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

Name of Entity: Dow Chemical Japan Limited
Name of Applicant: Michiro Kurita, Representative Director
Address: 2-24 Higashi Shinagawa 2-chome,Shinagawa-ku, Tokyo

Approved Type 1 Use Regulation

Names of types of living modified organisms	Cotton tolerant to Aryloxyalkanoate herbicide and glufosinate herbicide (modified *aad-12*, *pat*, *Gossypium hirsutum* L.) (DAS1910, OECD UI：DAS-8191Ø-7)
Content of Type 1 Use of living modified organisms	Use for provision as food, or animal feed purposes, processing, storage, transportation and disposal, and other acts attendant with these.
Method of Type 1 Use of living modified organisms	-

Summary of the Evaluation on Adverse Effect on Biological Diversity

I. Information Collected for the Evaluation for its Impact on Biodiversity

1. Information about Preparation of Living Modified Organisms

(1) Information of Donor Nucleic Acid

a. Constitution and Origin of Components

Table 1 (p.2) shows the constitution and origin of donor nucleic acid used to create cotton tolerant to aryloxyalkanoate herbicide and glufosinate herbicide (modified *aad-12*, *pat*, *Gossypium hirsutum* L.) (DAS1910, OECD UI：DAS-8191Ø-7) (to be called “this recombinant cotton” hereinafter.).

Table1 Constitution, Origin and Function of Components of Donated Nucelic Acid

Component	Size (bp)	Origin and Function
T-DNA Border B	24	T-DNA border sequence derived from *Agrobacterium tumefaciens*.
RB7 MAR	1166	Nuclear matrix attachment region derived from *Nicotiana tabacum* (Allen *et al.*, 1996). It stabilizes the gene expression.
Modified *aad-12* cassette		
AtUbi10	1322	Promotor of Polyubiquitin 10 (UBQ10) gene derived from *Arabidopsis thaliana*. It contains 5'-terminal untranslated region and intron (Norris *et al*., 1993). It makes gene express on the whole 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	The 3'-terminal untranslated region, which is consist of transcription termination point and polyadenylation site of ORF23 derived from plasmid pTi15955 of Agrobacterium (Barker *et al*., 1983). It terminates gene transcription.
pat cassette		
CsVMV	517	Promotor derived from Cassava vein mosaic virus. It contains the 5’-terminal untranslated region (Verdaguer *et al*., 1998). It expresses gene in the whole plant body.

Component	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	The 3'-terminal untranslated region, which is consist of transcription termination point and polyadenylation site of ORF1 derived from plasmid pTi15955 of Agrobacterium (Barker *et al*., 1983).。It terminates gene transcription.
T-DNA Border A	24	T-DNA border sequence derived from Agrobacterium.
T-DNA Border A	24	T-DNA border sequence derived from Agrobacterium.
T-DNA Border A	24	T-DNA border sequence derived from Agrobacterium.

(Dow Chemical Japan Limited has the rights and responsibilities relating to the information in this table.)

b. Function of Components

[1] Function of objective gene, expression regulatory region, localization signal, selection marker, and each component in other donor nucleic acid.

Donor nucleic acid contains *RB7 MAR* sequence, which is nuclear matrix attachment region. Nuclear matrix attachment region is often observed in genome DNA sequence, and considered to take a role to attach DNA to nuclear matrix in order to form the loop structure of DNA. It is reported that the transgene expression is enhanced and gene-silencing which inhibits the transgene expression is reduced when the nuclear matrix attachment region is adjacent to either side of transgene (Allen *et al*., 2000 ; Halweg *et al*., 2005). It is also reported that transgene expression is enhanced when the nuclear matrix attachment region is adjacent to either of the upstream or both sides of transgene, however, transgene expression will not be enhanced when it is adjacent to the downstream (Fukuda and Nishikawa, 2003).

[2] Function of protein produced by the expression of objective gene and selection marker, as well as the fact that the protein shares homology with protein which is revealed to have allergy properties, if applicable.

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 (Wright *et al*., 2007).

In this recombinant cotton, modified AAD-12 protein catalyzes the reaction introducing oxygen into Aryloxyalkanoate herbicide, converts it to a compound with no herbicidal activity, and exhibits herbicide tolerance. For example, modified AAD-12 protein catalyzes the reaction introducing oxygen into 2,4-dichlorophenoxyacetic acid herbicide (2,4-D), and converts it into 2,4-dichlorophenol (2,4-DCP) having no herbicidal activity and glyoxylic acid (see Figure 1, p.4) For reference, herbicides providing substrate for modified AAD-12 protein are shown in Appendix 1.

As a result of the research using Allergen Database (FARRP Allergen Database version 12, 2012) to see whether modified AAD-12 protein shares amino acid sequence with the known allergens, it was found that AAD-12 protein had no sequence structurally similar to the known allergens.

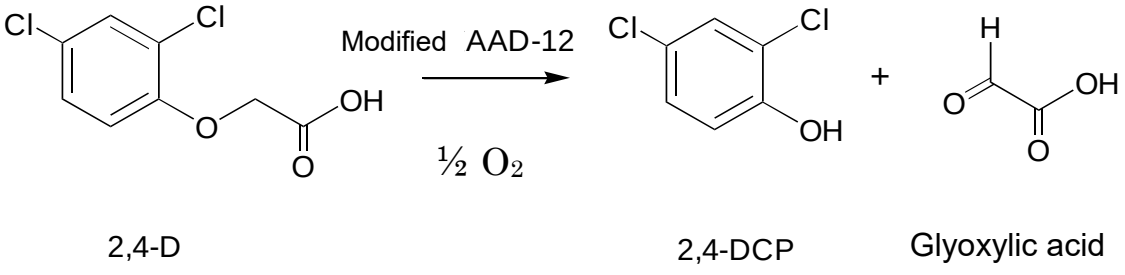

Figure1 Action mechanism of modified AAD-12 protein

(Dow Chemical Japan Limited has the rights and responsibilities relating to the information in this figure.)

Phosphinothricin Acetyl Transferase (to be called “PATprotein” hereinafter) rapidly converts glufosinate L-isomer into *N*-acetyl-L-glufosinate (2-acetoamid-4-

methylphosphinico-butanoic acid) which is a stable compound not toxic to plants.

Glufosinate L-isomer, which is a structural analog of glutamic acid works as an antagonistic inhibitor of glutamine synthetase in bacteria and plants, and has herbicidal activity. Therefore, in a plant that is not tolerant to glufosinate herbicide, a large amount of ammonia accumulates within the cells due to the glutamic acid synthetase inhibition, and it leads to eventual death of plant cells. 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).

