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 aryloxyalkanoate herbicide, glyphosate herbicide and glufosinate herbicide (modified *aad-12*, *2mepsps*, *pat*, *Glycine max* (L.) Merr.) (DAS44406, OECD UI: DAS-444Ø6-6)
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

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition and origins of donor nucleic acid used for production of Soybean tolerant to aryloxyalkanoate herbicide, glyphosate herbicide and glufosinate herbicide (modified *aad-12*, *2mepsps*, *pat*, *Glycine max* (L.) Merr.) (DAS44406, OECD UI: DAS-444Ø6-6) (hereinafter referred to as "this modified soybean") are shown in Table 1 (p.1-3).

Table 1 Composition of donor nucleic acid, origins and function of component elements

Component element	Size (bp)	Origin and function
RB7 MAR	1166	Nuclear matrix attachment region derived from *Nicotiana tabacum* (Hall *et al*., 1991). Stabilizes expression of the gene.
2mepsps cassette		
Histone H4A748 3' UTR	661	A 3' terminal untranslated region consisting of the transcription termination point of histone H4A748 gene derived from *Arabidopsis thaliana* and a polyadenylation site (Chaboute *et al*., 1987). Terminates transcription of the gene.

Table 1 Composition of donor nucleic acid, origins and function of component elements (cont.)

Component element	Size (bp)	Origin and function
2mepsps	1338	5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps* gene) derived from *Zea mays* where point mutation is induced for 2 points. Expresses double mutant 5-enol pyruvylshikimate-3-phosphate synthase (2mEPSPS protein). Regarding the amino acid sequence of the expressed 2mEPSPS protein, threonine at the 102nd position and proline at the 106th position are modified to isoleucine and serine, respectively (Lebrun *et al*., 1996; Lebrun *et al*., 2003).
TPotp C	372	Created based on the translated region of a chloroplast transit peptide derived from ribulose 1,5-bisphosphate carboxylase/oxygenase of *Zea mays* and *Helianthus annuus*. Bound to a *2mepsps* gene for the purpose of transporting 2mEPSPS protein synthesized at cytoplasm to chloroplast (Lebrun *et al*., 1996; Lebrun *et al*., 2003).
Histone H4A748 promoter	1430	Promoter derived from *Arabidopsis thaliana*. Contains a 5' terminal untranslated region of histone H4A748 gene and an intron region of histone H3 gene (Chaboute *et al*., 1987). Expresses the gene for the entire plant body.
Modified *aad-12* cassette		
AtUbi10 promoter	1322	Promoter of polyubiquitin 10 (UBQ10) gene derived from *Arabidopsis thaliana*. Contains a 5' terminal untranslated region and intron (Norris *et al*., 1993). Expresses the gene for the entire plant body.
Modified *aad-12*	882	Aryloxyalkanoate dioxygenase gene derived from gram-negative bacillus *Delftia acidovorans* which is modified to a codon that is suitable for expressing in a plant body. Expresses the modified AryloxyAlkanoate Dioxygenase (hereinafter referred to as "modified AAD-12 protein"). Regarding the amino acid sequence of the expressed modified AAD-12 protein, alanine is added to the second position for introduction of a cloning site (Wright *et al*., 2007).
AtuORF23 3' UTR	457	A 3' terminal untranslated region consisting of the transcription termination point of ORF23 derived from the plasmid pTi15955 of *Agrobacterium tumefaciens* and a polyadenylation site (Barker *et al*., 1983). Terminates transcription of the gene.
pat cassette		
CsVMV promoter	517	Promoter derived from Cassava vein mosaic virus. Contains a 5' terminal untranslated region (Verdaguer *et al*., 1996). Expresses the gene for the entire plant body.

Table 1 Composition of donor nucleic acid, origins and function of component elements (cont.)

Component element	Size (bp)	Origin and function
pat	552	Phosphinothricin acetyltransferase gene derived from *Streptomyces viridochromogenes* which is modified to a codon that is suitable for expressing in a plant body. Expresses the PAT protein. No modification is made to the amino acid sequence of the expressed PAT protein (Wohlleben *et al*., 1988).
AtuORF1 3' UTR	704	A 3' terminal untranslated region consisting of the transcription termination point of ORF1 derived from the plasmid pTi15955 of *Agrobacterium tumefaciens* and a polyadenylation site (Barker *et al*., 1983). Terminates transcription of the gene.
T-DNA Border A	24	T-DNA boundary sequence derived from *Agrobacterium tumefaciens* (Barker *et al*., 1983).
Exoskeleton region		
T-DNA Border A	24	T-DNA boundary sequence derived from *Agrobacterium tumefaciens* (Barker *et al*., 1983).
T-DNA Border A	24	T-DNA boundary sequence derived from *Agrobacterium tumefaciens* (Barker *et al*., 1983).
Ori Rep	1020	Sequence derived from the replication origin of broad host range plasmid RK2 (Stalker *et al*., 1981).
trfA	1149	Sequence derived from broad host range plasmid RK2. Codes replication initiator protein required for replication of plasmid (Stalker *et al*., 1981).
*Spec*R	789	Sequence derived from the *Spec*R gene of *E. coli*. Confers spectinomycin resistance (Fling *et al*., 1985).
T-DNA Border B	24	T-DNA boundary sequence derived from *Agrobacterium tumefaciens* (Barker *et al*., 1983).

2) Functions of component elements

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

Functions of individual elements of the inserted genes are shown in Table 1 (p.1-3).

The donor nucleic acid contains the *RB7 MAR* sequence as a nuclear matrix attachment region. A nuclear matrix binding region is a region frequently observed in genomic DNA sequences, and is considered to play a role in adhering DNA to the nuclear matrix for the purpose of forming a loop structure of the DNA. It has been reported that a nuclear matrix binding region flanking in the vicinity of an introduced gene stabilized the expression of

the gene by reducing the gene silencing (Allen *et al.*, 2000). It also has been reported that a nuclear matrix binding region enhanced the expression of the introduced gene (Halweg *et al.*, 2005), it was reported that it had no influence on the expression of endogenous genes (Chattopadhyay *et al.*, 1998) nor enhanced the expression of the introduced gene if flanking downstream (Fukuda and Nishikawa, 2003).

(b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein known to be allergic

Modified AAD-12 protein is an enzyme that catalyzes the reaction to introduce oxygen specifically to achiral and S-isomers of compounds with the aryloxyalkanoate structure.

This modified soybean exhibits herbicide tolerance by the modified AAD-12 protein catalyzing the reaction to introduce oxygen to aryloxyalkanoate herbicide and converting it to a compound with no herbicidal activity (Wright *et al.*, 2007). For instance, the modified AAD-12 protein catalyzes the reaction to introduce oxygen to herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and coverts it to 2,4-dichlorophenol (2,4-DCP) and glyoxylic acid that have no herbicidal activity (Figure 1, p.4). The herbicides that become the substrate of the modified AAD-12 protein are shown in Attachment 1.

Investigation on the FARRP Allergen Database version 13 (2013) identified that the modified AAD-12 protein has no amino acid sequence that is structurally related to known allergens.

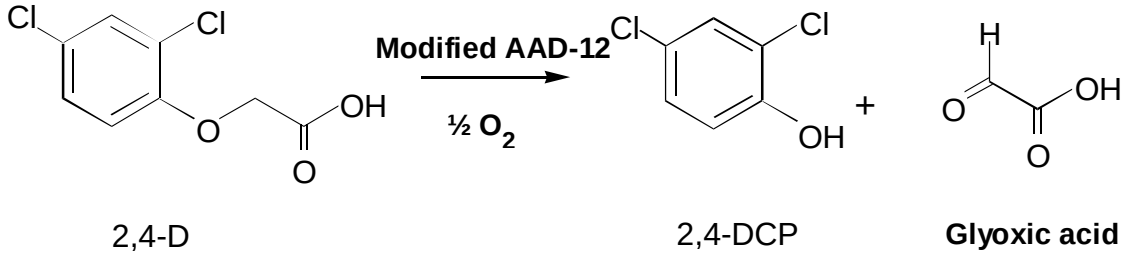


Figure 1 Action mechanism of modified AAD-12 protein

(All the rights pertinent to the information in the diagram above and the responsibility for content rest upon Dow Chemical Japan Limited.)

The 5-enolpyruvylshikimate-3-phosphate synthase (hereinafter referred to as “EPSPS protein”) is involved in the biosynthesis of aromatic amino acids, vitamins and many secondary metabolites in plants and microorganisms. In plants the EPSPS protein is localized inside plastids, and condenses phosphoenolpyruvate and 3-phosphoshikimate into 5-enolpyruvylshikimate-3-phosphate. This is one of reactions in the shikimate pathway as a biosynthesis pathway of aromatic amino acids, and is widely known to be inhibited by glyphosate herbicide that is a reversible competitive inhibitor of EPSPS protein against phosphoenolpyruvate. That is, plants sprayed with glyphosate undergo inhibition of biosynthesis of aromatic amino acids, resulting in inhibition of protein

synthesis and therefore wither and die. As a side note, this enzyme has a high specificity to its substrates, namely, phosphoenolpyruvate and 3-phosphoshikimate (OECD, 1999a).

