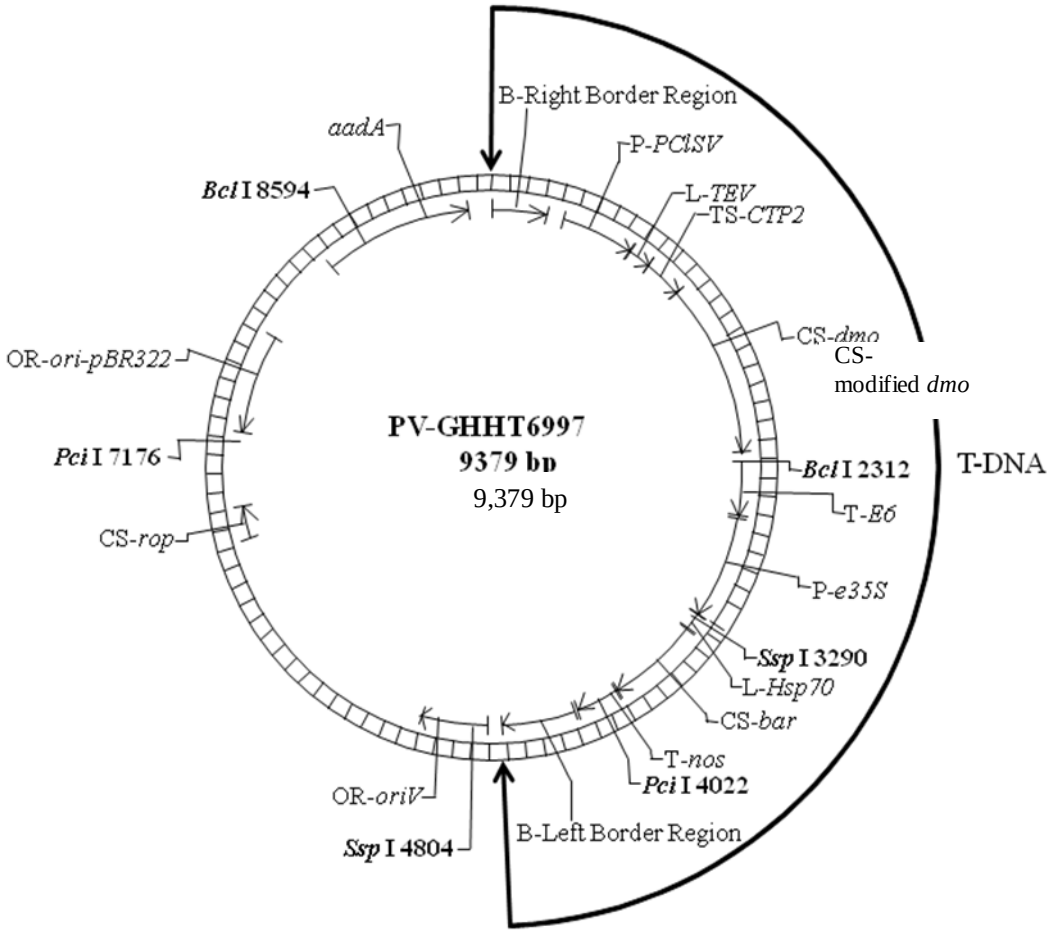

Figure 1 Plasmid map of PV-GHHT6997[1]

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

Table 1 Origins and functions of individual component elements of PV-GHHT6997 used for producing present recombinant cotton[2]

Component elements	Position in the plasmid (bp)	Origin and function
T-DNA		
B[*1]-Right Border Region	1-331	Sequence of a DNA region derived from *Agrobacterium tumefaciens* , containing right border sequence utilized for transmitting T-DNA ((Depicker et al., 1982; Zambryski et al., 1982)).
Intervening Sequence	332-433	Sequence as utilized for DNA cloning
P[*2]-*PClSV*	434-866	Promoter inducing the transcription of Full-Length Transcript (FLt) created from the transcription of full genome of Peanut chlorotic streak caulimovirus (PClSV) (Maiti and Shepherd, 1998).
Intervening Sequence	867-872	Sequence as utilized for DNA cloning
L[*3]-*TEV*	873-1,004	5'-terminal untranslated region derived from Tobacco Etch virus (TEV). Responsible for controlling the gene expression (Niepel and Gallie, 1999).
Intervening Sequence	1,005-1,005	Sequence as utilized for DNA cloning
TS[*4]-*CTP2*	1,006-1,233	Sequence encoding chloroplast transit peptide derived from 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) gene (ShkG) of *Arabidopsis thaliana* (arabidopsis) (Klee et al., 1987; Herrmann, 1995). Transports the modified DMO protein into chloroplast.
CS[*5]-改変 *dmo*	1,234-2,256	Coding sequence of dicamba mono-oxygenase (DMO protein) derived from *Stenotrophomonas maltophilia*, providing the tolerance to herbicides dicamba (Wang et al., 1997; Herman et al., 2005).
Intervening Sequence	2,257-2,310	Sequence as utilized for DNA cloning
T[*6]-*E6*	2,311-2,625	3'-terminal untranslated region derived from E6 gene encoding a fiber protein responsible for forming initial fiber of *Gossypium barbadense* (pima cotton) (John, 1996). Induces polyadenylation of mRNA.
Intervening Sequence	2,626-2,637	Sequence as utilized for DNA cloning
P-*e35S*	2,638-3,249	Promoter of cauliflower mosaic virus (CaMV), containing double enhancer (Kay et al., 1987) (Odell et al., 1985). Constantly induces the transcription in plant cells.

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

Table 1 Origins and functions of individual component elements of PV-GHHT6997 used for producing present recombinant cotton (continued)

Component elements	Position in the plasmid (bp)	Origin and function
T-DNA		
Intervening Sequence	3,250-3,252	Sequence as utilized for the DNA cloning
L-*Hsp70*	3,253-3,348	5'-terminal untranslated region derived from hsp70 gene encoding heat shock protein 70 (HSP70) of *Petunia hybrida* (petunia) (Winter et al., 1988; Rensing and Maier, 1994). Responsible for regulation of the gene expression.
Intervening Sequence	3,349-3,354	Sequence as utilized for DNA cloning
CS-*bar*	3,355-3,906	Sequence containing a gene encoding phosphinothricin N-acetyl transferase (PAT protein) derived from *Streptomyces hygroscopicus* (Thompson et al., 1987). Provides the tolerance to the herbicide glufosinate.
Intervening Sequence	3,907-3,911	Sequence as utilized for DNA cloning
T-*nos*	3,912-4,164	3'-terminal untranslated region of nopaline synthase (nos) gene derived from T-DNA of *A. tumefaciens*, inducing polyadenylation (Bevan et al., 1983; Fraley et al., 1983).
Intervening Sequence	4,165-4,183	Sequence as utilized for DNA cloning
B-Left Border Region	4,184-4,625	DNA region derived from *A. tumefaciens*, containing the left border sequence utilized for transmitting T-DNA (Barker et al., 1983).
Outside backbone sequence of the plasmid (not present in the present recombinant cotton)		
Intervening Sequence	4,626-4,711	Sequence as utilized for DNA cloning
OR 注7-*ori V*	4,712-5,108	Initiating region of replication derived from the broad-host range plasmid RK2. Permits autonomous replication of vector in Agrobacterium (Stalker et al., 1981).
Intervening Sequence	5,109-6,616	Sequence as utilized for DNA cloning
CS-*rop*	6,617-6,808	Coding sequence of primer repressor protein (rop) derived from ColE1 plasmid. Maintains a copy number of the plasmid in Escherichia coli (Giza and Huang, 1989).
Intervening Sequence	6,809-7,235	Sequence as utilized for DNA cloning
OR-*ori-pBR322*	7,236-7,824	Initiating region of replication derived from pBR322, permitting autonomous replication of vector in E.coli (Sutcliffe, 1979).