As a result of the research using Allergen Database (FARRP Allergen Database version 12、2012) to see whether PAT protein shares amino acid sequence with the known allergens, it was found that it had no sequence structurally similar to the known allergens.

③ Detail of changes made in the host's metabolic system if applicable

【Modified AAD-12 protein】

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 of modified AAD-12 protein to introduce oxygen into the substrate, α-ketoglutamic acid is converted to succinic acid in the presence of α-ketoglutamic acid (Figure 2, p.5).

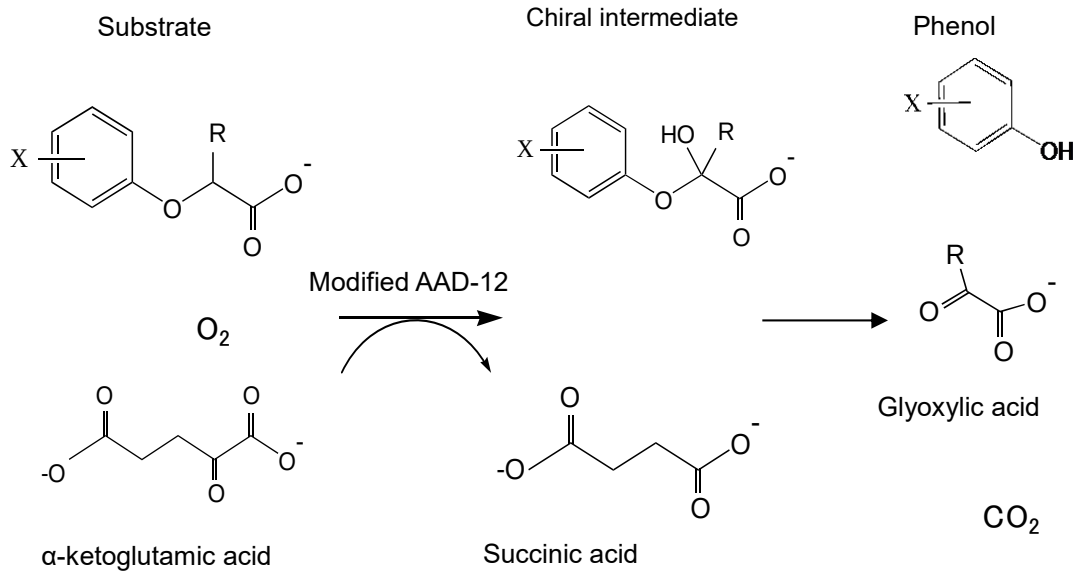

Figure 2 Enzymic Reaction of Modified AAD-12 Protein in the presence of α-ketoglutamic acid (Dow Chemical Japan Limited has the rights and responsibilities relating to the information in this figure.)

To see whether a compound existing in a plant body which is structurally and physiologically similar to an compound with Aryloxyalkanoate structure can be the substrate of modified AAD-12 protein, the formation of succinic acid was confirmed by a measurement of succinic acid in the reaction experiment of the modified AAD-12 protein with the addition of α-ketoglutamic acid. In the succinic acid measurement, its production amount was calculated by measuring by the absorbance of 340 nm the amount of oxidation type nicotinamide adenine dinucleotide (NAD) formed by the oxidation of reduced type nicotinamide adenine dinucleotide (NADH) in the reaction system using succinyl CoA synthetase, pyruvate kinase, and lactic acid dehydrogenase (Luo *et al*., 2006; Figure 3, p.6).

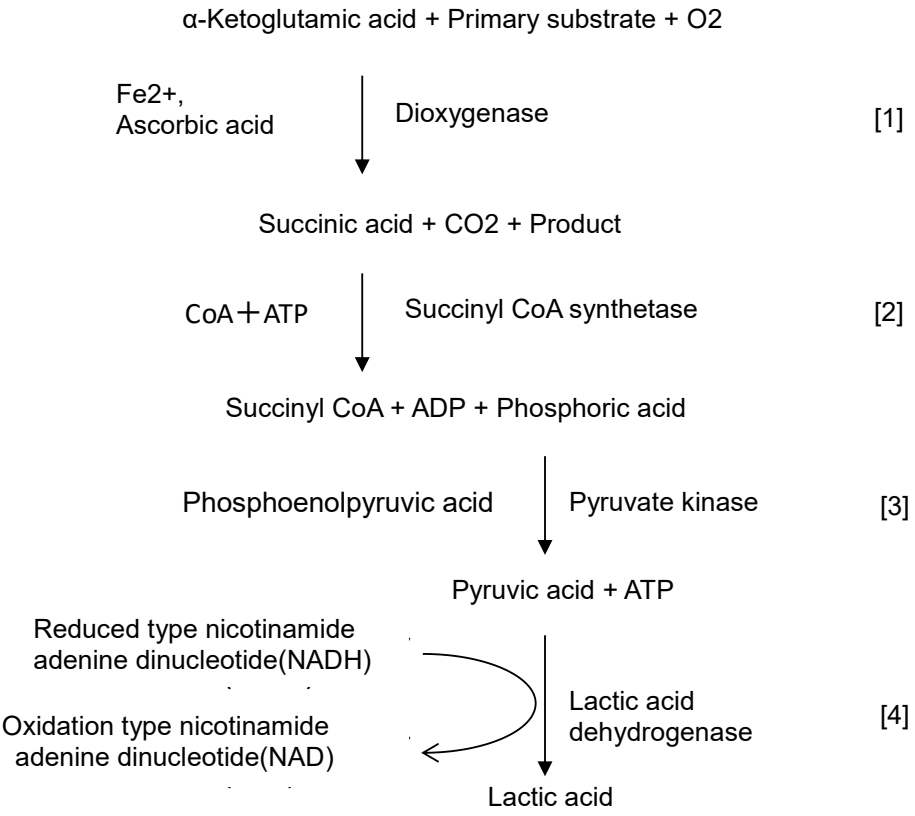

Figure 3 Reaction Path of Succinic Acid Measurement (Revision of Scheme 1 in Luo *et al*., 2006.)