To this modified soybean, *2mepsps* gene is introduced where point mutation is induced to 2 points of *epsps* gene that codes EPSPS protein of maize. In the amino acid sequence of 2mEPSPS protein produced by the *2mepsps* gene, threonine at the 102nd position and proline at the 106th position are modified to isoleucine and serine, respectively, compared to the amino acid sequence of the wild EPSPS protein. By this, 2mEPSPS protein becomes insensitive to glyphosate, and the shikimate synthesis functions without being affected by competitive inhibition by glyphosate. As a result, the relevant plants become able to grow under the presence of glyphosate (Lebrun *et al*., 2003).

Investigation on the FARRP Allergen Database version 13 (2013) identified that the 2mEPSPS protein has no functionally-important amino acid sequence that is structurally related to known allergens.

Phosphinothricin AcetylTransferase (hereinafter referred to as "PAT protein") swiftly coverts L-glufosinate to *N*-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid) which is a stable compound not toxic to plants.

L-glufosinate is a structural analog of glutamate and a competitive inhibitor of glutamine synthetase in microorganisms and plants, and has activity as an herbicide. Therefore, in plants that are not tolerant to herbicide glufosinate, a large amount of ammonia accumulates inside the cells due to inhibition of glutamine synthetase, which eventuates to plant cell death. Meanwhile, *N*-acetyl-L-glufosinate does not inhibit glutamine synthetase and genetically modified plants that express PAT protein do not receive effects of ammonia, and thus exhibits tolerance to herbicide glufosinate (OECD, 2002).

Investigation on the FARRP Allergen Database version 13 (2013) identified that PAT protein has no amino acid sequence that is structurally related to known allergens.

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

[Modified AAD-12 protein]

The modified AAD-12 protein is an enzyme that catalyzes the reaction to introduce oxygen specifically to achiral and S-isomers of compounds with the aryloxyalkanoate structure.

In the reaction where modified AAD-12 protein introduces oxygen to a substrate, if there is α-ketoglutarate, α-ketoglutarate is converted to succinate (Figure 2, p.6).

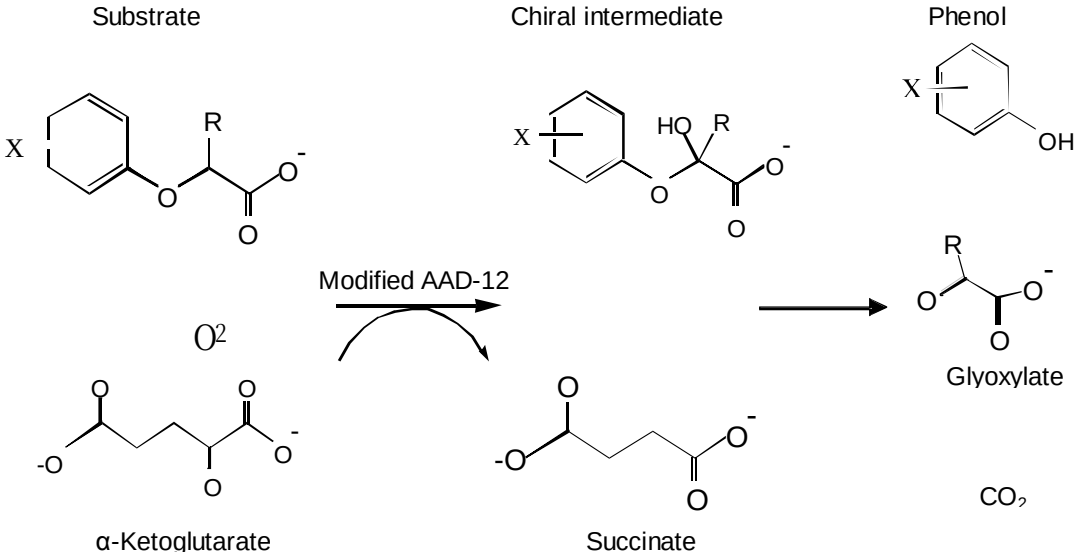


Figure 2 Enzyme reaction of modified AAD-12 protein under the presence of α-ketoglutarate

To that end, the possibility of compounds that exist inside plant bodies that are structurally or physiologically related to compounds with the aryloxyalkanoate structure becoming the substrates of modified AAD-12 protein was confirmed by formation of succinate through the assay of succinate in the reaction experiment of modified AAD-12 protein with α-ketoglutarate. For the assay of succinate, in the reaction system using succinyl-CoA synthetase, pyruvate kinase and lactate dehydrogenase as shown in Figure 3, the amount of oxidized nicotinamide adenine dinucleotide (NAD) generated from oxidation of reduced nicotinamide adenine dinucleotide (NADH) was obtained by measuring the absorbance at 340 nm and was converted to the generated amount of succinate (Luo *et al*., 2006; Figure 3, p.7).

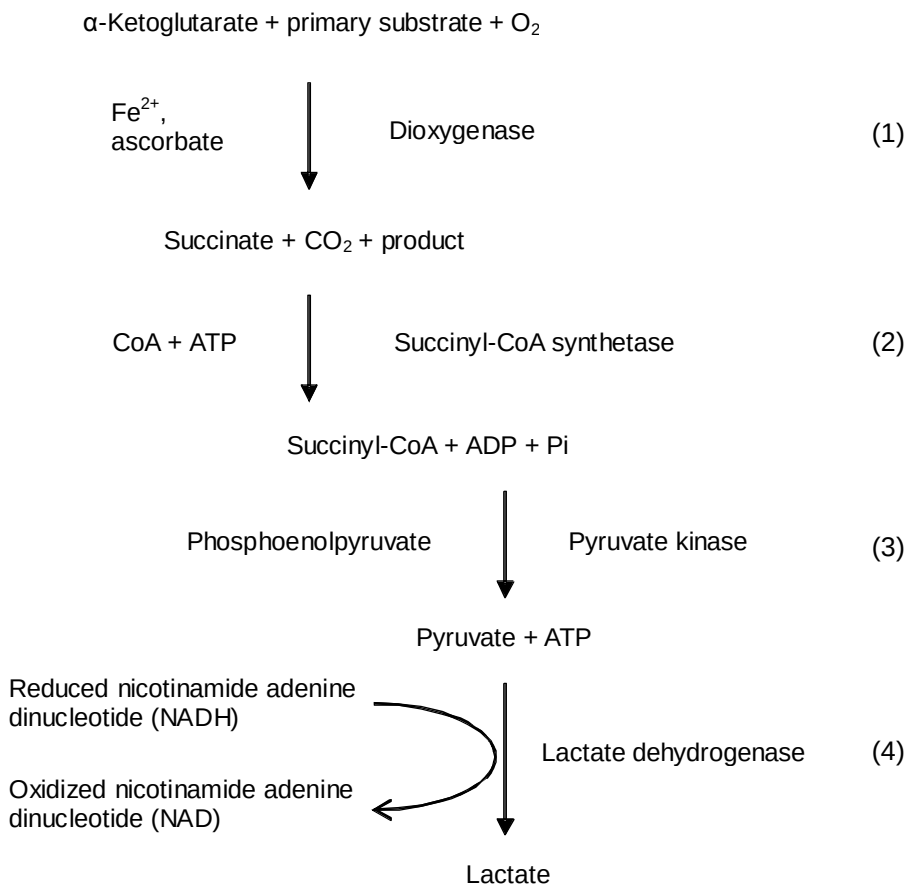


Figure 3 Reaction path of assay of succinate (modified Scheme 1 of Luo *et al*., 2006)

(1) In the reaction where dioxygenase introduces oxygen to the substrate, α-ketoglutarate is converted to succinate.

(2) Under the presence of coenzyme A (CoA) and adenosine-5'-triphosphate (ATP), succinate is converted to succinyl-CoA by succinyl-CoA synthetase, and adenosine-5'-diphosphate (ADP) and phosphate (Pi) are produced simultaneously.

(3) Pyruvate and ATP are generated from phosphoenolpyruvate and ADP by pyruvate kinase.

(4) Under the presence of reduced nicotinamide adenine dinucleotide (NADH), pyruvate is converted to lactate by lactate dehydrogenase, and NAD is produced simultaneously. The amount of NAD produced in this reaction is considered equal to the amount of succinate.

As the compounds that exist inside plant bodies that are structurally or physiologically related to compounds with the aryloxyalkanoate structure, some plant hormones (indole-3-acetic acid, abscisic acid, gibberellic acid (GA3) and aminocyclopropane-1-caoboxylic acid), some phenylpropanoid intermediates (*trans*-cinnamic acid, coumaric acid and sinapic acid), and 20 types of L-amino acids were used for the assay of succinate.

Regarding the 20 types of L-amino acids, no production of succinate was identified for modified AAD-12 protein at a concentration of 1 μM, and it was considered that modified AAD-12 protein does not react with the 20 types of L-amino acids (Attachment 2, Table 1, p.18). Meanwhile, when 10-μM modified AAD-12 protein was acted on plant hormones and phenylpropanoid intermediates, production of succinate was identified for indole-3-acetic acid, coumaric acid, *trans*-cinnamic acid and sinapic acid (Attachment 2, Table 4, p.21). In the assay of succinate, there is a possibility of uncoupling taking place that induces succinate products without undergoing oxidation of substrate (Hausinger, 2004). Therefore, in order to confirm oxidation of compounds that exhibited reaction in the assay of succinate, measurement of the primary oxides was carried out by the Fourier transform mass spectrometry (FT/MS). As a result, when 10-μM modified AAD-12 protein was acted, only the oxides of *trans*-cinnamic acid and indole-3-acetic acid were detected (Attachment 2, Figure 2 and Figure 3, p.24 and p.25).