Table 1 Origins and functions of individual component elements of PV-GHHT6997 used for producing present recombinant cotton (continued)

Component elements	Position in the plasmid (bp)	Origin and function
Outside backbone sequence of the plasmid (not present in the present recombinant cotton)		
Intervening Sequence	7,825-8,354	Sequence as utilized for DNA cloning
aadA	8,355-9,243	Bacterial promoter/coding sequence/3'-untranslated region of 3"(9)-O-nucleotidyl transferase (aminoglycoside modified enzyme) of transposon Tn7 (Fling et al., 1985). Provides resistances to spectinomycin and streptomycin.
Intervening Sequence	9,244-9,379	Sequence as utilized for DNA cloning

[*1]B – Border sequence

[*2]P - Promoter

[*3]L - Leader sequence

[*4]TS - Targeting Sequence

[*5]CS - Coding Sequence

[*6]T - Transcription Termination Sequence

[*7]OR - Origin of Replication

2. 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 allergenicity

【Modified MON88701 DMO protein】

The present recombinant cotton is transferred with the modified *dmo* gene derived from *S. maltophilia*, and expresses the modified MON88701 DMO protein. The modified MON88701 DMO protein provides the tolerance against the herbicide dicamba to the present recombinant cotton. *S. maltophilia* is a gram-negative bacteria eccentrically located in wet environments, soil and plants (Denton and Kerr, 1998), and DI-6 strain was isolated from the soil (Krueger et al., 1989).

DMO protein is an enzyme catalyzing a demethylating reaction from dicamba to DSCA (3, 6-dichlorosalicylic acid), which does not have herbicidal activity, and formaldehyde (HCHO) (Chakraborty et al., 2005), and this action provides the tolerance against dicamba to a plant (Figure 2, p.8). It has been reported that the tolerance to herbicide dicamba is provided for soybean, tomato, Arabidopsis and tobacco by transferring the modified dmo gene (Behrens et al., 2007).

DMO protein is a Rieske-type non-heme iron oxygenase, and constitutes three components, redox system, in addition to redox enzyme, and ferredoxin. These three proteins conjugatively act in the redox system, similar to several other oxygenases, and transport electrons from nicotinamide adenine nucleotide (NADH) to oxygen to catalyze demethylation of electron acceptor substrate (in this case, herbicide dicamba) (Chakraborty et al., 2005). This redox system is shown in Figure 2 (p.8).

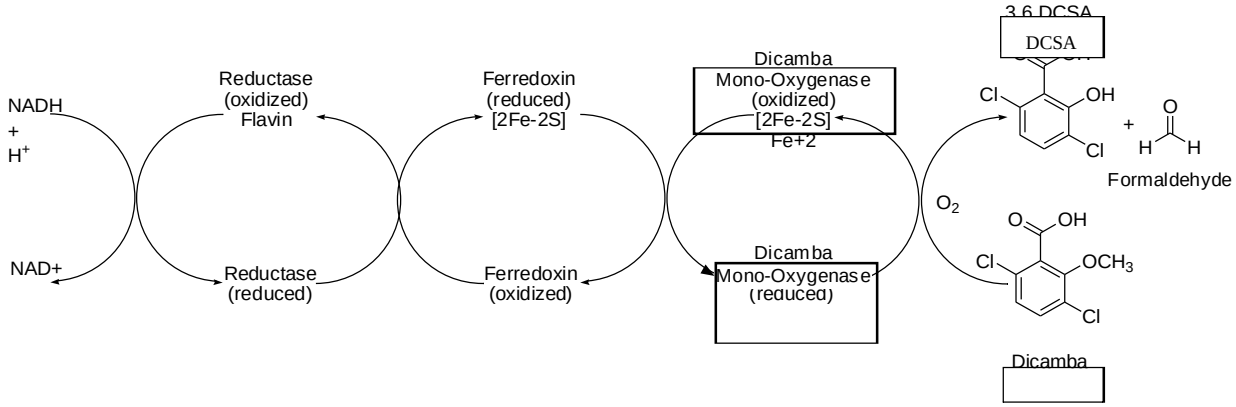

Figure 2 Redox system comprising three components of DMO protein[3]

The Figure indicates electron transfer system from NADH to DMO protein, and produces DSCA by demethylating reaction of dicamba.

The crystal structure of DMO protein is analyzed using DMO protein[4] in which a histidine tag is added in its C-terminal end (D'Ordine et al., 2009; Dumitru et al., 2009). It has been identified that the crystal structure of the DMO protein is a trimer comprising 3 monomers of the DMO protein (Figure 3, p.9). Each monomer Rieske [2Fe-2S] cluster domain contains Rieske [2Fe-2S] cluster and non-heme iron center domain containing nonheme iron center (D'Ordine et al., 2009; Dumitru et al., 2009). It has been known that these domains are commonly present in all Rieske type mono-oxyganases, and is the main domain responsible for electron transportation (Ferraro et al., 2005).

The electrons transported from NADH are transported to the terminal DMO protein through endogenous redox enzyme and ferredoxin (Figure 2, p.8). This electron reductively activates oxygen to catalyze the demethylating reaction of dicamba. Since the electron transportation is carried out between neighbouring monomers, it is necessary that the DMO protein forms the trimer such that the distance and arrangement between monomers are correctly arranged (D'Ordine et al., 2009). Since the distance between the Rieske [2Fe-2S] cluster domain and the nonheme iron center domain are apart from each other in the monomer, the electron transportation is not carried out (D'Ordine et al., 2009; Dumitru et al., 2009).

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

[4] DMO protein provided for analyzing the crystal structure has the same sequence to those of wild type DMO protein, except that a histidine tag is added to its C-terminal end, and that alanine is inserted at position 2 from N-terminal end since the restriction enzyme cleavable site is inserted during the process of the cloning.

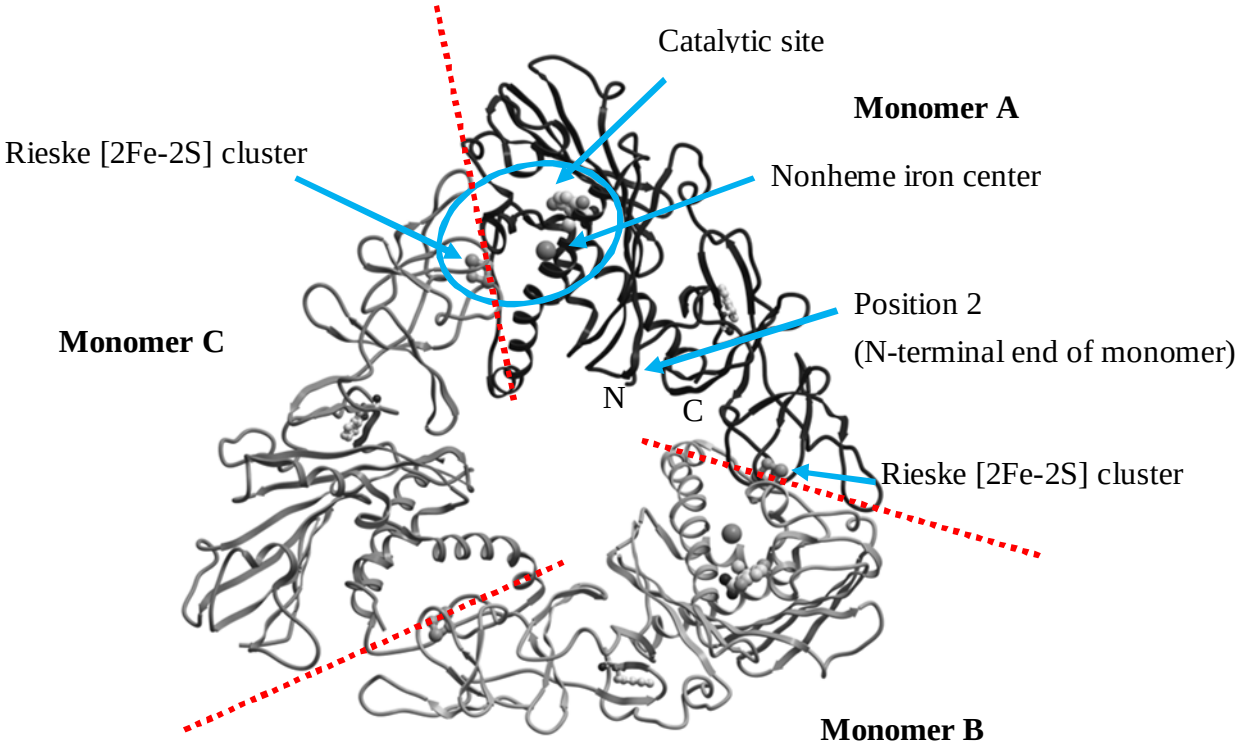

Figure 3 Crystal structure of DMO protein[5]

This figure is a schematic figure of the trimer which is the crystal structure of the DMO protein and its active form (D'Ordine et al., 2009). The characteristic structure including the nonheme iron center domain containing the nonheme iron center, N-terminal and C-terminal and the Rieske [2Fe-2S] cluster domain containing the Rieske [2Fe-2S] cluster is indicated as monomer A. The catalytic site formed by monomer A and monomer C is circled in blue. In addition, the Rieske [2Fe-2S] cluster of monomer C is indicated with an arrow. Since the electron transfer is carried out between neighboring monomers, the electron transfer is carried out between the Rieske [2Fe-2S] cluster domain of monomer C and the nonheme iron center domain of monomer A. The red dotted line indicates a junction at which the electron transfer is carried out between neighboring nonheme iron center domains subunits of the Rieske [2Fe-2S] cluster domain, and indicates the border line of the each monomer. For DMO protein provided for analyzing the crystal structure, histidine tag is added at its C-terminal end instead of the wild type DMO protein or modified MON88701 DMO protein and alanine is inserted at position 2 from the N-terminal end. (see Figure 4, p.14, regarding modification of the amino acid sequence).