[1] α-Ketoglutamic acid is converted to succinic acid in the reaction of dioxygenase introducing oxygen to the substrate.
[2] In the presence of coenzyme A (CoA) and adenosine 5'-triphosphate (ATP), succinic acid is converted to succinyl CoA by succinyl CoA synthetase, and adenosine 5'-diphosphate (ADP) and phosphoric acid are produced at the same time.
[3] Pyruvic acid and ATP are produced from phosphoenolpyruvic acid and ADP by pyruvate kinase.
[4] In the presence of reduced type nicotinamide adenine dinucleotide (NADH), pyruvic acid is converted to lactic acid by lactic acid dehydrogenase, and NAD is produced at the same time. The amount of NAD produced in this reaction is considered to be equivalent to that of succinic acid in quantity.

(Dow Chemical Japan Limited has the rights and responsibilities relating to the information in this figure.)

In the succinic acid measurement, plant hormones such as indole-3-acetic acid, abscisic acid, gibberellic acid (GA3), aminocycropropane -1-carboxylic acid and phenylpropanoid intermediates such as trans-cinammic acid, coumaric acid and sinapic acid as well as 20 different kinds of L-amino acids, which are compounds structurally and physiologically similar to Aryloxyalkanoate structured compounds existing in plant bodies, were used.

Regarding the 20 kinds of L-amino acids, no production of succinic acid was observed at the modified AAD-12 protein concentration of 1 μM, therefore, it was considered that the modified AAD-12 protein does not react with these 20 kinds of L-amino acids (Appendix 2, Table 1, p.18). On the other hand, as a result of reacting the modified AAD-12 protein at 1 μM with plant hormone and phenylpropanoid intermediate, it was observed that a small amount of succinic acid was produced with coumaric acid, trans-cinammic acid and aminocycropropane-1-carboxylic acid (Appendix 2, Table 2, p.19). In addition, as the result of reacting the modified AAD-12 protein at 5 μM and 10 μM, production of succinic acid was observed with indole-3-acetic acid, gibberellic acid, abscisic acid, coumaric acid, trans-cinammic acid, and sinapic acid (Appendix 2, Table 3 and Table 4, p.20 and p.21). As described above, the production of succinic acid was observed with several kinds of compounds at the different concentrations of the modified AAD-12 protein. However, in the succinic acid measurement, there is possibility that an uncoupling reaction inducing the production of succinic acid without substrate oxidation may happen (Hausinger, 2004). Consequently, the measurement of primary oxide by Fourier transformation mass spectrometric analysis (FT/MS) was carried out in order to see whether the compound which was confirmed to have a certain reaction in the succinic acid measurement was actually oxidized. As the result, only the oxides of trans-cinammic acid and indole-3-acetic acid were detected in the reaction of the modified AAD-12 protein at 10 μM.

In this context, the catalyst efficiency of the modified AAD-12 protein was investigated by the succinic acid measurement, regarding trans-cinammic acid and indole-3-acetic acid and *S*-dichlorprop[*1)] being a compound with aryloxyalkanoate structure and having similar chemical constitution to 2,4-D as reference. Then the catalyst efficiency (*Kcat/Km)* of trans-cinammic acid, indole-3-acetic acid and *S*-dichlorprop resulted in 156.7 $M^{-1}s^{-1}$, 8.2 $M^{-1}s^{-1}$ and 30175 $M^{-1}s^{-1}$ respectively (Appendix 2, Table 5, p.22). *Kcat/Km* for trans-cinammic acid and indole-3-acetic acid turned to be 0.52% and 0.027% of *Kcat/Km* of *S*-dichlorprop respectively, which indicated that the catalyst efficiency of trans-cinammic acid and indole-3-acetic acid is remarkably low compared to *S*-dichlorprop. It was also reported that *Kcat/Km* on 2,4-D of the modified AAD-12 protein is 18600 $M^{-1}s^{-1}$ (Wright *et al*., 2010), which means that 2,4-D of the modified AAD-12 protein and *S*-dichlorprop have the equivalent catalyst efficiency, thus it is considered that the catalyst efficiency of trans-cinammic acid and indole-3-acetic acid is much lower than that of 2,4-D.

*1) Optical *S*-isomer compound of dichlorprop herbicide. As only *R*-isomer is herbicidal active, *S-dichlorprop* does not have herbicidal activity.

Moreover, it was reported that *Kcat/Km* of *in vitro* trans-cinammic acid in cinnamate-4-hydroxylase of *Arabidopsis thaliana* is 3.4×10^6 $M^{-1}s^{-1}$ (Chen *et al*., 2007). It was also reported that *Kcat/Km* of *in vitro* indole-3-acetic acid in IAA amido synthetase of rice is 2.75×10^3 $M^{-1}s^{-1}$(Chen *et al*., 2009). As mentioned above, both of cinnamate-4- hydroxylase and IAA amido synthetase have high catalyst efficiency, and thus it is considered that trans-cinammic acid and indole-3-acetic acid are efficiently and specifically used in the existing metabolic pathway within a plant body. On the other hand, the *Kcat/Km* value of the modified AAD-12 protein against trans-cinammic acid is 0.005 % of the *Kcat/Km* value of cinnamate-4- hydroxylase againt trans-cinammic acid. And the *Kcat/Km* value of the modified AAD-12 protein against indole-3-acetic acid is 0.3 % of the *Kcat/Km* value of indole-3-acetic acid amido synthetase to indole-3-acetic acid, which means that the *Kcat/Km* value of the modified AAD-12 protein is very low in both cases.

Based on the above, although there is possibility that the modified AAD-12 protein oxidizes trans-cinammic acid and indole-3-acetic acid, its catalyst efficiency is very low, therefore, it is unlikely that the observed oxidation reaction influences the metabolic pathway of plants.

It is also considered that the modified AAD-12 protein does not change other metabolic systems in plant bodies because no aryloxyalkanoate structured compounds are known to exist within plant bodies.

Regarding the influence that 2,4-DCP which is a degradative product of 2,4-D herbicide gives to aquatic organisms, LC_{50}(Lethal concentration 50) on acute toxicity test is 1.7 mg/L for fresh water fish and 1.4 mg/L for *Daphnia magna,* and EC_{50} (Effective concentration 50) for Lemna is 1.5 mg/L. On chronic toxicity test, NOEC (Non-observed effect concentration) is 014 mg/L for Lemna and 0.21 mg/L for *Daphnia magna.* As for the influence on land creatures, LC50 for earthworms is 125 mg/kg and EC_{10} (Effective concentration 10%) for *Folsomia candida* is 0.7 mg/kg (OECD, 2006). On the other hand, regarding the influence of 2,4-D on aquatic creatures, LC_{50} on acute toxicity test is 0.26 mg/L for freshwater fish and 2.2 mg/L for *Daphnia magna*, and EC_{50} for *Lemna gibba* is 0.2992 mg/L. On chronic toxicity test, NOEC is 0.0476 mg/L for *Lemna gibba* and 0.20 mg/L for *Daphnia magna* (EPA, 2004).