Accordingly, the catalytic efficiency of modified AAD-12 protein was investigated by the assay of succinate for *trans*-cinnamic acid and indole-3-acetic acid, and, as a control, for *S*-dichlorprop* which is a substance with the aryloxyalkanoate structure and a chemical structure similar to 2,4-D. As a result, the catalytic efficiency, *Kcat/Km*, was 156.7 $M^{-1}s^{-1}$, 8.2 $M^{-1}s^{-1}$ and 30,175 $M^{-1}s^{-1}$ for *trans*-cinnamic acid, indole-3-acetic acid and *S*-dichlorprop, respectively (Attachment 2, Table 5, p.22). The values of *Kcat/Km* for *trans*-cinnamic acid and indole-3-acetic acid were 0.52% and 0.027% of that for *S*-dichlorprop, respectively, indicating substantially low catalytic efficiencies for *trans*-cinnamic acid and indole-3-acetic acid compared to that for *S*-dichlorprop. There was a report stating that the *Kcat/Km* value of modified AAD-12 protein for 2,4-D was 18,600 $M^{-1}s^{-1}$ (Wright *et al*., 2010). Since the catalytic efficiency of modified AAD-12 protein is equivalent for 2,4-D and *S*-dichlorprop, the catalytic efficiency for *trans*-cinnamic acid and indole-3-acetic acid is considered substantially low compared to that for 2,4-D.

Additionally, it was reported that the *in vitro Kcat/Km* value of cinnamate-4-hydroxylase of *Arabidopsis thaliana* for *trans*-cinnamic acid was 3.4×10^6 $M^{-1}s^{-1}$ (Chen *et al*., 2007). It also was reported that the *in vitro Kcat/Km* value of indole-3-acetic acid (IAA) amido synthetase of *Oryza sativa* for indole-3-acetic acid was 2.75×10^3 $M^{-1}s^{-1}$ (Chen *et al*., 2009). Like these, the catalytic efficiency of cinnamate-4-hydroxylase and that of IAA amido synthetase are both high, and it is therefore considered that *trans*-cinnamic acid and indole-3-acetic acid are efficiently and specifically used in the existing metabolic pathways in plant bodies. Meanwhile, the *Kcat/Km* value of modified AAD-12 protein for *trans*-cinnamic acid was 0.005% of that of cinnamate-4-hydroxylase for *trans*-cinnamic acid, and the *Kcat/Km* value of modified AAD-12 protein for indole-3-acetic acid was 0.3% of that of IAA amido synthetase for indole-3-acetic acid. Both of the *Kcat/Km* values of modified AAD-12 protein are substantially low.

* *S*-isomer of chiral herbicide dichlorprop. Only the *R*-isomer has herbicidal activity, and *S*-dichlorprop has no herbicidal activity.

Based on the abovementioned understanding, it is considered that while there is a possibility of modified AAD-12 protein oxidizing *trans*-cinnamic acid and indole-3-acetic acid, the catalytic efficiency is extremely low, and the possibility of identified oxidation reactions affecting the metabolic pathways of plants is low.

Since no compound with aryloxyalkanoate structure is known to exist in plant bodies to date, it is considered to be unlikely that modified AAD-12 protein alters other metabolic systems of plant bodies.

Regarding the effects of 2,4-DCP as a decomposition product of herbicide 2,4-D on aquatic organisms, LC_{50} (median lethal concentration) identified in acute toxicity studies was 1.7 mg/L for freshwater fish and 1.4 mg/L for *Daphnia magna*, and EC_{50} (half maximal effective concentration) was 1.5 mg/L for Lemna. The NOEC (no observed effect concentration) identified in chronic toxicity studies was 0.14 mg/L for Lemna and 0.21 mg/L for *Daphnia magna*. Additionally, regarding its effects on terrestrial organisms, LC_{50} was 125 mg/kg for earthworm and EC_{10} (10% effect concentration) was 0.7 mg/kg for *Folsomia candida* (OECD, 2006). Meanwhile, regarding the effects of 2,4-D on aquatic organisms, LC_{50} identified in acute toxicity studies was 0.26 mg/L for freshwater fish and 2.2 mg/L for *Daphnia magna*, and EC_{50} was 0.2992 mg/L for *Lemna gibba*. The NOEC identified in chronic toxicity studies was 0.0476 mg/L for *Lemna gibba* and 0.20 mg/L for *Daphnia magna* (EPA, 2004).

Like these, the toxicity of 2,4-DCP as a decomposition product of 2,4-D is equivalent to or lower than that of 2,4-D, and it is considered to be unlikely that the concentration of 2,4-DCP will reach levels that cause adverse effects more than the sprayed 2,4-D does, even if the maximum possible concentration of 2,4-DCP is assumed for when 2,4-D is sprayed.

The residual 2,4-DCP concentration in grains was investigated after spraying the upper limit amount of the appropriate use range of 2,4-D onto a soybean line (DAS68416, OECD UI: DAS-68416-4) (hereinafter referred to as “Soybean 68416 Line”) that has tolerance to aryloxyalkanoate herbicides similar to this modified soybean. As a result, the maximum average residual amount was 0.047 mg/kg (Attachment 3, Table 2, p.26). LD_{50} (median lethal dose) of 2,4-DCP identified in acute toxicity studies on mice was 1,276-1,352 mg/kg body weight, and NOAEL (no observed adverse effect level) identified in 2-year chronic toxicity studies on rats was 440 mg/kg body weight/day for males and >250 mg/kg body weight/day for females (OECD, 2006), which is substantially larger than the residual amount of 2,4-DCP in the Soybean 68416 Line. Therefore, it is considered to be unlikely that the imported seeds of this modified soybean that has tolerance to aryloxyalkanoate similar to the Soybean 68416 Line cause adverse effects on wild animals or plants.

[2mEPSPS protein]

EPSPS protein is an enzyme that reacts specifically with phosphoenolpyruvate and shikimate-3-phosphate, and indicated not the rate determining enzyme in the shikimate pathway as the synthesis pathway of aromatic amino acids (Weiss and Edwards, 1980;

Herrmann, 1983). It is also reported that aromatic amino acids as the final products were not produced excessively even in cultured plant cells that produce EPSPS protein 40 times than normal cells (Smart *et al*., 1985). Additionally, while it is known that shikimate reacts with EPSPS protein in addition to these substrates, its reactivity is about 1/2,000,000 of the reactivity with shikimate-3-phosphate, and the possibility of shikimate reacting with EPSPS protein as a substrate in the body is substantially low (Gruys *et al*., 1992).

Meanwhile, for mutant EPSPS protein of *E. coli* that underwent amino acid changes equivalent to 2mEPSPS protein where the 97th threonine and the 101st proline are changed to isoleucine and serine, respectively, it was reported that the mutant EPSPS protein exhibited a high affinity with phosphoenolpyruvate and 3-phosphokishimate similar to wild EPSPS protein (Funke *et al*., 2009). Additionally, 2mEPSPS protein is similar to EPSPS protein both structurally and functionally other than being insensitive to glyphosate herbicide, and they have the same mechanism of action (Herouet-Guicheney *et al*., 2009). Therefore, it is considered that 2mEPSPS protein has high substrate specificity similar to EPSPS protein and unlikely to alter other metabolic systems.

[PAT protein]

PAT protein is an enzyme that extremely specifically acetylates free amino groups of L-glufosinate that is the active ingredient of herbicide glufosinate, and never acetylates other amino acids or D-glufosinate (OECD, 1999b). Additionally, acetylation of L-glufosinate by PAT protein is never affected, even under overabundance of L-amino acids (OECD, 1999b). Therefore, it is considered to be unlikely that PAT protein alters other metabolic systems of plant bodies.

It has been confirmed that the toxicity (acute toxicity, subacute toxicity, chronic toxicity, carcinogenicity, and reproductive and developmental toxicity) of *N*-acetyl-L-glufosinate as a metabolic product of herbicide glufosinate on animals is lower than that of glufosinate (Food Safety Commission of Japan, 2010), and it is considered to be unlikely that the concentration of *N*-acetyl-L-glufosinate becomes to the level that causes adverse effects more than the sprayed glufosinate does, even if the maximum possible concentration of *N*-acetyl-L-glufosinate is assumed for when glufosinate is sprayed. As a side note, *N*-acetyl-L-glufosinate is included in the compounds subject to the residue standards for soybeans.

(2) Information concerning vector

1) Name and origin

The vector pDAB2407 which became the base of introduced pDAB8264 was derived from *A. tumefaciens* and *E. coli*.

2) Properties

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

The total number of base pairs in the expression vector pDAB8264 is 16,018 bp. The

nucleotide sequence of pDAB8264 is shown in Attachment 4.

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

The expression vector pDAB8264 possesses the *Spec*R gene that confers tolerance to spectinomycin. While the *Spec*R gene was used as a selective marker on establishment of the expression vector pDAB8264, it is located outside the T-DNA region and thus is not introduced into this modified soybean.