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

As mentioned above (p.7), it is necessary that the modified MON88701 DMO protein expressed in the present recombinant cotton forms the trimer in the present recombinant cotton, in order to possess the tolerance to the herbicide dicamba for the present recombinant cotton. Since it was confirmed that the present recombinant cotton possesses tolerance to the herbicide dicamba and the demethylating enzyme activity to dicamba is found in the modified MON88701 DMO protein produced and purified from E.coli (Figure 2, p.8 of Attachment 4), it was considered that the trimer of the modified MON88701 DMO protein is formed in the present recombinant cotton to function it.

While whether or not the modified MON88701 DMO protein shares a similar amino acid sequence with the existence allergen is compared by FASTA algorithm and consecutive 8 amino acids using AD_2013[6], no similarity with the existence allergen was found.

【PAT protein】

The present recombinant cotton is transferred with the *bar* gene derived from *S. hygroscopicus*, and expresses PAT protein. PAT protein provides tolerance to the herbicide glufosinate (Thompson et al., 1987).

Glufosinate exerts its herbicidal activity by binding it with glutamine synthase. The glutamine synthase is the main enzyme assimilating ammonium produced by photorespiration to a plant. Glufosinate is bound with glutamine synthase to inhibit glutamine synthase, accumulate the toxicity of ammonium within plant body and discharge the plant (Wild and Manderscheid, 1984; Manderscheid and Wild, 1986).

PAT protein produced from the present recombinant cotton is acetyltransferase acetylating glufosinate. Glufosinate is changed to N-acetyl glufosinate upon acetylating it by the action of this enzyme. N-acetyl glufosinate does not inhibit photorespiration, since it cannot be bound with glutamine synthase. So, accumulation of ammonium is avoided. The present recombinant cotton is not discharged by the production of PAT protein, even when the herbicide glufosinate is sprayed.

While whether or not PAT protein shares a similar amino acid sequence with the existence allergen is compared by FASTA algorithm and consecutive 8 amino acids 8 amino acids using AD_2013[7], no

[6] Database comprising sequences registered in FARRP (Food Allergy Research and Resource Program) Allergen Online database (FARRP, 2013) , containing 1,630 amino acid sequences in January 2013.
[7] Database comprising sequences registered in FARRP (Food Allergy Research and Resource Program) Allergen Online database (FARRP, 2013) , containing 1,630 amino acid sequences in January 2013.

similarity with the existence allergen was found.

3. Contents of any change caused to the metabolic system of recipient organism

Whether or not the metabolic system contained in the recipient organism is changed by expressing the modified MON88701 DMO protein and PAT protein is examined.

【Modified MON88701 DMO protein】

Generally, substrate specificity is determined whether or not necessary structure for an enzymatic reaction is present. The specificity of DMO protein to dicamba is based on a specific interactive action carried out at the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). According to the result of analyzing the crystal structure of DMO protein for the metabolism of dicamba, carboxyl group and chlorine atom of dicamba react with amino acid located at the catalytic site of DMO protein (Dumitru et al., 2009). The carboxyl group forms 6 hydrogen bonds with amino acid at the catalytic site of the DMO protein. The hydrogen bond plays an important role for binding the enzyme and the substrate. On the other hand, the chlorine atom has a role that the substrate is stably located at a proper position. These interactive actions are confirmed in crystal analysis of DMO protein when dicamba is present at the catalytic site of DMO protein. Accordingly, it has been indicated that not only benzene ring but also these chemical groups of dicamba play an extremely important role for properly locating the substrate necessary for the enzymatic action (D'Ordine et al., 2009; Dumitru et al., 2009). For DMO protein analyzed by D'Ordine et al. (2009) and Dumitru et al. (2009), alanine is, inserted at position 2 from its N-terminal end of the wild type DMO protein and histidine tag is added at its C-terminal end (hereinafter, referred to as "C-terminal his-DMO protein") (Figure 4, p.14).

According to research relating to the catalytic site of DMO protein as mentioned above, there is a possibility that compounds structurally similar to dicamba (phenylcarboxylic acid containing methoxy group) is a substrate for DMO protein. In order to confirm the substrate specificity for the modified MON88701 DMO protein, 1) substrate reactivity test between various types herbicides and DMO protein (test A), and 2) substrate reactivity test between endogenous compound in cotton and the DMO protein (test B) are conducted in US.

Since materials to be provided are different in each test, the materials to be tested are primarily

described in the following I. Next, in the following II, outline of the result of the test is described. In the following III, it is discussed regarding the substrate specificity of the modified MON88701 DMO protein, and whether or not the metabolic system contained in the recipient organism is changed by expressing the modified MON88701 DMO protein.

I. Concerning materials to be provided

The present recombinant cotton and a control cotton without any recombination Coker130 were provided in test A (*in vivo*). DMO protein provided in test B (*in vitro*) (hereinafter, referred to as "N-terminal his-DMO protein") has an identical amino acid sequence to the DMO protein isolated from *S. maltophilia* DI-6 strain except for the his-tag at N terminus (Figure 4, p.14). Accordingly, a difference between DMO protein provided in the in vitro test and the modified DMO protein expressed in the present recombinant cotton is only that the histidine tag is present or absent, and leucine at position 2 from the N-terminal end of the amino acid sequence is present or absent (Figure 4, p.14). Since the amino acid at the position 2 and the position of the histidine tag are steric structurally apart from the catalytic site of DMO protein, and since it has been said that the histidine tag does not generally affect the structure of a protein (Carson et al., 2007), it was considered that the difference of the amino acid sequence does not affect the substrate specificity of DMO protein and the result of the in vitro test. In order to evaluate whether or not the modified MON88701 DMO protein has the same specificity with those of DMO protein used for analyzing the substrate specificity, the modified MON88701 DMO protein produced and purified in E.coli was also provided in test B.

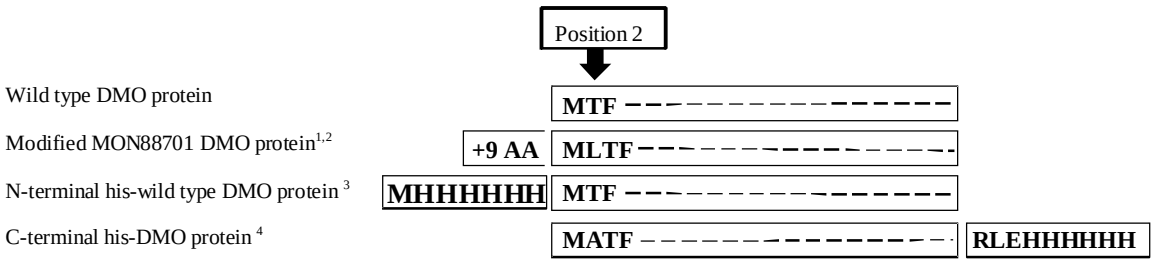

Figure 4 Comparison between amino acid sequences of wild type DMO protein, modified MON88701 DMO protein and DMO proteins provided in tests A and B[8]

This figure indicates various amino acid sequences of DMO proteins described in this evaluation report.
[1]Modified MON88701DMO protein expressed in the present recombinant cotton is in that leucine is inserted just next to methionine at position 1 from the N-terminal end. In addition, 9 amino acids derived from CTP2 are bound at the N-terminal end.
[2]Modified MON88701 DMO protein provided in test B is produced in E.coli and purified.
[3] N-terminal his-wild type DMO protein provided in test B is produced in E.coli and purified.
[4] C-terminal his-DMO protein is provided for analyzing the crystal structure (D'Ordine et al., 2009;

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Dumitru et al., 2009).