As described above , 2,4-DCP which is a degradative product of 2,4-D has the equivalent or less toxicity compared to 2,4-D, therefore it is considered that even the highest concentration of 2,4-DCP in sprayed 2,4-D will not have as much impact as the sprayed 2,4-D itself.

【PAT Protein】

PAT protein is an enzyme that acetylates very specifically the free amino group of L-glufosinate, an active ingredient of glufosinate herbicide, but does not acetylate any other amino acids or D-glufosinate (OECD, 1999). And even if there is excessive amount of L-type amino acid, it will not affect the acetylation reaction of L-glufosinate by PAT protein.

Therefore, it is considered that PAT protein does not change the other metabolic system in a plant body.

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, 2010), and it is considered that even the highest concentration of *N*-acetyl-L-glufosinate in sprayed glufosinate will not have as much impact as the sprayed glufosinate itself.

(2) Information of Vector

a Name and Origin

Vector pDAB2407, the basis of the introduced pDAB4468 was derived from Agrobacterium and *Escherichia coli*.

b Characteristics

① Base number and sequence of vector

The number of bases of expression vector pDAB4468 is 12154 bp. The nucleotide sequence of pDAB4468 is shown in Appendix 3.

② Specific function of nucleotide sequence if any

Expression vector pDAB4468 has *spec*R gene imparting spectinomycin tolerance. *spec*R gene was used as the selection marker for constructing an expression vector pDAB4468, however, *spec*R gene is not introduced to this recombinant cotton because it is located outside of T-DNA region.

Meanwhile, as a result of southern blot analysis to see the presence of *spec*R gene in this recombinant cotton, it was confirmed that no *spec*R gene is present (Table 2 in Appendix 4, p.5).

③ Whether or not vector has infectivity, and the host range information if it has infectivity

T-DNA region of the vector which became the basis of expression vector pDAB4468, is replaced by donor nucleic acid shown in Table 1 (p.2), and it neither contains any sequence enabling Agrobacterium infection nor has any known infectivity.

(3) Preparation Method for Living Modified Organisms

a. Constitution of whole nucleic acid transferred into the host

The map of expression vector pDAB4468 is shown in the upper diagram in Figure 4 (p.10).
Production process of expression vector pDAB4468 is shown in Appendix 5.

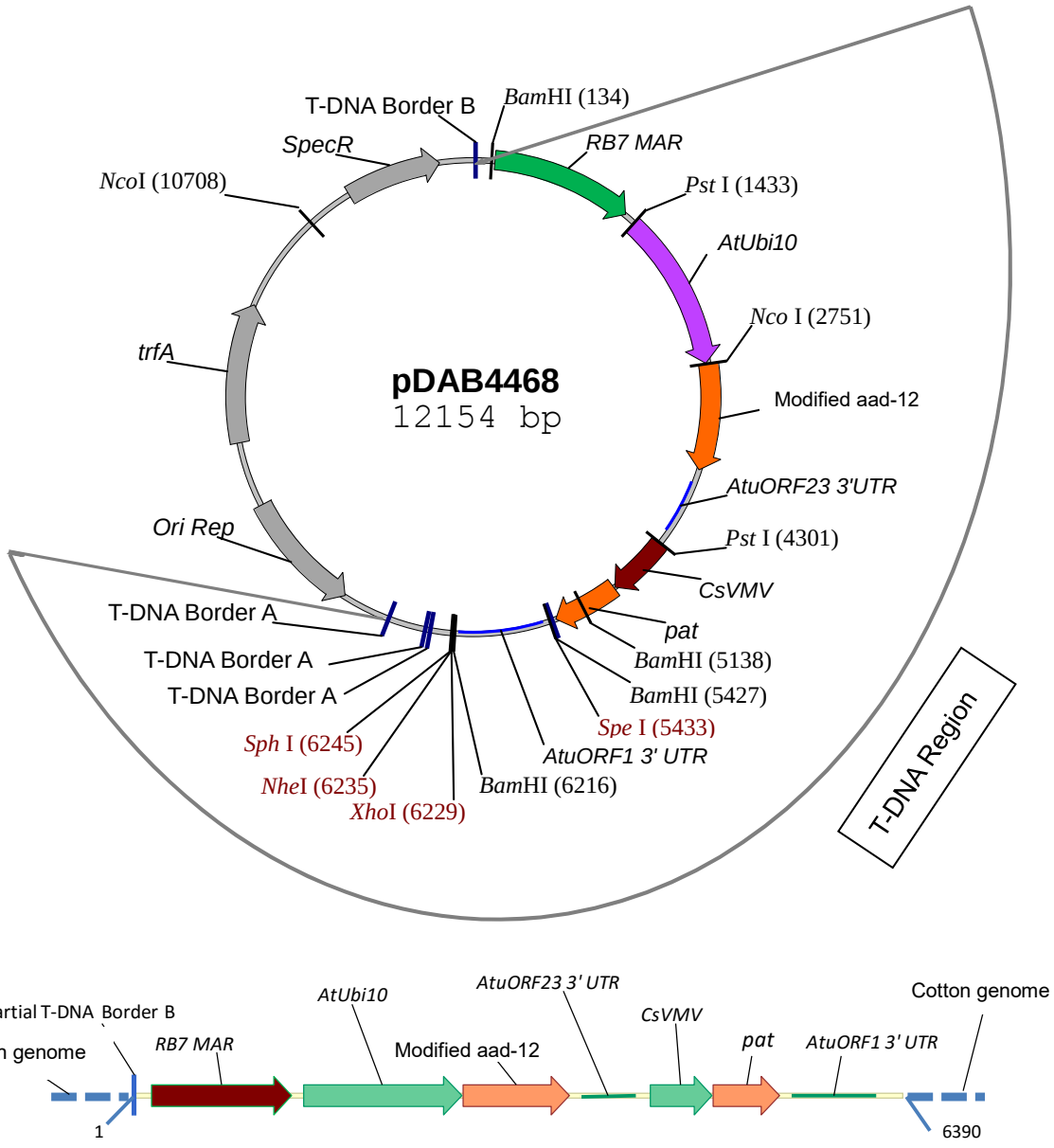

Figure 3 Map of Expression Vector pDAB4468 (Upper) and Outlined Diagram of T-DNA Region (Lower)

* The numbers in parenthesis in the upper diagram indicate the cutting positions of restriction enzyme on plasmid, starting at T-DNA Border B. The number (6390) in the lower diagram indicates the size of nucleic acid actually transferred.