Southern blot analysis of this modified soybean confirmed no existence of the *Spec*R gene (Attachment 5, Table 2, p.7).

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

The T-DNA region of the vector which became the base of the expression vector pDAB8264 is rewritten to the donor nucleic acid shown in Table 1 (p.1-3), and does not contain a sequence that allows for infection of *Agrobacterium tumefaciens*; infectivity has not been identified yet.

(3) Method of preparing living modified organisms

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

The map of the expression vector pDAB8264 is shown in Figure **4** (p.13). The production process of the expression vector pDAB8264 is shown in Attachment 6.

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

Introduction of nucleic acid into the recipient organism was carried out by *Agrobacterium* method.

3) Processes of rearing living modified organisms

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

Selection was made by culturing adventitious buds and shoots formed from cultured *Agrobacterium*-infected tissues in a medium containing herbicide glufosinate.

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

Absence of remaining *Agrobacterium* in this modified soybean was confirmed by no detection of the exoskeleton region in the T0 and T1 generations using the PCR method.

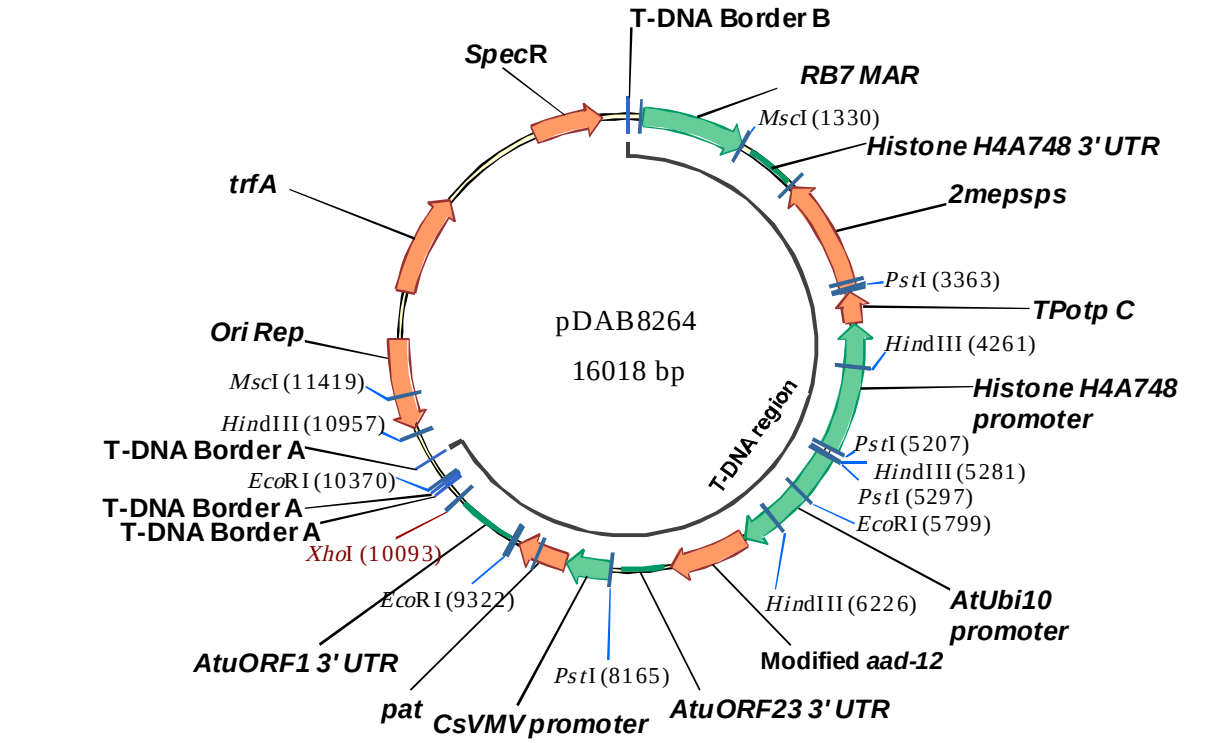
(c) Process of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effects on Biological Diversity

Individual bodies with tolerance were selected by applying glufosinate to the re-differentiated plant bodies. For the selected plant bodies, plant bodies with a copy of the

introduced gene was selected (T0 and T1 generations) by analyzing the number of copies of modified *aad-12* gene, *2mepsps* gene and *pat* gene by the quantitative PCR method using an internal reference. Additionally, at outdoor fields in the US, progenies (from T2 generation onward) underwent existence confirmation of modified *aad-12* gene, *2mepsps* gene and *pat* gene by the PCR method as well as analysis of modified *aad-12* cassette, *2mepsps* cassette, *pat* cassette and *RB7 MAR* sequence for their number of copies by the Southern blot analysis. This modified soybean was selected by comprehensively assessing these results, confirmation of protein expression (AAD-12 protein, 2mEPSPS protein and PAT protein), herbicide tolerance and agronomic traits identified. The range of application is progenies from T2 generation onward. Details of rearing is shown in Figure 5 (p.14).

The status of application and approval of this modified soybean in Japan is summarized below (as of December 2013).

September 2011	Approved for Type 1 Use Regulation (isolated field test) by the Ministry of Agriculture, Forestry and Fisheries (MAFF) and the Ministry of the Environment based on the “Law Concerning the Conservation and Sustainable Use of Biological Diversity Through Regulations on the Use of Living Modified Organisms” (Period of use: September 2, 2011 to March 31, 2013).
2014	Planning to apply to the Ministry of Health, Labour and Welfare for safety confirmation as use for food based on the “Food Sanitation Act”.
2014	Planning to apply to MAFF for safety confirmation as use for feed based on the “Act on Safety Assurance and Quality Improvement of Feeds”.



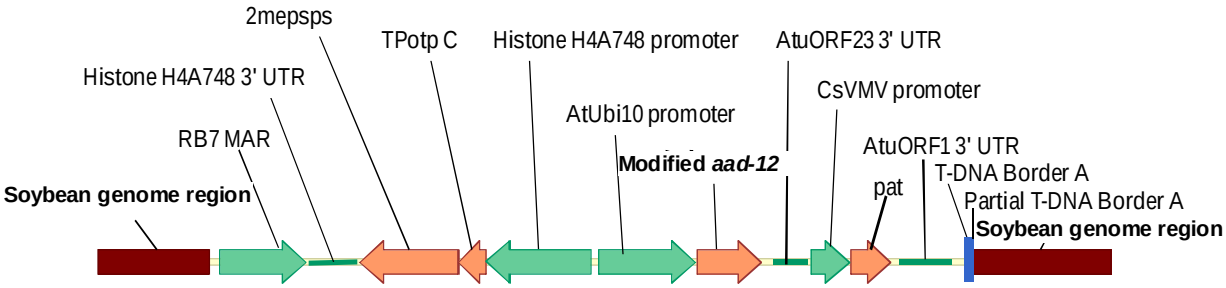


Figure 4 Map of expression vector pDAB8264 (top) and schematic of insertion of T-DNA region (bottom)

* The numbers in parenthesis in the top figure refer to the restriction enzyme cleavage site on the plasmid with T-DNA Border B as the starting point.

Confidential and Non-disclosed

Figure 5 Process of rearing of this modified soybean

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

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

Transferred nucleic acid obeys the law of Mendelian inheritance once incorporated into the plant chromosome. Segregation of traits introduced into this modified soybean in the group of F2 generation (Figure 5, p.14) was analyzed (2011, Indiana, USA). One F1 generation individual body that was obtained by crossing the T2 generation line and non-modified soybean was self-pollinated, and the presence or absence of PAT protein expression in the F2 group was investigated using the lateral flow strip method†. Additionally, the presence or absence of transferred nucleic acids by the PCR method using primer specific to this modified soybean.

As a result, all the transferred nucleic acids were detected by the PCR method for the individual bodies for which PAT protein was detected by the lateral flow strip method. It was confirmed that transferred nucleic acids exist on the soybean chromosome since the observed values were consistent with the Mendel's law of segregation for nuclear genes (Table 2, p.14).

Table 2 Segregation analysis of F2 generation of this modified soybean [1)]

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

In order to confirm the number of copies of transferred nucleic acids and their genetic stability over multiple generations, Southern blot analysis was carried out for F2, T2, T3, T4 and T6 generations. As a result, it was confirmed that one copy each of modified *aad-12* cassette, *2mepsps* cassette, *pat* cassette and *RB7 MAR* sequence were introduced to this modified soybean, and are inherited stably over multiple generations (Attachment 5, Table 2, p.5-7).

In order to confirm the insertion of individual component elements to the T-DNA region, cloning and sequencing were performed for the entire inserted gene for this modified soybean including the host genome border region. As a result, the base sequences were determined for the total of 13,659 bp including the transferred nucleic acid region with 10,280 bp, sequence flanking the 5' terminal with 1,494 bp and the sequence flanking the 3' terminal with 1,885 bp (Attachment 7). Regarding T-DNA Border, it was clarified that

† Method to visually identify accumulation of a marker that is an immune complex consisting of the antigen in the specimen, a labelled antibody and capture antibody while the specimen moves on a membrane by the capillary phenomenon. In the present test, presence or absence of expression of PAT protein by this modified soybean was confirmed by capturing the protein with the antibody on a membrane and visual inspection of the band.