II. Result of tests

II-1. Substrate reactivity test between various types of herbicide and DMO protein (test A)

A. Spraying test of various types of herbicide (*in vivo*)

The present recombinant cotton and control cotton without any recombination Coker130 were provided to conduct the spraying test for 8 groups 10 types of herbicide which have different mechanisms of action (Table 2, p.15). Each herbicide was sprayed with 2 steps of an amount to be sprayed on 10 individuals of cotton within each line, at prior to germination or 2 to 5 leaves stage, cultivated in a US greenhouse to evaluate the tolerance to herbicide by investigating harmful effects by the herbicide at days 20 to 22 after spraying.

As a result, the present recombinant cotton showed a strong tolerance to herbicide dicamba among the 10 types of herbicides (Table 2, p.15) (Table 2, p.6 of Attachment 2). No difference between the present recombinant cotton and the control cotton without any recombination was found in the harmful effects by the herbicides except dicamba.

Table 2 Herbicides provided in the spraying test for the various types of herbicide against the present recombinant cotton[9]

Active component of herbicide	Type of herbicide (Mechanism of action) [1]
Dicamba	Phenoxy carboxylates (Artificial auxin)
2,4-D	Phenoxy carboxylates (Artificial auxin)
2,4-DB	Phenoxy carboxylates (Artificial auxin)
Acetochlor	Chloro acetoamides (Inhibition of synthesizing very long chain fatty acid)
Atrazine	Triazines (Inhibition of photochemical II)
Oxyfluorfen	Diphenyl ethers (Inhibition of protoporphilinogen oxydase)
Halosulfuron	Sulfonyl ureas (Inhibition of acetolactate synthase)
Trifluralin	Dinitro anilines (Inhibition of forming spindle microtubule)
Paraquat	Bipyridiliums (Electron conversion on photochemical I)
Glyphosate	Glycines (Inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase)

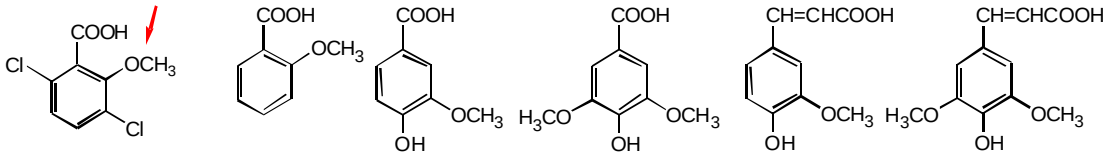
[1]Prepared from RAC (2011)

II-2. Substrate reactivity test between endogenous compound in cotton and DMO protein (test B)

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

B. Metabolic test of endogenous compound in cotton using DMO protein (*in vitro*)

As mentioned above (p.18), it was considered that a possible compound as substrate for DMO protein is only a compound structurally similar to dicamba (phenyl carboxylate containing methoxy group). Accordingly, 5 types of compounds (o-anisic acid, vanillic acid, syringic acid, ferulic acid and sinapinic acid) which could be specified as structurally similar to dicamba and having methoxy and phenyl carboxyl portion among the endogenous compound in cotton were provided to conduct a metabolic test using DMO protein (Figure 1, p4; Figure 5, p. 16 of Attachment 3).



Dicamba ***o*-anisic acid** **Vanillic acid** **Syringic acid** **Ferulic acid** **Sinapinic acid**

Figure 5 Dicamba and Endogenous compounds provided *in vitro* test using DMO protein[10]

The red arrow indicates methyl group removed by DMO protein.

The endogenous compounds as mentioned above were added to a reacting solution containing N-terminal his-wild type DMO protein and a reacting solution not containing N-terminal his-wild type DMO protein to incubate it. Then, LC-UV analysis and LC-MS analysis were conducted on the reacting solutions to investigate whether or not the compounds as added decreased, or other compounds were produced from the compound as added. As a result, while dicamba decreased, the 5 endogenous compounds did not decrease, and other compounds were not detected (Figure 3 to 5, p.10 to 12 of Attachment 3).

The modified MON88701 DMO protein produced and purified from E.coli was reacted with o-anisic acid, which is the endogenous compound structurally similar to dicamba. As a result, o-anisic acid was not metabolized by the modified MON88701 DMO protein (Figure 3, p.9 of Attachment 4).

According to the above-mentioned result, it was considered that the modified MON88701 DMO protein metabolizes the endogenous compound in cotton and produces a new metabolite.

III. Discussion of the effects on substrate specificity of the modified MON88701 DMO protein and the effects of the modified MON88701 DMO protein on the metabolic system contained in the recipient organism

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

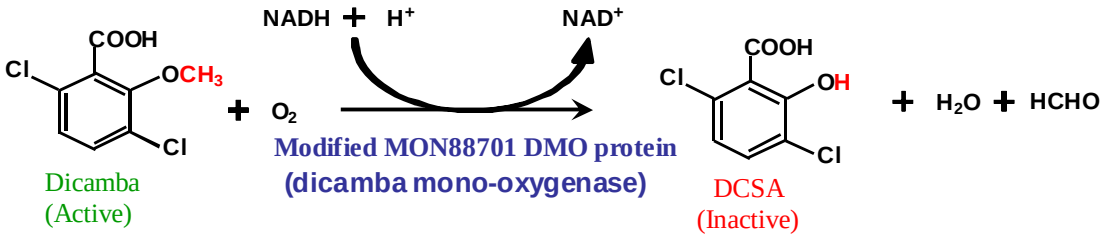
According to the results of the substrate reactivity test between the various types of herbicide and the modified MON88701 DMO protein (test A), and the substrate reactivity test between the endogenous compound in cotton, DMO protein and the modified MON88701 DMO protein (test B), it was considered to be extremely less possibility that the modified MON88701 DMO protein metabolizes the compounds except dicamba and provides any effect on the metabolic system of the recipient organism cotton.

【PAT protein】

PAT protein shows a high specificity to glufosinate in the presence of acetyl CoA. While glufosinate is categorized as L-amino acid, PAT protein never acetylates other L-amino acids. In addition, it was indicated in a competitive assay that the acetylation of glufosinate by PAT protein was not inhibited, even in the presence of various types of amino acid with high concentration. Further, it has been reported that the acetylation of glufosinate by PAT protein was not inhibited in the presence of L-glutamic acid similar to glufosinate (Wehrmann et al., 1996). According to the above-mentioned matters, it was considered that PAT protein has high substrate specificity against glufosinate and does not affect the metabolic system of the recipient organism.

As mentioned above, since each enzymatic reaction is different, and the structure of each substrate is quite different, it is hard to consider that the modified MON88701 DMO protein and PAT protein expressed in the present recombinant cotton interactively affect each other in the plant body.

＜Inactivation of dicamba＞



＜Inactivation of glufosinate＞

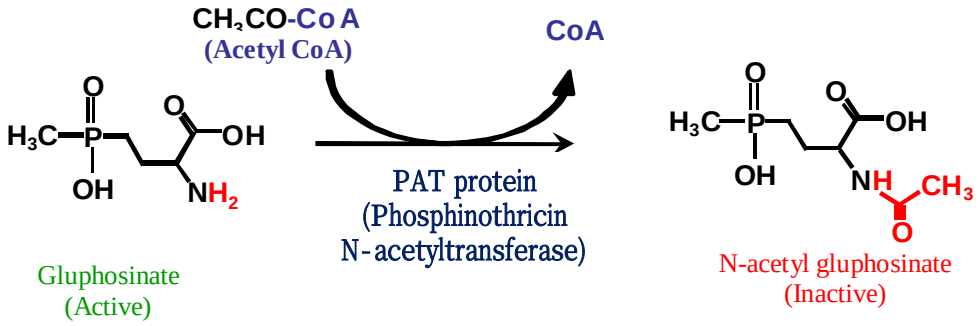


Figure 6 Substrates and metabolites of the modified MON88701 DMO protein and PAT protein[11]

(2) Information concerning vectors

1) Name and origin

The vector PV0GHHT6997 used for producing the present recombinant cotton was assembled from plasmids including the vector pBR322 and the like (Sutcliffe, 1979).