(Dow Chemical Japan Limited has the rights and responsibilities relating to the information in this figure.)

b. Method to transfer nucleic acid into the host

Nucleic acid was transferred into the host by Agrobacterium method.

c. Growing process of living modified organisms

① How to select cells to which nucleic acid is transferred

Callus were selected on the culture media containing gulfosinate herbicide.

② Presence or absence of remaining Agrobacterium cells if Agrobacterium method is taken to transfer nucleic acid

We confirmed that there was no Agrobacterium fungus remaining by adding antibiotics to callus-inducing medium and embryonic callus culture medium, and after sterilizing Agrobacterium, making sure that no exoskeleton region was detected in T0 and T1 generations by PCR method.

③ The breeding process regarding the cell with nucleic acid transferred the line in which the existing condition of a copy of the transferred nucleic acid was confirmed and the line subjected to a Confined Field Trial, as well as the line used for collecting the information necessary to other the Biological Diversity Risk Assessment Tests.

Glufosinate was applied to re-differentiated plant bodies, and tolerant individual organisms were selected. Regarding the selected plant bodies, we analyzed the copy number of modified *aad-12* gene and *pat*-gene by quantitative PCR method using the internal standard. Especially as for *G. hirsutum*, we took account that it is allotetraploid cotton and selected the plant body having single-copied transgenes (T0 and T1 generations). Furthermore, in an outdoor field in the United States, we performed a presence check of modified *aad-12* gene and *pat* gene in the progeny lines (since T2 generation) by PCR method, and analyzed the copy number of modified *aad-12* cassette, pat cassette and *RB7 MAR* sequence by southern blot analysis. Judging comprehensively from these results, the confirmation of protein expression, herbicide tolerance and agricultural characters, we selected this recombinant cotton. The scope of this application is about the progeny lines since T2 generation.

Detail is shown in Figure 5 (p.12).

The following explains about the approvals and the application status for this recombinant cotton in Japan (as of July 2013).

May 2012	Approved for Type 1 use regulation (Confined Field Trials) by MAFF and MOE based on *"Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms"* (Duration: May 29 2012 to March 31 2014).
2013	Planning to apply to Ministry of Health, Labor and Welfare for safety assessment for food products, based on "Food Sanitation Act".
2013	Planning to apply to MAFF for safety assessment for animal feeding products, based on *"the Act on Safety Assurance and Quality Improvement*

of Feeds”.

Confidential Information / Not Open to the Public

Figure 4　Breeding Chart of This Recombinant Cotton

(4)　Existing condition of nucleic acid transferred into a cell and stability of phenotypic expression by this nucleic acid

① Place where the copy of transferred nucleic acid exists

Having integrated to plant chromosome, nucleic acid will follow Mendel’s Law.　To see how the traits introduced into this recombinant cotton will segregate in the group of T1 generation (Fig. 5, p.12), we confirmed the presence of modified *aad-12* gene by PCR method, and studied the presence or absence of its herbicide tolerance by spraying herbicide 2,4-D and glufosinate herbicide (Indiana State U.S. in 2009).

As a result, all the individual organisms with modified *aad-12* gene confirmed by PCR method, exhibited tolerance to both herbicides. Based on this result, we have confirmed that the observation value was not contradictory to Mendel’s Law on intranuclear gene, therefore the transferred nucleic acid exists on the chromosome (Table 2, p.12).

Table 2　Trait segregation of this recombinant cotton in T1 generation

Confidential Information / Not Open to the Public

② Copy number of transferred nucleic acid and transmitting stability of nucleic acid copy through generations

To confirm the copy number of transferred nucleic acid, southern blot analysis was performed from T2 to T5 generations as well as in BC1F2 generation, and as a result it was confirmed that *RB7 MAR* sequence introduced to this recombinant cotton, modified *aad-12* cassette and *pat* cassette are single-copied and transmitted stably in multiple generations (Table 2, p.4, Appendix 4).

Also, we cloned the entire inserted gene and decided the nucleotide sequence in this recombinant cotton including the host genome border region in order to confirm the insertion of individual components in T-DNA region. Then we determined 8834 bp in total of nucleotide sequences, including transferred nucleic acid region 6390 bp, 5’ end flanking sequence 1373 bp and 3’ end flanking sequence 1071 bp (Appendix 6). As a result, it was found that not T-DNA Border A but only a part of T-DNA Border B was transferred, and all the other components

were transferred in its entirety (Lower diagram in Figure 4, p.10).

③ If there are multiple copies existing on chromosome, whether they are neighboring or separately placed to each other

Multiple copies do not exist on the chromosome.

④ Stability of expression between individuals and generations under the natural conditions, regarding the characteristics specified in ① in (6).

From T3 to T5 generations of this recombinant cotton, the expression level of modified AAD-12 protein and PAT protein on a leaf was investigated using the ELISA method. And as a result, it was confirmed that both of modified AAD-12 protein and PAT protein expressed stably in multiple generations (Table 3, p.13).

Table 3 Expression level of modified AAD-12 protein and PAT protein in a leaf of this recombinant cotton from T3 to T5 generations

Confidential Information / Not Open to the Public

⑤ If there is possibility that the transferred nucleic acid will be transmitted to wild plants and animals by a virus infection or via any other routes, whether it is transmissible or not and the level of transmission if it is.

This recombinant cotton does not contain any transmissible sequence; therefore, there is no possibility of transferred gene to be transmitted to wild animals or wild plants.

(5) Method to detect and identify the living modified organisms as well as its detection sensitivity and reliability

As a method to detect and identify this recombinant cotton, PCR method is developed, which enables us to set transgene sequence and adjacent genome sequence as primers, and detect this recombinant cotton specifically to each event. Regarding the mixing ratio of this recombinant cotton to non-recombinant cotton, the detection limit vale in this PCR method is 0.04% in terms of genome DNA concentration ratio. About reproducibility, it is confirmed that inter-laboratory transferability was secured in *Dow AgroSciences USA* and *Eurofins GeneScan USA* (Appendix 7).