T-DNA Border B was not transferred and one of T-DNA Border A and a part of another T-DNA A were transferred, while all other component elements were transferred in a complete form (Figure **4**, bottom, p.13). Meanwhile, it also was clarified that 3 bp was newly inserted at the 5' terminal of the inserted sequence, and 4,383 bp was lost from the soybean genome. Additionally, it was clarified that there was no destruction of the endogenous genes or the control region because of the insertion of introduced gene and loss in the soybean genome (Attachment 7).

3) Positional relationship in the case of multiple copies existing in chromosome

No multiple copies exist in the chromosome.

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

For the T5 generation and T6 generation of this modified soybean, expression level of modified AAD-12 protein, 2mEPSPS protein and PAT protein in leaves in the 7 leaf stage was investigated using the ELISA method (2012, Indiana, USA). As a result, it was confirmed that the expression of modified AAD-12 protein, 2mEPSPS protein and PAT protein was stably conferred to multiple generations (Table 3-Table 5, p.15).

Table 3 Expression level of modified AAD-12 protein
in leaves of T5 and T6 generations of this modified soybean

Confidential and Non-disclosed

Table 4 Expression level of 2mEPSPS protein
in leaves of T5 and T6 generations of this modified soybean

Confidential and Non-disclosed

Table 5 Expression level of PAT protein
in leaves of T5 and T6 generations of this modified soybean

Confidential and Non-disclosed

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

Modified soybean does not include transmittable sequences and there is no risk of the nucleic acids introduced to this modified soybean being transferred to wild animals or wild plants.

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

A PCR method that uses a primer specific to this modified soybean has been developed as a method to detect and identify this modified soybean. Regarding the contamination ratio of this modified soybean to non-modified soybean, the limit of detection using the current PCR is 0.04% as the genome DNA level ratio (Attachment 8, Table 6, p.22). Regarding the reproducibility, assurance of inter-laboratory transferability was confirmed at US Dow AgroSciences and US Eurofins GeneScan (Attachment 8, Table 12, p.27)

(6) Difference between modified organism and recipient organism or species to which recipient organism belongs

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

For this modified soybean, modified *aad-12* gene, *2mepsps* gene and *pat* gene are introduced, and they confer tolerance to aryloxyalkanoate herbicide, herbicide glyphosate, and herbicide glufosinate by expressing modified AAD-12 protein, 2mEPSPS protein and PAT protein, respectively. By growing this modified soybean conferred with the tolerance to aryloxyalkanoate herbicide and herbicide glyphosate, farmers will have a wider range of herbicide products to choose from and will be able to prevent weeds that gain resistance to other herbicides. Additionally, the tolerance to herbicide glufosinate was used as the marker for selection.

During isolated field tests conducted in 2012 at the Ogori Development Center, Dow Chemical Japan Limited, herbicide 2,4-D, herbicide glyphosate and glufosinate tolerance test was conducted for this modified soybean (T7 generation) and non-modified soybean as a control (Maverick). Seventeen days after seeding (around the 2 leaf stage of true leaf), the modified soybean and non-modified soybean (12 individual bodies each) were sprayed with 2,4-D at 1,120 g a.e.[‡]/ha (normal application). In 2 weeks after application, all non-modified soybeans withered, while all of this modified soybean plants exhibited adequate herbicide tolerance with no chemical damage observable (“Isolated Field Test Results Report”, Figure 1, p.2). Additionally, seventeen days after seeding (around the 2 leaf stage of true leaf), the modified soybean and non-modified soybean (12 individual bodies each) were sprayed with glyphosate at 1,260 g a.e.[‡]/ha (normal application). In 2 weeks after application, all non-modified soybeans withered, while all of this modified soybean plants exhibited adequate herbicide tolerance with no chemical damage observable (“Isolated Field Test Results Report”, Figure 1, p.2). Furthermore, seventeen days after seeding (around the 2 leaf stage of true leaf), the modified soybean and non-modified soybean (12 individual bodies each) were sprayed with glufosinate at 374 g a.i.[‡]/ha (normal application). In 2 weeks after application, all non-modified soybeans withered, while all of this modified soybean plants exhibited adequate herbicide tolerance with no chemical damage observable (“Isolated Field Test Results Report”, Figure 1, p.2).

[‡] Active ingredient content of herbicide is expressed with active ingredient (a.i.) or acid equivalent (a.e.). Active ingredient (a.i.) content refers to the component weight, and acid equivalent (a.e.) refers to the amount of free acids. Herbicide preparations contain active ingredient(s) in the form of salt or as is. When the active ingredient exists in the form of salt, the active component is an acid, and the base part varies depending on preparation. Since various preparations exist that contain 2,4-D or glyphosate with different base parts, acid equivalent was used in order to accurately apprehend the amount of active component.

2) 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

In 2012, isolated field tests were conducted at the Ogori Development Center, Dow Chemical Japan Limited, and the differences between this modified soybean (T7 generation) and non-modified soybean as a control (Maverick) were investigated.

(a) Morphological and growth characteristics

As morphological and growth characteristics, comparison between this modified soybean and non-modified soybean was carried out referencing the items in the Screening Standards by Agricultural, Forestry and Fishery Plant Species for agricultural soybean species (Ministry of Agriculture, Forestry and Fisheries, 2012) as the standards for seed/seedling registration, regarding germination rate, uniformity of germination, beginning period of flowering, ending period of flowering, mature period, lobular form, amount of trichome, growth type, main stem length, position of lowest pod shooting node, number of nodes on the main stem, number of branches, aboveground fresh weight during harvest period, number of fertile pods, total grain weight per plant, mature grain weight per plant, weight of a hundred grains, and properties (size, shape, seed coat color and hilum color) of grain.

At the isolated field, both this modified soybean and non-modified soybean started germinating 4 days after seeding. Regarding the germination rate, there was no statistically significant difference observed between this modified soybean and non-modified soybean ("Isolated Field Test Results Report", Table 1, p.3). Regarding the uniformity of germination, there was no difference observed between this modified soybean and non-modified soybean. There was no difference observed between this modified soybean and non-modified soybean for the beginning period of flowering, ending period of flowering, and the mature period ("Isolated Field Test Results Report", Table 2, p.4), or for the lobular form, amount of trichome, growth type, and the properties of grain ("Isolated Field Test Results Report", Table 3 and Table 6, p.4 and 6). Additionally, there was no statistically significant difference observed between this modified soybean and non-modified soybean for any item of main stem length, position of lowest pod shooting node, number of nodes on the main stem, number of branches, aboveground fresh weight during harvest period, number of fertile pods, total grain weight per plant, mature grain weight per plant, and weight of a hundred grains ("Isolated Field Test Results Report", Table 4 and Table 5, p.5).

(b) Cold tolerance at the early stage of growth

The cold tolerance of this modified soybean and non-modified soybean at an early stage of growth was investigated. This modified soybean and non-modified soybean that grew to the first leaf developing period (6 individuals each) were grown in an incubator set to 4°C and 16-hour day length, and their growing conditions were observed. As a result, both this modified soybean and non-modified soybean exhibited symptoms of leaf whitening, plant body shrinkage and severe growth inhibition after 39 days, and there was no difference observed in the level of the symptoms ("Isolated Field Test Results Report", Figure 3, p.7).

(c) Wintering ability of the mature plant

The wintering ability of the mature plant of this modified soybean and non-modified soybean was investigated. Mature plants grown in the field (16 plants) were left untouched without harvesting until the following year, and the conditions of the plant bodies under the natural winter conditions were observed. As a result of observation on individuals served for testing in January 2013, both this modified soybean and non-modified soybean were found to be withered and were identified to have no wintering ability ("Isolated Field Test Results Report", Figure 4, p.7).

(d) Fertility and size of the pollens

No difference was observed in pollen shape ("Isolated Field Test Results Report", Figure 5, p.8). The fertility (adequacy) and size of the pollens of this modified soybean and non-modified soybean that were stained with iodine-potassium iodine solution were investigated. As a result, no statistically significant difference was observed between this modified soybean and non-modified soybean ("Isolated Field Test Results Report", Table 7, p.9).

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

Regarding the production of the seed, comparison between this modified soybean and non-modified soybean was made on the number of fertile pods, total grain weight per plant, mature grain weight per plant, and the weight of a hundred grains. As a result, no statistically significant difference was observed for all items, and it was judged that there was no difference between this modified soybean and non-modified soybean in the production of the seed ("Isolated Field Test Results Report", Table 5, p.5).

Regarding pod dehiscence, the level of dehiscence during the mature period was observed for this modified soybean and non-modified soybean. As a result, both this modified soybean and non-modified soybean were non-dehiscent, and there was no observable difference ("Isolated Field Test Results Report", Table 8, p.9).

The dormancy of this modified soybean and non-modified soybean was assessed by germinating the harvested seeds in a petri dish immediately after harvesting without conducting dormancy breaking and investigating the germination rate. As a result, both this modified soybean and non-modified soybean exhibited a 100% germination rate, and it was judged that the dormancy was extremely shallow ("Isolated Field Test Results Report", Table 7, p.9).