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

2) Properties

1. The number of base pairs and nucleotide sequence of vector

The total number of base pairs in PV-GHHT6997 used for the production of the present recombinant cotton was 9,379 bp.

2. Presence or absence of nucleotide sequences containing specific functions, and those functions

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

3. Presence or absence of infectious characteristics of vector and, if present, information concerning the host range

The present vector is not known to be infectious.

(3) Method of preparing living modified organisms

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

Component elements of the present plasmid vector transferred to the recipient organism are described in Table 1 (p.4 to 6). In addition, the location of the component element of the donor nucleic acid within the vector and the section to be cleaved by restriction enzymes are shown in Figure 1 (p.3).

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

The T-DNA region of the plasmid vector PVGHHT6997 is transferred to the embryo axis of the cotton without any recombination Coker130 by *Agrobacterium* method.

3) Process of growing living modified organisms

1. Mode of selecting cells containing the transferred nucleic acid

After the dividing tissue collected from the embryo axis of the cotton without any recombination Coker130 and *A. tumefaciens* ABI strain comprising the plasmid vector PV-GHHT6997 were concurrently cultivated, the transformed cells were selected in the tissue culture supplemented with the herbicide glufosinate.

2. Presence or absence of remaining *Agrobacterium* in the case of using the *Agrobacterium* method for transferring nucleic acid

The *Agrobacterium* bacterium which is used for transformation was removed by the tissue culture medium supplemented with carbenicillin and cefotaxime. Further, that the *Agrobacterium* did not remain in the present recombinant cotton was confirmed, and that after the present recombinant cotton moved to a medium not supplemented with carbenicillin and cefotaxime, no colony of *Agrobacterium* formed on the medium was observed.

3. Process of growing and pedigree trees of the following lines: cells to which the nucleic acid were transferred; the line in which the state of existence of replication products of transferred nucleic acid was confirmed; the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effects on Biological Diversity

The re-differentiated individual (R0) as transformed was sprayed with the herbicide dicamba (0.56 kg a.e.[12]/ ha) and the herbicide glufosinate (0.59 kg a.i.[13]/ha) to select an individual comprising the modified *dmo* gene and the *bar* gene. Then, the R0 individual, as not discharged, was inbred to select an individual having 1 copy of T-DNA region (the modified *dmo* gene expression cassette and the *bar* gene expression cassette) as homo in its progeny, and R1 generation as homo using TaqMan PCR

[12] a.e.; acid equivalent. Herbicide products contains a salt of active ingredient or itself as the active ingredient. In the case of the active ingredient in salt form, the active ingredient is an acid, and the base portion is different, depending on the product. In the case of indicating an amount of the salt of the active ingredient in the product as a sprayed amount of the herbicide, a converted amount of acid as an active ingredient was used as a unit to be described, since correct amounts of the active ingredient cannot be compared between the products containing different base portions.

[13] a.i.; active ingredient. In the case of the herbicide glufosinate, the amounts of the active ingredient can be compared based on the amount of the salt of the active ingredient, since the base portion is the same in the different products.

method. In addition, the present recombinant cotton was finally selected with a superior phenotype and presence state of the transferred gene as indices.

The process of growing of the present recombinant cotton is shown in Figure 7 (p.21). The scope to be approved for Type 1 Use Regulation includes R3 generation and the progeny lines of R3 generation of the present recombinant cotton.

【Confidential: not disclosed to unauthorized persons】

Figure 7 Process of growing of the present recombinant cotton

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

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

In order to investigate whether or not T-DNA (the modified *dmo* gene expression cassette and the *bar* gene expression cassette) of the present recombinant cotton is present on the chromosome, the segregation ratio of T-DNA was analyzed in R1, BC1F1 and BC1F2 generations of the present recombinant cotton with a chi-square test.

In order to produce 3 generations (R1、BC1F1、BC1F2; Figure 7, p.21) to be provided in the test, the transformed re-differentiated individual (R0) was primarily inbred to confirm the segregation ratio of T-DNA in its progeny R1 generation using the Real-Time TaqMan PCR method. Further, 8 individuals of the present recombinant cotton containing T-DNA as homo possessed from R3 generation was bred with a cotton line DP0949 not having T-DNA to produce a F1 generation. 15 individuals containing T-DNA as homo possessed from F1 generation were selected using the End-Point TaqMan PCR method and bred with DP0949 to produce BC1F1 generation. The individual containing T-DNA as hetero was selected by spraying it in the BC1F1 generation with the herbicide glufosinate to confirm it using the End-Point TaqMan PCR method. 25 individuals containing T-DNA as homo possessed from BC1F1 generation were inbred to produce BC1F1 generation. The individual in BC1F1 generation was sprayed with the herbicide glufosinate and whether or not the individual possesses T-DNA using the End-Point TaqMan PCR method was confirmed. A chi-square test was conducted using the obtained segregation ratio.

As a result of the chi-square test, no statistical significant difference between the observed value and the expected value in the three generations as analyzed was confirmed (Table 3 to

Table 4, p.23; Table 1 to 2, p.7 of Attachment 5). Accordingly, it was considered that T-DNA of the present recombinant cotton is present on the chromosome and it is inherited and consistent with the Mendel law.

Table 3 Segregation ratio of T-DNA of the present recombinant cotton in R1 and BC1F2 generations[14]

		Observed values			Expected values having 1:2:1 in segregation ratio				
Generation	Number of Individuals to be provided[1]	Positive/homo Number of Individuals	Positive/hetero Number of Individuals	Negative/homo Number of Individuals	Positive/homo Number of Individuals	Positive/hetero Number of Individuals	Negative/homo Number of Individuals	χ^2	p value[4]
R1[2]	173	33	99	41	43.25	86.50	43.25	4.35	0.114
BC1F2[3]	118	36	56	26	29.50	59.00	29.50	2.00	0.368

[1] 173 individuals in R1 generation were obtained from 1 individual in parent generation and 118 individuals in BC1F2 generation were obtained from 25 individuals in parent generation.

[2] Observed value was confirmed whether or not T-DNA (the modified *dmo* gene expression cassette and the *bar* gene expression cassette) was present using the Real-Time TaqMan PCR method.

[3] Observed value was confirmed whether or not T-DNA (the modified *dmo* gene expression cassette and the *bar* gene expression cassette) was present using the End-Point TaqMan PCR method.

[4] The segregation ratio obtained from the above-mentioned 2 generations was analyzed with the chi-square test ($p \leq 0.05$).

Table 4 Segregation ratio of T-DNA of the present recombinant cotton in BC1F1 generation[15]

		Observed value		Expected value having 1:1 in segregation ratio			
Generation	Number of Individuals to be provided[1]	Positive/hetero Number of Individuals	Negative/homo Number of Individuals	Positive/hetero Number of Individuals	Negative/homo Number of Individuals	χ^2	p value[3]
BC1F1[2]	261	123	138	130.50	130.50	0.86	0.353

[1] 261 individuals in BC1F1 generation was obtained from 15 individuals in parent generation.

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

[2]Observed value was obtained by spraying the herbicide glufosinate (0.59 kg a.i./ha) and was confirmed whether or not T-DNA (the modified *dmo* gene expression cassette and the *bar* gene expression cassette) was present using the End-Point TaqMan PCR method.

[3] Segregation ratio obtained from BC1F1 generation was analyzed with the chi-square test ($p \leq 0.05$).

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

As a result of analyzing the transferred gene using the Southern blotting analysis, it was confirmed that one copy of T-DNA region was introduced in one place in the nuclear genome of the present recombinant cotton (Figure 4 to 6, p.37 to 39 of Attachment 6), and that it was stably inherited across multiple generations (R2 to R6 generations) (Figure 4, p.24 of Attachment 7). In addition, it was confirmed that the outside backbone was not transferred (Figure 7, p.40 of Attachment 6).

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

This item is not applicable, since there is one copy for all genes (Figure 4 to 6, p.37 to 39 of Attachment 6).

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

The Western blotting analysis was conducted for multiple generations (R2 to R6 generations) of the present recombinant cotton, and it was confirmed that the modified MON88701 DMO protein and PAT protein were stably expressed (Figure 5 to 6, p.25 to 26 of Attachment 7).