(6) Difference from the host or taxonomic species the host belongs to

① Detail of physiological and ecological properties imparted by expression of the copy of transferred nucleic acid

As modified *aad-12* gene and *pat* gene were introduced to this recombinant cotton, and modified AAD-12 protein and PAT protein are expressed respectively; therefore, tolerance to Aryloxyalkanoate herbicide and glufosinate herbicide have been imparted to this recombinant cotton.

In the Confined Field Trial conducted in 2012 at the Ogori Development Center of *The Dow Chemical Company Japan*, we carried out tolerance tests of this recombinant cotton (BC1F3 generation) and the control non-recombinant cotton (confidential information not to be disclosed) to 2,4-D and glufosinate herbicides. In these tests, we sprayed 1120g a.e./ha (normal usage) of 2,4-D to this recombinant cotton and non-recombinant cotton (10 individuals each) in 3 leaf stage, and examined the result after 2 weeks. It was found that all this recombinant cotton grew even after spraying 2,4-D, but the growth of the non-recombinant cotton was inhibited due to phytotoxicity (Figure 1, p.2 of "*Confined Field Trial Report*" (*Kakuri ho jou shiken kekka houkokusho*)). In addition, we sprayed 596g a.i./ha (normal usage) of glufosinate to this recombinant cotton and non-recombinant cotton (10 individuals each) in 3 leaf stage, and examined the result after 1 week. It was found that all this recombinant cotton grew even after spraying glufosinate, but the growth of the control non-recombinant cotton was inhibited due to phytotoxicity (Figure 2, p.3 of "*Confined Field Trial Report*" (*Kakuri ho jou shiken kekka houkokusho*)).

② Whether there is physiological or ecological difference between genetically modified crops and the taxonomic species that their hosts belong to, and the degree of difference if any

In 2012, we conducted a Confined Field Trial at the Ogori Development Center of *The Dow Chemical Company Japan*, and examined the difference between this recombinant cotton and the control non-recombinant cotton.

a Morphological and Growth Characteristics

Regarding the morphological and growth characteristics, we made comparison between this recombinant and non-recombinant cottons in terms of germination rate, uniformity of germination, flowering period, boll opening period, leaf shape, leaf length and width, the number of flower buds, flower color, plant type, trunk length, the number of nodes, total number of branches, weight of aerial part and underground part during the harvest period, the number of harvested bolls per seedling, the total number of bolls per seedling, boll shape, length and width of boll, weight of boll, the number of rooms in a boll, seed number per boll, weight of hundred seeds, seed shape, seed color, color of fluffy seed.

In the Confined Field, both of the recombinant and non-recombinant cottons started germination 5 days after seeding. There was no statistically significant difference between this recombinant cotton and non-recombinant cotton in terms of germination rate (Table 1, p.4 of "*Confined Field Trial Report*"). No difference was observed between this recombinant cotton and non-recombinant cotton regarding the uniformity of germination. No difference was observed either in terms of the flowering period and boll opening period (Table 2, p.4 of "*Confined Field Trial Report*") nor in leaf shape, flower color, plant type, boll shape, boll's room number, seed shape, seed color, and color of fluffy seed (Table 3 and 4, p.7 of "*Confined Field Trial Report*"). Furthermore, we observed no statistically significant difference

between this recombinant cotton and non-recombinant cotton in terms of leaf length and width, number of flower buds, trunk length, number of nodes, total number of branches, weight of aerial part and underground part during the harvest period, number of harvested bolls per seedling, total number of bolls per seedling, length and width of boll, weight of boll, seed number per boll, hundred-grain weight (Table 3 ~ 5, p.5~ 6 of "*Confined Field Trial Report*").

b Low-temperature Tolerance in Initial Growth Stage

We examined the low-temperature tolerance of this recombinant cotton and non-recombinant cotton in their initial growth stage. We cultivated this recombinant cotton and non-recombinant cotton (6 individuals each) that have grown to 1-leaf stage of true leaf, in the thermostat controlled at the temperature of 4 degrees C and the day-length of 16 hours, then observed their growing condition. After 16 days, both cottons exhibited body shrinkage and remarkable symptoms of growth disorder, and did not differ in degree (Figure 5, p.8 of "*Confined Field Trial Report*").

c Overwintering Ability of Full-Grown Plant

We examined the overwintering ability of full-grown plant of this recombinant cotton and non-recombinant cotton. We grew 10 seedlings in the Confined Field and left them without harvesting even after maturation, while observing the status of plant bodies under natural winter conditions. As a result of observing the test individuals in March 2013, no overwintering ability was confirmed because all the seedlings of both cottons had withered and died. (Figure 6, p.9 of "Confined Field Trial Report").

d Fertility and Size of Pollen

No difference was observed in the shape of pollen (Figure 7, p.10 of "Confined Field Trial Report"). We examined the fertility and size of pollen of this recombinant cotton and non-recombinant cotton that were stained with acetocarmine solution. As a result, no statistically significant difference was observed between this recombinant cotton and non-recombinant cotton (Table 6, p.10 of "Confined Field Trial Report").

e Production Output, Shattering Habit, Dormancy and Germination Rate of Seeds

As for seed productive amount, we compared the recombinant and non-recombinant cottons in terms of the number of harvested bolls per seedling, total number of bolls per seedling, number of seeds per boll and hundred-grain weight. As a result, no statistically significant difference was observed in all the above items, and we judged that there was no difference in the seed productive amount between this recombinant cotton and the non-recombinant cotton (Table 5, p.6 of "Confined Field Trial Report").

About shattering habit, we observed whether the seeds of the recombinant and non-recombinant cottons have shattered from opened bolls. As a result, no shattering habit was observed because the seeds of the recombinant and non-recombinant cottons were covered with fuzz (Figure 3, p.7 of "Confined Field Trial Report").

Regarding the germination rate of seeds immediately after harvesting and seeds air-dried at room temperature for 3 months after harvesting, no statistically significant difference was observed between this recombinant cotton and the non-recombinant cotton (Table 7, p.10 of "Confined Field Trial Report"). In addition, no statistically significant difference was observed between this recombinant cotton and the non-recombinant cotton in terms of germination rate of tested seeds and harvested seeds (Table 1 and 7, p.4 and

10 of “Confined Field Trial Report”), therefore it was considered that they have no difference in dormancy.

f Crossing Rate

We did not conduct a crossing test because there are no closely-related species which are crossable with cotton growing naturally in Japan.

g Harmful Substance Productivity

We conducted succeeding crop test, plowing test and soil microflora test to compare harmful substance productivity between this recombinant cotton and the non-recombinant cotton.