(f) Crossability

This modified soybean and non-modified soybean were planted alternately to the crossability test plot ("Isolated Field Test Results Report", Attachment Figure 2, p.14) with an interval of 25 cm, and 3,200 seeds obtained from non-modified soybeans were again sowed inside the isolated field. Among the 3,200 seeds that were sowed, 3,080 seeds germinated (germination rate 96.3%). The survivability was then investigated by treating them with 1,120g a.e./ha of herbicide 2,4-D at the 2 leaf stage of true leaf and counting the survived individuals. As a result, survival of 7 individuals out of the 3,080 individuals was confirmed, with a survival rate of 0.23% ("Isolated Field Test Results Report", Table 9, p.10).

(g) Productivity of harmful substances

In order to compare the productivity of harmful substances for this modified soybean and non-modified soybean, a succeeding crop test, plow-in test and soil microflora test were conducted.

< Succeeding crop test>

Root area soil of this modified soybean and non-modified soybean in the harvest period was collected from 8 locations per plot, mixed (8 plants/plot, 4 repeated plots) and filled into a 25-cell seedling growing tray. Single radish seed was sowed to each cell. The germination rate was investigated 7 days after seeding, and the plant height and dry weight were investigated 29 days after seeding.

As a result, regarding the germination rate, plant height and dry weight of assay plant radish, no statistically significant differences were observed between the test plots of this modified soybean and non-modified soybean (“Isolated Field Test Results Report”, Table 10, p.11).

<Plow-in test>

Aboveground part of plant bodies of this modified soybean and non-modified soybean in the harvest period were reaped (4 plants/plot, 4 repeated plots), dried, crushed, and mixed well with horticultural soil, where 4 plants were regarded as 1 sample (the weight ratio of dried powder: approx. 0.6%). The mixed soil was placed into a 25-cell seedling growing tray, and a single radish seed was sowed to each cell. The germination rate was investigated 7 days after seeding, and the plant height and the dry weight were investigated 22 days after seeding.

As a result, regarding the germination rate, plant height and dry weight of assay plant radish, no statistically significant differences were observed between the test plots of this modified soybean and non-modified soybean (“Isolated Field Test Results Report”, Table 11, p.11).

<Soil microflora test>

Soil of this modified soybean and non-modified soybean after harvesting was collected from 3 locations per plot (3 samples/plot, 4 repeated plots). Bacterial count, actinomycetal count, and filamentous fungal count were measured using the dilution plate technique. As a result, no statistically significant differences were observed between the test plots of this modified soybean and non-modified soybean (“Isolated Field Test Results Report”, Table 12, p.12).

3. Information concerning the use of living modified organisms

(1) Content of use

Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.

(2) Method of use

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(3) Method of collecting information after commencement of Type I Use by the corporation applying for approval

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(4) Measures to prevent potential Adverse Effects on Biological Diversity

See the “Emergency Measure Plan”.

(5) Results of use in an environment similar to the environment planned for use or Type I Use at a laboratory etc.

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(6) Information on the use overseas

Tests were carried out at a total of 497 fields in the USA (Indiana, Illinois, etc., 2009-2013), and no report has been made on differences from non-modified soybean that may pose a risk in causing Adverse Effects on Biological Diversity.

The application status of this modified soybean overseas is as shown below (Table 6, p.20).

Table 6 Application status of this modified soybean overseas (as of December 2013)

Country	Organization	Purpose	Status
USA	United States Department of Agriculture (USDA)	Cultivation	Aug 2011
	United States Food and Drug Administration (FDA)	Food, feed	Sep 2011 [1)]
Canada	Health Canada	Food	Sep 2011 [2)]
	Canadian Food Inspection Agency (CFIA)	Cultivation, feed	Sep 2011 [3)]
Australia and New Zealand	Food Standards Australia New Zealand (FSANZ)	Food	Apr 2012 [4)]
EU	European Food Safety Authority (EFSA)	Food, feed	Feb 2012
Korea	Ministry of Food and Drug Safety (MFDS) [5)]	Food	Jun 2012
	Rural Development Administration (RDA)	Feed, environment	Jun 2012

[1)] In December 2013, safety confirmed.
[2)] In June 2013, safety confirmed.
[3)] In June 2013, safety confirmed.
[4)] In April 2013, safety confirmed.
[5)] Former Korea Food & Drug Administration (KFDA)

II. Item-by-Item Assessment of Adverse Effects on Biological Diversity

1 Competitiveness

(1) Identification of wild animals and plants that has a potentially to be affected

Soybean has been grown in Japan over a long period of time, yet there has been no report made stating that soybean became a weed under the natural environment.

As shown in I. 2 (6), isolated field tests were conducted in 2012 at the Ogori Development Center, Dow Chemical Japan Limited, where differences in various traits involved with the competitiveness were investigated between this modified soybean and non-modified soybean by observing morphological and growth characteristics, cold tolerance in the early stage of growth, wintering ability of mature plant, fertility and size of the pollens, as well as production, shedding habit, dormancy and germination rate of seed. As a result, no difference was observed between this modified soybean and non-modified soybean.

For this modified soybean, modified *aad-12* gene, *2mepsps* gene and *pat* gene are introduced, and they confer tolerance to aryloxyalkanoate herbicide, herbicide glyphosate and herbicide glufosinate by expressing modified AAD-12 protein, 2mEPSPS protein and PAT protein, respectively. It is unthinkable that such herbicide tolerance enhances the competitiveness under natural conditions where application of such herbicides is unlikely to take place.

The donor nucleic acid of this modified soybean contains the *RB7 MAR* sequence, a nuclear matrix binding region derived from *Nicotiana tabacum*. It is reported that a nuclear matrix binding region enhances the expression of an introduced gene if flanking upstream or both sides of the introduced gene yet it does not enhance if flanking downstream (Fukuda and Nishikawa, 2003), and the *RB7 MAR* sequence is considered to have a possibility to affect the expression of the introduced gene only. From the results of the isolated field tests, it was considered that there was no difference between this modified soybean and non-modified soybean on physiological and ecological characteristics as mentioned above. Additionally, tests have been carried out at a total of 497 fields in the USA (2009-2013), yet no report has been made on differences that may pose a risk in causing Adverse Effects on Biological Diversity for this modified soybean compared to non-modified soybean. Therefore, it is considered to be unlikely that the *RB7 MAR* sequence alters other metabolic systems of plant bodies and thereby induces a difference from the recipient organism in physiological or ecological characteristics involved with the competitiveness.

Therefore, it was judged that no wild animal or plant is identifiable to have a possibility to be affected.

(2) Assessment of the specific content of adverse effects

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(3) Assessment of the possibility of adverse effects

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(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, no wild animal or plant was identified to have a possibility to be affected, and it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its competitiveness.

2 Productivity of harmful substances

(1) Identification of wild animals and plants that has a potentially to be affected

No productivity of harmful substances (e.g., allelopathic substances) that may affect the living or growth of wild animals or plants is known to exist in soybean.

This modified soybean produces modified AAD-12 protein that confers tolerance to aryloxyalkanoate herbicide, 2mEPSPS protein that confers tolerance to glyphosate herbicide and PAT protein that confers tolerance to glufosinate herbicide. None of modified AAD-12 protein, 2mEPSPS protein and PAT protein is known as a harmful substance.

In 2012, in the isolated field tests conducted at the Ogori Development Center, Dow Chemical Japan Limited, a succeeding crop test, plow-in test and soil microflora test were carried out for the purpose of comparing the productivity of harmful substances for this modified soybean and non-modified soybean. As a result, no statistically significant difference was identified between the test plots of this modified soybean and non-modified soybean for any of the succeeding crop test, plow-in test or the soil microflora test (“Isolated Field Test Results Report”, Tables 10-12, p.11-12).

As described in I. 2 (1) 2) (c), while the modified AAD-12 protein has a possibility to oxidize *trans*-cinnamic acid and indole-3-acetic acid, its catalytic efficiency is extremely low and the possibility of them affecting the metabolic pathways of plants is considered low. Since no compound with the aryloxyalkanoate structure is known to exist in plant bodies to date, it is considered to be unlikely that modified AAD-12 protein alters other metabolic systems of plant bodies. EPSPS protein is an enzyme that reacts specifically with phosphoenolpyruvate and shikimate-3-phosphate, and indicated not the rate determining enzyme in the shikimate pathway as the synthesis pathway of aromatic amino acids (Weiss and Edwards, 1980; Herrmann, 1983). It is also reported that aromatic amino acids as the final products were not produced excessively even in cultured plant cells that produce