In addition, samples of leaves (over-season leaf; OSL-1 to 4), roots, pollen and cotton seeds were collected from the present recombinant cotton which were grown with 4 repeats in the farm fields of 8 places in the US (Desha County, Arkansas, Tift County, Georgia, Pawnee County,Kansas, Rapides Parish, Louisiana, Perquimans County, North Carolina, Dona Ana County, New Mexico, Barnwell County,South Carolina and Hale County, Texas) were collected to analyze the expressed amount of the modified MON88701 DMO protein and PAT protein with the ELISA method (Table 5~Table 6 p.26 to 28; Attachment 8). As a result, it was confirmed that the modified MON88701 DMO protein and PAT protein were expressed in the leaf (over-season leaf; OSL-1 to 4), root, pollen and cotton seed of the present recombinant cotton (Table 5~Table 6, p.26 to 28; Table 1 to 2, p.19 to 20 of Attachment 8).

Table 5 Expressed amount of the modified MON88701 DMO protein in each tissue of the present recombinant cotton (2010, US)[16]

Tissue[1]	Growing stage [3]	Day after seeding [2]	Average value (standard deviation) Range (μg/g FW)[4]	Average value (standard deviation) Range (μg/g DW)[5]	Lower limit for detection/ Lower limit for quantification [6] (μg/g FW)
Leaf (OSL[8]-1)	2 to 4 leaves stage	14-25	27 (7.6) 13 – 42	180 (52) 110 – 280	0.168/0.313
Leaf (OSL-2)	4 to 7 leaves stage	25-37	41 (12) 19 – 65	240 (69) 110 – 380	0.168/0.313
Leaf (OSL-3)	9 leaves to time of flower initiation	35-99	52 (17) 24 – 97	240 (75) 91 – 410	0.168/0.313
Leaf (OSL-4)	Time of flower initiation to cutout [9]	70-121	57 (18) 0.70 – 91	230 (59) 2.8 – 310	0.168/0.313
Root	Time of 50% flowering to Time of flowering	62-99	14 (3.7) 8.2 – 21	43 (12) 26 – 72	0.136/0.313
Pollen [7]	Time of 50% flowering to Time of flowering	68-99	14 (28) 0.31 – 110	NA (NA) NA	0.043/0.125
Cotton seed	Matured stage	148-183	20 (4.6) 8.2 – 29	21 (5.0) 8.9 – 33	0.059/0.313

[1]Samples of leaves (over-season leaf; OSL-1 to 4), roots, pollen and cotton seeds were collected from the present recombinant cotton which were grown with 4 repeats in the farm fields of 8 places in US (Desha County,Arkansas, Tift County, Georgia, Pawnee County, Kansas, Rapides Parish, Louisiana, Perquimans County, North Carolina, Dona Ana County, New Mexico, Barnwell County and Hale County, Texas) were collected to analyze the expressed amount of the modified MON88701 DMO protein and PAT protein with the ELISA method.

[2] Indicates range of days after seeding at sampling in the 8 farm fields indicated in note 1.

[3]Growing step at which each tissue was collected. Described in Ritchie et al. (2007).

[4]Expressed amount of the protein was indicated as arithmetic mean and standard deviation (indicated in parenthesis). In addition, the weight of the protein was indicated as μg per 1 g of fresh weight of the tissue. The average value, the standard deviation and the range (lower limit and upper limit) of each tissue was calculated based on the data obtained from all farm fields. (The number of samples (n) is n=32, except for the following samples. The leaf of OSL-3 is n=31, since the data in one sample was less than LOD, and the leaves of OSL-1 and OSL-4 is n=28, since the samples could not be collected

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from 1 farm field, pollen is n=29, since the data in 2 samples is less than LOD and the data in one sample could not be determined.)

[5]Weight of the protein is indicated as μg per 1 g of dry weight of the tissue. The dry weight was obtained by dividing the fresh weight with a conversion coefficient for dry weight obtained from the data of water analysis. NA=Not Applicable (no correspondence).

[6]Lower limit for quantification=limit of quantification (LOQ); Lower limit for detection=limit of detection (LOD)

[7]Water amount of pollen cannot be measured, obtained sample was limited. Due thereto, the expressed amount in pollen is indicated only as fresh weight.

[8] OSL= over-season leaf (Leaf)

[9] Cutout indicates a period that the distribution of the nutrient element changed from the growth of flower to the growth of boll, after the time of flowering generally passed. The indication therefor is the period that the upper flower blossomed with 4 to 5 nodes from the upper side.

Table 6 Expressed amount of PAT protein in each tissue of the present recombinant cotton (2010, US)[17]

Tissue[1]	Growing stage[3]	Day after seeding[2]	Average value (standard deviation) Range (μg/g FW)[4]	Average value (standard deviation) Range (μg/g DW)[5]	Lower limit for detection/ Lower limit for quantification[6] (μg/g FW)
Leaf (OSL[8]-1)	2 to 4 leaves stage	14-25	0.84 (0.21) 0.46 – 1.4	5.5 (1.5) 3.7 – 9.1	0.162/0.188
Leaf (OSL-2)	4 to 7 leaves stage	25-37	1.1 (0.26) 0.68 – 1.6	6.4 (1.4) 3.8 – 9.4	0.162/0.188
Leaf (OSL-3)	9 leaves to time of flower initiation	35-99	1.0 (0.34) 0.34 – 1.7	4.8 (2.0) 1.3 – 10	0.162/0.188
Leaf (OSL-4)	Time of flower initiation to cutout[9]	70-121	0.78 (0.29) 0.42 – 1.7	3.2 (1.2) 2.0 – 6.7	0.162/0.188
Root	Time of 50% flowering to Time of flowering	62-99	0.56 (0.18) 0.27 – 0.89	1.8 (0.75) 0.93 – 3.3	0.096/0.188
Pollen[7]	Time of 50% flowering to Time of flowering	68-99	0.56 (0.24) 0.27 – 0.90	NA (NA) NA	0.021/0.188
Cotton seed	Matured stage	148-183	6.1 (0.95) 4.8 – 8.8	6.6 (1.1) 5.2 – 9.6	0.032/0.188

[1]Samples of leaves (over-season leaf; OSL-1 to 4), roots, pollen and cotton seeds were collected from the present recombinant cotton which were grown with 4 repeats in the farm fields of 8 places in US (Desha County, Arkansas, Tift County, Georgia, Pawnee County, Kansas, Rapides Parish, Louisiana, Perquimans County, North Carolina, Dona Ana County, New Mexico, Barnwell County and Hale County, Texas) were collected to analyze the expressed amount of the modified MON88701 DMO protein and PAT protein with the ELISA method.

[2] Indicates range of days after seeding at sampling in the 8 farm fields indicated in note 1.

[3]Growing step at which each tissue was collected. Described in Ritchie et al. (2007).

[4] Expressed amount of the protein was indicated as arithmetic mean and standard deviation (indicated in parenthesis). In addition, the weight of the protein was indicated as μg per 1 g of fresh weight of the tissue. The average value, the standard deviation and the range (lower limit and upper limit) of each tissue was calculated based on the data obtained from all farm fields. (The number of samples (n) is n=32, except for the following samples. The leaf of OSL-1 is n=28, since the sample could not be

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collected in one farm field, the leaf of OSL-4 is n=27, since the sample could not be collected in one farm field, and since the date of one sample is less than LOD, the leaf of OSL-3 is n=31, since the data of one sample is less than LOD, and pollen is n=6, since the data in 26 samples is less than LOD)

[5] Weight of the protein is indicated as μg per 1 g of dry weight of the tissue. The dry weight was obtained by dividing the fresh weight with a conversion coefficient for dry weight obtained from the data of water analysis. NA=Not Applicable (no correspondence).

[6] Lower limit for quantification=limit of quantification (LOQ); Lower limit for detection=limit of detection (LOD)

[7] Water amount of pollen could not be measured and the obtained sample was limited. Due thereto, the expressed amount in pollen was indicated only as fresh weight.

[8] OSL= over-season leaf (Leaf)

[9] Cutout indicates the period that the distribution of the nutrient element is changed from the growth of flower to the growth of boll, after the time of flowering is generally passed. The indication therefor is the period that the upper flower blossomed with 4 to 5 nodes from the upper side.