<Succeeding Crop Test>

After taking the root zone soil of this recombinant cotton and the non- recombinant cotton in harvest at 4 positions in each plot and mixing them, we removed foreign objects such as rocks by straining them and put them into a cell tray with 25 cells. Then we sowed a piece of seed of radish in each cell, and examined their germination rate after 8 days and their plant height and dry weight after 29 days.

As a result, no statistically significant difference was observed between the experimental plots of this recombinant cotton and the non-recombinant cotton, in terms of the germination rate, plant height or dry weight of the tested plant radish (Table 8, p.12 of “Confined Field Trial Report”)

<Plowing Test>

We cut off the aerial plant parts of this recombinant cotton and the non-recombinant cotton (6 seedlings per plot) during the harvest and made a sample unit including 6 seedlings, then dried and crushed them into powder, and mixed the powder well into horticultural soil (weight ratio of the dried powder is approx.0.6%). Next, we put the mixed soil in a 25-celled tray and sowed a seed of radish in each cell, then examined the germination rate after 9 days, and the plant height and dry weight after 21 days.

As a result, no statistically significant difference was observed between the experimental plots of this recombinant cotton and the non-recombinant cotton in terms of germination rate, plant height and dry weight of the tested radish, (Table 9, p.12 of “Confined Field Trial Report”).

< Soil Microflora Test>

We collected postharvest soil of this recombinant cotton and the non-recombinant cotton at three positions in each plot, then measured the number of bacteria, antinomycetes and filamentous fungus by dilution plate technique. As a result, no statistically significant difference was observed between the experimental plots of this recombinant cotton and the non-recombinant cotton (Table 10, p.12 of “Confined Field Trial Report”)

II Results of review meeting for Adverse Effect Assessment on Biological Diversity

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

(1) Results of Assessment on Adverse Effect on Biological Diversity

This recombinant cotton is created by introducing T-DNA region of pDAB4468 which was constructed by Agrobacterium method, based on plasmid pDAB2407 derived from *Agrobacterium tumefaciens* and *Escherichia coli*.

It is confirmed by the segregation pattern of genes and the southern blot analysis that this recombinant cotton has been transmitted stably through multiple generations and a single copy of modified *aad-12* gene coding for modified AAD-12 protein derived from *Delftia acidovorans, pat*-gene coding for PAT protein derived from *Streptomyces viridochromogenes*, and T-DNA region including nuclear matrix attachment region *RB7 MAR* (stabilizing the expression of introduced gene) derived from *Nicotiana tabacum*, is incorporated chromosomally.

(A) Competitiveness

It is not reported that wild cotton belonging to host's species was found in natural conditions in Japan.

In 2012, an investigation was conducted in one of the Confined Fields in Japan, on various characteristics concerning the competitiveness of this recombinant cotton. As a result, no difference was observed beween this recombinant cotton and the non-recombinant control cotton.

Modified *aad-12* gene and *pat* gene are introduced into this recombinant cotton, and the tolerance to Aryloxyalkanoate herbicide and glufosinate herbicide is provided by producing modified AAD-12 protein and PAT protein, however, it is hard to consider that having such tolerance improves its competitiveness under the natural conditions where it is inconceivable that those herbicides are sprayed.

From all of the above, no wild animals and plants that could be affected were specified; therefore, we have reached the judgement that the applicant's conclusion stating that there is no risk of affecting on the biological diversity, which is derived from the competitiveness of this recombinant cotton, is reasonable.

(B) Productivity of harmful substances

The cotton belonging to a host species contains Gossypol which is toxic to non-ruminant

animals, and Cyclopropane fatty acid that causes decolorization of hen's eggs or a decrease of its hatching rate. However, it is not reported that wild mammals eat cottonseeds. In addition, it is not known that cotton produces harmful substances such as allelopathic substance that disrupts the habitat or growth of wild animals and plants. Although this recombinant cotton produces modified AAD-12 protein that provides tolerance to Aryloxyalkanoate herbicide and PAT protein that provides tolerance to glufosinate herbicide, those proteins have never been reported as harmful substances and it is also confirmed that they have no sequences similar to the known allergens. Additionally it is confirmed that *N*-acetyl-L-glufosinate which is a metabolite of glufosinate herbicide, has less toxicity to animals than glufosinate itself.

In order to compare the productivity of harmful substances between this recombinant cotton and the non-recombinant cotton, succeeding crop test, plowing test, and soil microflora test were conducted at the Confined Fields in Japan in 2012. As a result of all these tests, no statistically significant difference was observed between the experimental plots of this recombinant cotton and the non-recombinant cotton.

From the above, no wild animals or plants that can be affected were specified, and we have reached the judgement that the applicant's conclusion stating that there is no risk of affecting on the biological diversity, which is derived from the productivity of harmful substances, is reasonable.

(C) Crossability

As no wild plants capable of cross-fertilization with cotton are living in the natural environment in Japan, any wild plant which may possibly be affected was not specified, therefore, we have reached the judgement that the applicant's conclusion stating that there is no risk of affecting on the biological diversity, which is derived from the crossability, is reasonable.

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

Based on all of the abovementioned facts, we have reached the judgment that the conclusion of the Assessment on Adverse Effect on Biological Diversity, stating that there shall be no risk of affecting on the biological diversity in Japan if this recombinant cotton is used according to Type 1 Use Regulations, is reasonable.

Reference List

Allen, George C.; Hall Jr., Gerald; Michalowski, Susan; Newman, Winnell; Spiker, Steven; Weissinger, Arthur K.; Thompson, William F. High-level transgene expression in plant cells: Effects of a strong scaffold attachment region from tobacco. The Plant Cell. 1996, 8(5), p.899-913.

Allen, George C.; Spiker, Steven; Thompson, William F. Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Molecular Biology. 2000, 43(2-3), p.361-376.

Barker, R.F.; Idler, K.B.; Thompson, D.V.; Kemp, J.D. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Molecular Biology. 1983, 2(6), p.335-350.

Chen, Hao; Jiang, Hanxiao; Morgan, John A. Non-natural cinnamic acid derivatives as substrates of cinnamate 4-hydroxylase. Phytochemistry. 2007, 68(3), p.306-311.

Chen, Qingfeng; Zhang, Baichen; Hicks, Leslie M.; Wang, Shiping; Jez, Joseph M. A liquid chromatography–tandem mass spectrometry-based assay for indole-3-acetic acid–amido synthetase. Analytical Biochemistry. 2009, 390(2), p.149-154.