EPSPS protein 40 times than normal cells (Smart *et al*., 1985). Additionally, while it is known that shikimate reacts with EPSPS protein in addition to these substrates, its reactivity is about 1/2,000,000 of the reactivity with shikimate-3-phosphate, and the possibility of shikimate reacting with EPSPS protein as a substrate in the body is substantially low (Gruys *et al*., 1992). Meanwhile, for mutant EPSPS protein of *E. coli* that underwent amino acid changes equivalent to 2mEPSPS protein where the 97th threonine and the 101st proline are changed to isoleucine and serine, respectively, it was reported that the mutant EPSPS protein exhibited a high affinity with phosphoenolpyruvate and 3-phosphokishimate similar to wild EPSPS protein (Funke *et al*., 2009). Additionally, 2mEPSPS protein is similar to EPSPS protein both structurally and functionally other than being insensitive to glyphosate herbicide, and they have the same mechanism of action (Herouet-Guicheney *et al*., 2009). Therefore, it is considered that 2mEPSPS protein has high substrate specificity similar to EPSPS protein and unlikely to alter other metabolic systems. PAT protein is an enzyme that extremely specifically acetylates free amino groups of L-glufosinate that is the active ingredient of herbicide glufosinate, and never acetylates other amino acids or D-glufosinate (OECD, 1999b). Additionally, acetylation of L-glufosinate by PAT protein is never affected, even under overabundance of L-amino acids (OECD, 1999b). Therefore, it is considered to be unlikely that PAT protein alters other metabolic systems of plant bodies. The donor nucleic acid of this modified soybean contains the *RB7 MAR* sequence, a nuclear matrix binding region derived from *Nicotiana tabacum*. While it has been reported that a nuclear matrix binding region enhances the expression of the introduced gene (Halweg *et al*., 2005), it was reported that it had no influence on the expression of endogenous genes (Chattopadhyay *et al*., 1998) nor enhanced the expression of the introduced gene if flanking downstream (Fukuda and Nishikawa, 2003). Therefore, the *RB7 MAR* sequence is considered to have a possibility to affect the expression of the introduced gene only. As shown in I. 2 (6), from the results of isolated field tests conducted in 2012 at the Ogori Development Center, Dow Chemical Japan Limited, it was considered that there was no difference between this modified soybean and non-modified soybean in morphological and ecological characteristics. Additionally, tests have been carried out at a total of 497 fields in the USA (2009-2013), yet no report has been made on differences that may pose a risk in causing Adverse Effects on Biological Diversity for this modified soybean compared to non-modified soybean. Therefore, it is considered to be unlikely that the *RB7 MAR* sequence alters other metabolic systems of plant bodies.

Investigation on the FARRP Allergen Database version 13 (2013) identified that the modified AAD-12 protein, 2mEPSPS protein and PAT protein had no amino acid sequence that is structurally related to known allergens.

Regarding the effects of 2,4-DCP as a decomposition product of herbicide 2,4-D on aquatic organisms, LC_{50} (median lethal concentration) identified in acute toxicity studies was 1.7 mg/L for freshwater fish and 1.4 mg/L for *Daphnia magna*, and EC_{50} (half maximal effective concentration) was 1.5 mg/L for Lemna. The NOEC (no observed effect concentration) identified in chronic toxicity studies was 0.14 mg/L for Lemna and 0.21 mg/L for *Daphnia*

magna. Additionally, regarding its effects on terrestrial organisms, LC_{50} was 125 mg/kg for earthworm and EC_{10} (10% effect concentration) was 0.7 mg/kg for *Folsomia candida* (OECD, 2006). Meanwhile, regarding the effects of 2,4-D on aquatic organisms, LC_{50} identified in acute toxicity studies was 0.26 mg/L for freshwater fish and 2.2 mg/L for *Daphnia magna*, and EC_{50} was 0.2992 mg/L for *Lemna gibba*. The NOEC identified in chronic toxicity studies was 0.0476 mg/L for *Lemna gibba* and 0.20 mg/L for *Daphnia magna* (EPA, 2004).

Like these, the toxicity of 2,4-DCP as a decomposition product of 2,4-D is equivalent to or lower than that of 2,4-D, and it is considered to be unlikely that the concentration of 2,4-DCP will reach levels that cause adverse effects more than the sprayed 2,4-D does, even if the maximum possible concentration of 2,4-DCP is assumed for when 2,4-D is sprayed.

The residual 2,4-DCP concentration in grains was investigated after spraying the upper limit amount of the appropriate use range of 2,4-D onto a soybean line (DAS68416, OECD UI: DAS-68416-4) (hereinafter referred to as “Soybean 68416 Line”) that has tolerance to aryloxyalkanoate herbicides similar to this modified soybean. As a result, the maximum average residual amount was 0.047 mg/kg (Attachment 3, Table 2, p.26). LD_{50} (median lethal dose) of 2,4-DCP identified in acute toxicity studies on mice was 1,276-1,352 mg/kg body weight, and NOAEL (no observed adverse effect level) identified in 2-year chronic toxicity studies on rats was 440 mg/kg body weight/day for males and >250 mg/kg body weight/day for females (OECD, 2006), which is substantially larger than the residual amount of 2,4-DCP in the Soybean 68416 Line. Therefore, it is considered to be unlikely that the imported seeds of this modified soybean that has tolerance to aryloxyalkanoate similar to the Soybean 68416 Line cause adverse effects on wild animals or plants.

It has been confirmed that the toxicity (acute toxicity, subacute toxicity, chronic toxicity, carcinogenicity, and reproductive and developmental toxicity) of *N*-acetyl-L-glufosinate as a metabolic product of herbicide glufosinate on animals is lower than that of glufosinate (Food Safety Commission of Japan, 2010), and it is considered to be unlikely that the concentration of *N*-acetyl-L-glufosinate becomes to the level that causes adverse effects more than the sprayed glufosinate does, even if the maximum possible concentration of *N*-acetyl-L-glufosinate is assumed for when glufosinate is sprayed. As a side note, *N*-acetyl-L-glufosinate is included in the compounds subject to the residue standards for soybeans.

Therefore, it was judged that no wild animal or plant is identifiable to receive effects attributable to the productivity of harmful substances.

(2) Assessment of the specific content of adverse effects

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(3) Assessment of the possibility of adverse effects

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(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, no wild animal or plant was identified to have a possibility to be affected, and it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its productivity of harmful substances.

3 Crossability

(1) Identification of wild animals and plants that has a potentially to be affected

As a wild relative that is crossable with soybean, *Glycine soja* grows wild in Japan (OECD, 2000). Therefore, *Glycine soja* was identified as a wild plant that has a possibility to be affected due to the crossability.

(2) Assessment of the specific content of adverse effects

Soybean and *Glycine soja* have the same chromosome number as 2n = 40 and are crossable (OECD, 2000). The specific effect related to the crossability is the possibility of raising the competitiveness of a *Glycine soja* group after the modified *aad-12* gene, *2mepsps* gene or the *pat* gene originating from this modified soybean propagating from a relevant crossbreed into the group.

(3) Assessment of the possibility of adverse effects

In Japan, *Glycine soja* is distributed in Hokkaido, Honshu, Shikoku and Kyushu, and grows wild on fields and wastelands (Numata *et al.*, 1978). Therefore, when this modified soybean is used in Japan in accordance with the Type 1 Use Regulation, undeniably there are opportunities for this modified soybean to crossbreed with *Glycine soja*.

However, soybean and *Glycine soja* are self-fertilizing plants, and it is known that in general the flowering season of *Glycine soja* is later than that of soybean and their flowering seasons rarely overlap (Nakayama and Yamaguchi, 2002). Therefore, it is considered to be unlikely that crossing of soybean and *Glycine soja* occurs. In an actual example, the average crossing rate of Tanba Black (soybean specific to Japan that has a relatively late flowing season) and *Glycine soja* was reported to be 0.73% (Nakayama and Yamaguchi, 2002). Additionally, under conditions where *Glycine soja* was twining around modified soybean and the flowering season overlapped, only one individual body was reported to be crossbred out of 11,860 individual bodies germinated from seeds collected from *Glycine soja* (Mizuguti *et al.*, 2009). On top of that, in an experiment where two types of modified soybean with much later flowering seasons (AG6702RR and AG5905RR) were used (i.e. flowering peak much closer to *Glycine soja*) and *Glycine soja* was twining around modified soybeans, the number of crossbreeds out of 25,741 individual bodies was 25 individual bodies (0.097%) for

AG6702RR and 10 individual bodies (0.039%) for AG5905RR. When *Glycine soja* was grown 2, 4, 6, 8, and 10 m away from modified soybean (AG6702RR and AG5905RR), the number of crossbreeds was reported to be 1 (out of 7,521 individual bodies), 1 (7,485), 1 (14,952), 0 (14,964), and 0 (21,749), respectively (Mizuguti *et al*., 2010). Like these examples, although crossing of soybean and *Glycine soja* can occur under the conditions of them growing nearby and their flowering seasons overlapping, the possibility of soybean crossbreeding with *Glycine soja* was considered extremely low even under such special conditions.

In the isolated field tests, this modified soybean and non-modified soybean were planted alternately with an interval of 25 cm, and seeds were collected from the non-modified soybean. Of which 3,200 seeds were again sowed inside the isolated field, and treated with herbicide 2,4-D at the 2 leaf stage of true leaf. When the survivability was investigated by counting the individuals that exhibited herbicide tolerance, the survival rate was 0.23% ("Isolated Field Test Results Report", Table 9, p.10). The normal cross-pollination rate of soybean is known to be less than 1% (OECD, 2000), and it was considered that the crossing rate estimated from the survival rate of the crossbreed of this modified soybean and non-modified soybean (0.23%) would not exceed the normal crossing rate of soybean.

Regarding the adequacy of pollens investigated at the isolated fields, no difference was observed between this modified soybean and non-modified soybean. Similarly, no difference was observed for the morphology or size of the pollens. Therefore, it was considered that the crossability of this modified soybean is unlikely to exceed that of normal soybean, and the reproductive traits of this modified soybean are equivalent to that of non-modified soybean.