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

There is no possibility that the transferred nucleic acid is transmitted to wild animals and wild plants through infection of viruses and other routes, since the transferred nucleic acid sequence does not have any function of transmission.

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

The present recombinant cotton can be detected and identified with the End-Point TaqMan PCR method by utilizing a primer set which can specifically bind to the present recombinant cotton (Attachment 9). It is recommended that the concentration of DNA used for detection is a range of 5 to 10 ng per single reaction of PCR, and it can be detected using one seed.

A confirmation test for reproducibility was conducted using 90 seeds of the present recombinant cotton and 89 seeds of the cotton without any recombination (p.6 of Attachment 9).

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

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

The modified *dmo* gene and the *bar* gene transferred to the present recombinant cotton express the modified MON88701 DMO protein and PAT protein to provide tolerance to the herbicide dicamba and the herbicide glufosinate.

For conventional cotton, the spraying of the herbicide dicamba and the herbicide glufosinate was limited for treating the weed stem and leaf prior to seeding. However, since the present recombinant cotton has resistance to the herbicide dicamba and the herbicide glufosinate, applicable range of spraying dicamba and glufosinate was elongated. It is possible for dicamba to treat the present recombinant cotton from germination and the growth period at 7 days before harvesting in addition to the treatment of the weed stem and leaf from after seeding to germination. It is possible for glufosinate to treat the present recombinant cotton from germination to the growth

period until the early stage of flowering, in addition to the treatment of the weed stem and leaf from after seeding to germination. Systemic usage of the herbicide dicamba and the herbicide glufosinate against the present recombinant cotton, as scheduled upon commercialization of the present recombinant cotton is shown in Table 7 (p. 322).

Table 7 Systemic usage of the herbicide dicamba and the herbicide glufosinate on the present recombinant cotton[18]

	Period of spraying				
	Treatment prior to germination	Stem and leaf treatment			
		4 nodes	8 nodes	12 nodes	7 days prior to harvesting
Maximum systemic usage[1]	1.12 kg a.e./ha[3] Dicamba	0.56 kg a.e./ha Dicamba; 0.89 kg a.i./ha Glufosinate	0.56 kg a.e./ha Dicamba; 0.59 kg a.i./ha Glufosinate	0.56 kg a.e./ha Dicamba; 0.59 kg a.i./ha Glufosinate	0.56 kg a.e./ha Dicamba
Recommended systemic usage against the routine weed species	0.56 kg a.e./ha Dicamba	0.56 kg a.e./ha Dicamba	0.59 kg a.i./ha Glufosinate	No spraying	No spraying
Recommended systemic usage against perennial weed species [2]	0.56 kg a.e./ha Dicamba	0.56 kg a.e./ha Dicamba	0.59 kg a.i./ha Glufosinate	0.56 kg a.e./ha Dicamba or 0.59 kg a.i./ha Glufosinate	0.56 kg a.e./ha Dicamba

[1]Indicated as maximum amount for usage in each growing step. The upper limit of total amount for usage of dicamba is 2.24 kg a.e./ha, and 1.79 kg a.i./ha for glufosinate.

[2]Including cases where the time of growing the weed is late.

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The herbicide dicamba effectively controls 95 or more types of the yearly plant and biennial plant, and suppresses the growth of 100 or more types of perennial plant species of board leaf and woody plants. The herbicide glufosinate is a broad contact type non-selective herbicide controlling 120 or more types of broad leaf plants and bent grass. In addition, the herbicide dicamba and the herbicide glufosinate can control blackweed (*Ambrosia artemisiifolia*), horseweed (*Conyza canadensis*), pigweed (*Amaranthus palmeri*) and the like which are resistance to the herbicide glyphosate.

2. With respect to the physiological or ecological characteristics listed below, presence or absence of differences between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

At present, commercial cultivation of cotton is rarely conducted in Japan, only for decoration. In addition, it has not been reported that the seed of cotton imported for oil or for feed into Japan is grown natively in the natural environment of Japan through spillage during transportation.

In an isolated field (hereinafter, referred to as "present isolated field) of Kawauchi research farm of Japan Monsanto Limited, the isolated field test of the present recombinant cotton was performed. The R5 generation of the present recombinant cotton was provided for the test (Figure 7, p.21). For the control cotton without any recombination, Coker130 was used as the parent line to be transferred with genes for the present recombinant cotton being used. The low temperature resistance test (I. 2-(6)-(b)-b, p36) in the initial period of growth was performed in an artificial climate laboratory in the US in 2010.

a Morphological and growth characteristics

In order to evaluate the morphological and growth characteristics, 9 items (flower initiation, dry weight on the aerial part, shape of boll before opening, mature period, culm length, number of nodes, color in fiber (floccus), and color in the episperm) were evaluated.

The statistical analysis was conducted for the dry weight on the aerial part, culm length and the number of nodes, and no statistical analysis were conducted for the shape of boll before opening, mature period, color in fiber (floccus) and color in the episperm.

As a result, no statistical significant differences between the present recombinant cotton and the control cotton without any recombination were found in the items which were statistically analyzed. In the items which were not statistically analyzed, a difference between the present recombinant cotton and the control cotton without any recombination was found in the flower initiation (Table 3, p.13 of Attachment 10).

The flower initiation was July 28 for the present recombinant cotton and July 31 for the control cotton without any recombination (Table 3, p.13 of Attachment 10).

b Cold-tolerance at the early stage of growth

The cold-tolerance test in early stage of growth was conducted in an artificial climate laboratory of US Monsanto Company. Young seedlings of the present recombinant cotton, the control cotton without any recombination Coker130 and 4 conventional commercial products at day 20 after seeding were grown at 15°C in daytime/10°C at nighttime for 12 days to compare growth stage, plant vigour, plant length, fresh weight and dry weight.

As a result, the difference between the present recombinant cotton and the control cotton without any recombination was small for the items which were not statistically analyzed (growing stage and plant vigour), and the growth response against low temperature was similar (Table 2, p.6 of Attachment 11). On the other hand, a statistically significant difference between the present recombinant cotton and the control cotton without any recombination was confirmed in the fresh weight and dry weight among the items which were statistically analyzed (plant length, fresh weight and dry weight) (Table 2, p.6 of Attachment 11).

The average value of the fresh weight was 18.5 g for the present recombinant cotton and 21.0 g for the control cotton without any recombination, and those of the present recombinant cotton was lower (Table 2, p.6 of Attachment 11). In addition, the average value of the dry weight was 3.6 g for the present recombinant cotton and 4.4 g for the control cotton without any recombination, and those of the present recombinant cotton was lower (Table 2, p.6 of Attachment 11).

c Wintering ability of the mature plant

The present recombinant cotton and the control cotton without any recombination which were grown in the isolated field were continuously grown after the mature stage to observe the growing status during the winter season in Japan. As a result of observing the individual which was cultivated in the wintering ability test site on January 9, 2013, both the present recombinant cotton and the control cotton without any recombination were discharged (Figure 8, p.16 of Attachment 10).

d Fertility and pollen size

Pollen collected from the present recombinant cotton and the control cotton without any recombination which were grown in the isolated field were stained with Alexander solution to measure the fertility and the size of the pollen. As a result of statistically analyzing these items, no statistically significant difference between the present recombinant cotton and the control cotton without any recombination was found in either fertility or the size of the pollen (Figure 9 and Table 4, p.17 of Attachment 10).

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

Produced amount:

For the present recombinant cotton and the control cotton without any recombination which were grown in the isolated field, items relating to the produced amount of the seed (number of bolls per single individual, number of seeds per boll, weight of seeds per boll and weight of 100 seeds) were investigated. As a result of statistically analyzing these items, a statistically significant difference between the present recombinant cotton and the control cotton without any recombination was found in the weight of 100 seeds (Table 5, p.18 of Attachment 10). The average value of 100 seeds was 8.05g for the present recombinant cotton and 8.79g for the control cotton without any recombination, and those of the present recombinant cotton was lower (Table 5, p.18 of Attachment 10).

Shedding habit:

The seeds at harvesting for the present recombinant cotton and the control cotton without any recombination were hard to separate since the fibers were entangled, and

shedding under the natural condition was not found.