EPA. Environmental Fate and Effects Division's Risk Assessment for the Reregistration Eligibility Document for 2,4-Dichlorophenoxyacetic Acid (2,4-D). 2004, http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2004-0167-0003, (Refer to 2013-2-12).

FAO. FAOSTAT, 2013, http://faostat.fao.org/default.aspx?lang=en, (Refer to 2013-5-15).

Fukuda, Yuji; Nishikawa, Satoshi. Matrix attachment regions enhance transcription of a downstream transgene and the accessibility of its promoter region to micrococcal nuclease. Plant Molecular Biology. 2003, 51, p.665-675.

Halweg, Christopher; Thompson, William F.; Spiker, Steven. The Rb7 matrix attachment region increases the likelihood and magnitude of transgene expression in tobacco cells: A flow cytometric study. The Plant Cell. 2005, 17(2), p.418-429.

Hausinger, Robert P. Fe (Ⅱ) /α-ketoglutarate-dependent hydroxylases and related enzymes. Critical Reviews in Biochemistry and Molecular Biology. 2004, 39(1), p.21-68.

Jenkins, Johnie N. Cotton. In: Traditional crop breeding practices: an historical review to serve as a baseline for assessing the role of modern biotechnology. OECD. 2003, p.61-70.

Luo, Lusong; Pappalardi, Melissa B.; Tummino, Peter J.; Copeland, Robert A.; Fraser, Marie E; Grzyska, Piotr K.; Hausinger, Robert P. An assay for Fe(II)/2-oxoglutarate-dependent dioxygenases by enzyme- coupled detection of succinate formation. Analytical Biochemistry. 2006, 353(1), p.69-74.

Norris, Susan R.; Meyer, Sandra E.; Callis, Judy. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Molecular Biology. 1993, 21(5), p.895-906.

OECD. Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. Series on Harmonization of Regulatory Oversight in Biotechnology No.11. 1999. http://www.oecd.org/env/ehs/biotrack/46815628.pdf, (Refer to 2013-7-29).

OECD. Module Ⅱ：Phosphinothricin. Series on Harmonization of Regulatory Oversight in Biotechnology No.25. 2002. http://www.oecd.org/env/ehs/biotrack/46815748.pdf, (Refer to 2013-7-29).

OECD. Consensus document on compositional considerations for new varieties of Cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and Anti-nutrients. Series on the Safety of Novel Foods and Feeds No.11. 2004. http://www.oecd.org/env/ehs/biotrack/46815236.pdf, (Refer to 2013-7-29).

OECD. '2,4-Dichlorophenol'. OECD Existing Chemicals Database. 2006, http://webnet.oecd.org/HPV/UI/handler.axd?id=3d43ed35-5ef5-4323-bddd-4aa527db0765, (Refer to 2013-2-12).

OECD. Consensus document on the Biology of Cotton (*Gossypium* spp.). Series on Harmonisation of Regulatory Oversight in Biotechnology No.45. 2008. http://www.oecd.org/env/ehs/biotrack/46815918.pdf, (Refer to 2013-7-29).

Umbeck, Paul F.; Barton, Kenneth A.; Nordheim, Erik V.; McCarty, Jack C.; Parrott, WilliamL.; Jenkins, JohnieN. Degree of pollen dispersal by insects from a field test of genetically engineered cotton. Journal of Economic Entomology, 1991, 84(6), p.1943-1950.

Van Deynze, Allen E.; Sundstrom, Frederick J.; Bradford, Kent J. Pollen-mediated gene flow in California cotton depends on pollinator activity. Crop Science, 2005, 45, p.1565-1570.

Verdaguer, Bertrand; de Kochko, Alexandre; Fux, Charles I.; Beachy, Roger N.; Fauquet, Claude. Functional organization of the cassava vein mosaic virus (CsVMV) promoter. Plant Molecular Biology. 1998, 37(6), p.1055-1067.

Wohlleben, W.; Arnold, W.; Broer, I.; Hillemann, D.; Strauch, E.; Pühler, A. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene. 1988, 70(1), p.25-37.

Wright, Terry R.; Lira, Justin M.; Walsh, Terence, Authony; Merlo, Donald J.; Jayakumar, Pon Samuel; Lin, Gaofeng. Novel herbicide tolerance genes. 2007, WO 2007/053482 A2.

Wright, Terry R.; Shan, Guomin; Walsh, Terence A.; Lira, Justin M.; Cui, Cory; Song, Ping; Zhuang, Meibao; Arnold, Nicole L.; Lin, Gaofeng; Yau, Kerrm; Russell, Sean M.; Cicchillo, Robert M.; Peterson, Mark A.; Simpson, David M.; Zhou, Ning; Ponsamuel, Jayakumar; Zhang, Zhanyuan. Robust crop tolerance to broadleaf and grass herbicides provided by Aryloxyalkanoateioxygenase transgenes. Proceedins of the National Academy of Sciences of the United Sta

Shigeo Harada, 1981. pp.26-42. Kougei Sakumotsu Gaku, Ⅱ Seniryo, Wata (Cotton, Fiber Material Ⅱ, Industrial Crop Science) Edited by Hiroshi Kurihara. Published by Rural Culture Association Japan.

Trade Statistics. “Statistics by commodity and country”. Ministry of Finance Japan. http://www.customs.go.jp/toukei/srch/index.htm?M=01&P=0 (Refer to 2013-5-14).

Glufosinate. 2010. Pesticides Risk Assessment Report. Food Safety Commission Japan. http://www.fsc.go.jp/fsciis/assessmentDocument/show/kya20070717010 (Refer to 2013-2-12).

Appendix List

Confidential Information / Not Open to the Public

1. Assessment Report of Confined Field Trial on Cotton Tolerant to Aryloxyalkanoate Herbicide and Glufosinate Herbicide (Modified *aad-12*, *pat*, *Gossypium hirsutum* L.) (DAS1910, OECD UI：DAS-8191Ø-7)

2. Appendix 1: Herbicides to Which the Modified AAD-12 Protein Exhibits Activity

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

4. Appendix 3： Nucleotide Sequence of pDAB4468

5. Appendix 4： Introduced Gene Copy Number and Stability Between or Within Generations

6. Appendix 5： Construct Process of pDAB4468

7. Appendix 6： Cloning and Characterization of the DNA Sequence for the Insert and Its Flanking Border Regions of DAS-81910-7 Cotton

8. Appendix 7： Development and Validation of an Event-Specific Real-Time PCR System for the Quantitative Detection of DAS-81910-7 Cotton