Assuming this modified soybean crossbred with *Glycine soja*, propagation of the modified *aad-12* gene, *2mepsps* gene or the *pat* gene originating from this modified soybean into *Glycine soja* groups requires the crossbreed progenies to survive under the natural environment and repeatedly crossbreed with *Glycine soja*. Regarding the crossbreed formation of soybean and *Glycine soja* and the gene propagation from soybean to *Glycine soja*, investigations have been conducted in Japan. In the investigation conducted in 2003, morphological "intermediate" often observed in crossbreed progenies of soybean and *Glycine soja* was searched at 8 locations in Hiroshima Prefecture and 9 locations in Akita Prefecture where *Glycine soja* grows wild, and 1 intermediate individual was found at 1 location in Akita Prefecture (Kaga *et al*., 2005). In 2004, investigation was conducted for *Glycine soja* groups (adjacent to soybean cultivating fields) at a total of 57 locations, namely, 8 locations in Akita Prefecture, 6 locations in Ibaraki Prefecture, 4 locations in Aichi Prefecture, 6 locations in Hiroshima Prefecture, and 33 locations in Saga Prefecture, and 11 intermediate individuals were found at 3 locations in Saga Prefecture. However, no intermediate was found at the place where an intermediate was found in the investigation conducted in 2003 (Kuroda *et al.*, 2005). From these results, crossbreed formation of soybean and *Glycine soja* occurs at the places where *Glycine soja* grows wild, yet the frequency is considered to be low. Additionally, no new soybean intermediate was found in the investigation conducted in 2005 for a total of 39 locations in Akita Prefecture, Ibaraki Prefecture, Kochi Prefecture and Saga Prefecture. On top of that, among the 12 intermediate individuals found until 2004 at 1 location in Akita Prefecture and 3 locations in Saga Prefecture, survival of progenies was identified only for 1 individual at 1 location in Saga Prefecture. For the fact that almost no intermediates were found in 2005 although

intermediates were producing many seeds in 2004, it was assumed that the crossbreeds are likely to be eliminated at the wild growth areas even if seeds are produced (Kuroda *et al*., 2006). In 2006, wild growth monitoring investigation for progenies at 1 location in Akita Prefecture and 3 locations in Saga Prefecture where intermediates were found till 2005 as well as investigation for intermediates at new 40 locations in Akita Prefecture, Hyogo Prefecture and Saga Prefecture were conducted. As a result, in the progeny monitoring, only 1 intermediate individual was found at 1 location in Saga Prefecture. In the investigation conducted for new 40 locations, only 1 intermediate individual each was found at 2 locations in Saga Prefecture (Kuroda *et al*., 2007).

Regarding 468 *Glycine soja* individuals, 17 intermediate individuals and 12 soybean individuals collected during the period from 2003 to 2006 at 1 location in Akita Prefecture and 5 locations in Saga Prefecture, analysis of polymorphism patterns was conducted using 20 types of microsatellite markers and 2 types of chloroplast dCAPS markers. As a result, it was clarified that all the intermediates were generated by crossbreeding of *Glycine soja* and late-season soybean, occurred through gene propagation of soybean to *Glycine soja*. However, the secondary gene propagation from intermediates to *Glycine soja* groups was not identified. Like this, while there is a possibility of gene propagation from soybean to *Glycine soja* occurring, the possibility of further gene propagation occurring under the natural environment of Japan was considered extremely low (Kuroda *et al*., 2010).

From the above, the natural crossing rate of this modified soybean is at the same level as the crossing rate of non-modified soybean, the flowering seasons of soybean and *Glycine soja* are unlikely to overlap according to the existing knowledge, and the crossing rate is low. Therefore, it is considered that the rate of crossing between this modified soybean and *Glycine soja* under natural conditions is extremely low.

Furthermore, it can be assumed that crossbreed progenies of soybean and *Glycine soja* do not survive for a long period of time at locations where *Glycine soja* grows wild. Therefore, it is considered that the possibility of gene propagation from soybean to *Glycine soja* occurring is extremely low. From II. 1 (1), it is considered that the competitiveness of this modified soybean is not raised, and it is considered that the possibility of this modified soybean crossing with *Glycine soja* and the introduced genes preferentially propagating into a group of *Glycine soja* is extremely low.

(4) Judgment on the likelihood of causing Adverse Effects on Biological Diversity

Based on the abovementioned understanding, it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its crossability.

4 Other properties

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III. Comprehensive Assessment of Adverse Effects on Biological Diversity

Soybean has been grown in Japan over a long period of time, yet there has been no report made stating that soybean became a weed under the natural environment. Regarding the traits that involve with the competitiveness (morphological and growth characteristics, cold tolerance in the early stage of growth, wintering ability of mature plant, fertility and size of the pollens, as well as production, shedding habit, dormancy and germination rate of seed), investigations were conducted at isolated fields. As a result, no trait indicative of the possibility of raising the competitiveness of this modified soybean was observed. While this modified soybean has tolerance to aryloxyalkanoate herbicide, glyphosate herbicide and herbicide glufosinate, it is unthinkable that such herbicide tolerance enhances the competitiveness under natural conditions where application of such herbicides is unlikely to take place.

Based on the abovementioned understanding, it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its competitiveness.

No productivity of harmful substances (e.g., allelopathic substances) that may affect the living or growth of wild animals or plants is known to exist in soybean. None of modified AAD-12 protein, 2mEPSPS protein and PAT protein is known as a harmful substance. No homology is identified between these proteins and known allergens in the amino acid sequences. Regarding the productivity of harmful substances, a succeeding crop test, plow-in test and soil microflora test were conducted. As a result, no statistically significant differences were observed for any item between the test plots of this modified soybean and non-modified soybean.

From the results of investigation on the effects on aquatic organisms, the toxicity of 2,4-DCP as a decomposition product of 2,4-D is equivalent or lower than the toxicity of 2,4-D, and it is considered to be unlikely that the concentration of 2,4-DCP will reach levels that cause adverse effects more than the sprayed 2,4-D does, even if the maximum possible concentration of 2,4-DCP is assumed for when 2,4-D is sprayed. It is considered to be unlikely that the imported seeds of this modified soybean grown with 2,4-D sprayed cause adverse effects on wild animals or plants. It has been confirmed that the toxicity of *N*-acetyl-L-glufosinate as a metabolic product of herbicide glufosinate on animals is lower than that of glufosinate, and it is considered to be unlikely that the concentration of *N*-acetyl-L-glufosinate becomes to the level that causes adverse effects more than the sprayed glufosinate does, even if the maximum possible concentration of *N*-acetyl-L-glufosinate is assumed for when glufosinate is sprayed.

Based on the abovementioned understanding, it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its productivity of harmful substances.

Soybean and its wild relative *Glycine soja* have the same chromosome number as 2n = 40 and are crossable. Therefore, *Glycine soja* was identified as a wild plant that has a possibility to be affected due to the crossability.

However, in the investigation on the fertility and size of the pollens conducted in isolated field tests, the reproductive traits of this modified soybean were considered equivalent to that of non-modified soybean. Additionally, the flowering seasons of soybean and *Glycine soja* are

unlikely to overlap according to the existing knowledge, and the crossing rate is low. Therefore, it is considered that the rate of crossing between this modified soybean and *Glycine soja* under natural conditions is extremely low. Furthermore, it can be assumed that crossbreed progenies of soybean and *Glycine soja* do not survive for a long period of time at locations where *Glycine soja* grows wild. Therefore, it is considered that the possibility of gene propagation from soybean to *Glycine soja* occurring is extremely low. On top of that, the competitiveness of this modified soybean is assumed unraised as mentioned above, and it is considered that the possibility of this modified soybean crossing with *Glycine soja* and the introduced genes preferentially propagating into a group of *Glycine soja* is extremely low.

Based on the abovementioned understanding, it was judged that this modified soybean would pose no risk in causing Adverse Effects on Biological Diversity attributable to its crossability.

Therefore, as comprehensive assessment, it was concluded that the use of this modified soybean in accordance with the Type 1 Use Regulation would pose no risk in causing Adverse Effects on Biological Diversity in Japan.

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Attachment List

Confidential and Non-disclosed

1. Isolated Field Test Results Report of Soybean Tolerant to Aryloxyalkanoate Herbicide, Glyphosate Herbicide and Glufosinate Herbicide (modified *aad-12*, *2mepsps*, *pat*, *Glycine max* (L.) Merr.) (DAS44406, OECD UI: DAS-444Ø6-6)

2. Attachment 1: Herbicides to which the modified AAD-12 protein exhibits activity

3. Attachment 2: Substrate Specificity of Aryloxyalkanoate Dioxygenase-12 (AAD-12)

4. Attachment 3: A Risk Assessment Overview of 2,4-D Treated Soybean Containing the DAS AAD-12 Trait
5. Attachment 4: Nucleotide Sequence of pDAB8264

6. Attachment 5: Number of Copies of Introduced Gene and Inter- and Intra-generation Stability

7. Attachment 6: Production Process of pDAB8264

8. Attachment 7: Cloning and Characterization of the DNA Sequence for the Insert and Its Flanking Border Regions of DAS-44406-6 Soybean

9. Attachment 8: Detection Method of This Modified Soybean