Dormancy and germination rate:

In order to use harvested seeds of the present recombinant cotton and the control cotton without any recombination which were grown in the isolated field for a germination test, floccus was removed from the seeds by hand, short fibers (linter) remaining on the surface were removed with sulfuric acid, dried and stored at normal temperature. Then, the seeds were arranged on a germination sheet wetted with water, and the germination sheet was wound with a roll shape for storage in an isothermal vessel set at 25°C for 10 days. The number of germinated seeds was counted after 10 days.

As a result, both germination rates for the present recombinant cotton and the control cotton without any recombination were high (Table 6, p.19 of Attachment 10).

f Crossability

A test for crossability was not conducted, since an alliance of *Gossypium* genus, which can be bred with *G.hirsutum,* to which the present recombinant cotton belongs, is not grown in Japan.

g Productivity of harmful substances

In order to confirm that substances which provide an effect on the soil microflora and other plants are not produced from the present recombinant cotton, soil microflora test, plow-in test and succeeding crop test were conducted. As a result, statistically significant differences were found in a number of filamentous bacteria in the soil microflora test. The number of filamentous bacteria was 1.4×10^6 CFU/g in the site for the present recombinant cotton and 1.6×10^6 CFU/g in the site for the control cotton without any recombination, and those in the site for the present recombinant cotton was lower (Table 7, p.20 of Attachment 10). However, the range of the number of filamentous bacteria in the site for the present recombinant cotton (1.2×10^6 CFU/g to 1.6×10^6 CFU/g) is encompassed within the range of the number of filamentous bacteria in the site for the control cotton without any recombination (1.1×10^6 CFU/g to 1.9×10^6 CFU/g) (Table 7, p.20 of Attachment 10).

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

A review was made by persons with specialized knowledge and experience concerning Adverse 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 Organisms based on the Law Concerning the Conservation and Sustainable Use of Biological Diversity Through Regulations on the Use of Living Modified Organisms. Results of the review are listed below.

（1）Results of the assessment of Adverse Effects on Biological Diversity

The present recombinant cotton is produced by transferring PV-GHHT6997 constituted from the plasmid pBR322 derived from *E.coli* (*Escherichia coli*) by *Agrobacterium* method.

It was confirmed with the method of separating genes and the Southern blotting analysis for the present recombinant cotton that one copy of T-DNA region comprising the modified *dmo* gene encoding the modified DMO protein derived from *Stenotrophomonas maltophilia* and the *bar* gene encoding PAT protein derived from *Streptomyces hygroscopicus* is incorporated on the chromosome and the region is stably transmitted across multiple generations. In addition, it was confirmed with the Western blotting analysis that the target gene is stably expressed across multiple generations.

a. Competitiveness

While the biological species cotton to which the recipient organism belongs has an actual achievement for use during a long term in Japan, it has not been reported that the cotton is natively grown under natural conditions in Japan.

In an isolated field in Japan in 2012, several traits in the competition of the present recombinant cotton were investigated. As a result, a difference between the present recombinant cotton and the control cotton without any recombination was found in the flowering initiation. In addition, statistically significant differences between the

present recombinant cotton and the control cotton without any recombination were found in the produced amount of seed (weight of 100 seeds). However, the differences were small, and it was considered that these differences do not increase the competitiveness of the present recombinant cotton. No difference between the present recombinant cotton and the control cotton without any recombination was found in the other items.

On the other hand, as a result of investigating the cold-tolerance in the early stage for growth in 2010 in the US, while statistically significant differences between the present recombinant cotton and the control cotton without any recombination were found in the fresh weight and e dry weight, these differences were small, and it was considered that these differences do not increase the competitiveness of the present recombinant cotton.

While the tolerance to the herbicide dicamba by the production of the modified DMO protein and the tolerance to glufosinate by the production of PAT protein is provided for the present recombinant cotton, it can be hardly considered that the tolerance to these herbicides increase the competitiveness under the natural conditions in which these herbicides are not possibly sprayed.

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

b. Productivity of harmful substances

The biological species cotton to which the recipient organism belongs contains gossypol indicating toxicity to mammals and cyclopropene fatty acid causing the bruising of eggs and a decrease in extrication rates. However, at present, commercial cultivation of cotton has been rarely conducted in Japan, and it has not been reported that cotton is natively grown in Japan. Accordingly, there is an extremely low possibility that wild animals, wild plants and the like will be harmed by feeding. In addition, there is no report that cotton produces harmful substances which prevent the inhabitation and growth of the wild animals, animal plants and the like, similar to other sensitive substances.

While the present recombinant cotton produces the modified DMO protein providing tolerance to the herbicide dicamba and PAT protein providing tolerance to the herbicide glufosinate, it was confirmed that both proteins do not have a similar sequence with the existing allergen. In addition, both proteins have high substrate

specificity, and it was considered that the effects of both proteins on the metabolic system of the recipient organism due thereto, do not produce a new harmful substance, and do not affect the content of gossypol and cyclopropene fatty acid.

Whether or not the present recombinant cotton produces a harmful substance (which is secreted from the roots to affect other plants or soil microflora, and which is contained in the plant body and affects other plants after the plant body is discharged) was investigated in the plow-in test and the succeeding crop test. As a result, while statistically significant differences between the test site for the present recombinant cotton and the control cotton without any recombination were found in the number of filamentous bacteria in the soil microflora test, the difference was small and it was considered that the present recombinant cotton produces harmful substances based on this difference. No statistically significant difference between the present recombinant cotton and the control cotton without any recombination was found in the other items relating to the producibility of harmful substances between the site for the present recombinant cotton and the site for the control cotton without any recombination.

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

c. Crossability

As mentioned above, it was determined that the conclusion made by the applicant that wild animals and wild plants which can be bred with the cotton are not specified in the natural environment in Japan and there is no possibility of Adverse Effects on Biological Diversity that is attributable to crossability was reasonable.

(2) Conclusion based on the Biological Diversity Risk Assessment

Based on the above understanding, the conclusion described in the Biological Diversity Risk Assessment Report that the use of the present recombined cotton in accordance with the type 1 Use Regulation causes no Adverse Effects on Biological Diversity in Japan has been judged to be reasonable.

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List of Attachments for Cotton tolerant to dicamba and glufosinate herbicides (modified *dmo*, *bar*, *Gossypium hirsutum* L.) (MON88701, OECD UI : MON-887Ø1-3)

Attachment 1 Deduced amino acid sequences of the modified MON88701 DMO protein and PAT protein from the modified *dmo* gene and the *bar* gene (Confidential)

Attachment 2 Specificity of MON 88701 Dicamba Mono-Oxygenase (DMO) When Ten Different Herbicides are Applied to MON 88701 and Conventional Cotton (RPN-2011-0078) (Confidential)

Attachment 3 Specificity of Dicamba Mono-Oxygenase for Potential Endogenous Substrates (RPN-10-365) (Confidential)

Attachment 4 Amended Report: Specificity of *E. coli*-produced MON 88701 Dicamba Mono-Oxygenase (DMO) Enzyme Using o-Anisic Acid as a Substrate (RPN-2011-0079) (Confidential)

Attachment 5 Segregation Analysis of the Coding Sequences Present in Herbicide-Tolerant Cotton MON 88701 Across Multiple Generations (RPN-2011-0089) (Confidential)

Attachment 6 Molecular Characterization of Dicamba Glufosinate-Tolerant Cotton MON 88701 (MSL0023280) (Confidential)

Attachment 7 Stability of DNA Insert and Expression of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701 (MSL0023322) (Confidential)

Attachment 8 Amended Report for MSL0024006: Assessment of MON 88701 DMO and PAT (*bar*) Protein Levels in Tissues from Dicamba Glufosinate Tolerant Cotton (MON 88701) Produced in U.S. Field Trials during 2010 (MSL0024523) (Confidential)

Attachment 9 Cotton GH_S26695 EndPoint TaqMan PCR with *ACP* Internal Control for Single Seed (BQ-QC-10842-02) (Confidential)

Attachment 10 Biological Diversity Risk Assessment Report of the cotton tolerance to the herbicides dicamba and glufosinate (modified *dmo*, *bar*, *Gossypium hirsutum* L.) (MON88701, OECD UI: MON-887Ø1-3) in an isolated field (Confidential)

Attachment 11 Assessment of the Effect of Cold Temperature on Dicamba and Glufosinate Tolerant Cotton MON88701 under Growth Chamber Conditions in 2010 (PLC-2010-0623) (Confidential)