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

Name: Bayer CropScience K.K.
Applicant: Gavin Marchant, President
Address: 1-6-5, Marunouchi, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (modified *bar*, modified *cry1Ab*, *Gossypium hirsutum* L.) (T304-40, OECD UI: BCS-GHØØ4-7)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal, and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Component elements of the donor nucleic acid used for the development of cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (modified *bar*, modified *cry1Ab*, *Gossypium hirsutum* L.) (T304-40, OECD UI: BCS-GHØØ4-7) (hereinafter referred to as "T304-40") are shown in Table 1.

In addition, the amino acid sequences of the modified PAT protein expressed by the modified *bar* gene and the modified Cry1Ab protein expressed by the modified *cry1Ab* gene are shown in Annex 1 (Confidential: Not made available or disclosed to unauthorized persons).

Table 1 Position, size, origin, and function of component elements in vector pTDL008

Component elements	Position in vector	Size (bp)	Origin and function
Modified *cry1Ab* gene expression cassette			
3'me1	8792-9728	937	The 3′ untranslated region (Marshall *et al*., 1996) of the NADP-malic enzyme (EC.1.1.1.40) gene derived from yellowtop (*Flaveria bidentis*). It terminates transcription and directs 3′ polyadenylation.
Modified *cry1Ab*	9729-11582	1854	A gene encoding a Bt protein derived from *Bacillus thuringiensis berliner* 1715 confering resistance to Lepidoptera. An alanine (Ala) is inserted at the 2nd position from the N terminal (Met<u>Ala</u>Asp2-Asp616) in the active region of the *cry1Ab5* gene (Höfte *et al*., 1986) encoding the wild-type Cry1Ab protein. The codon is modified to enhance its expression in plants, but the amino acid sequence remains unchanged.
5'e1	11583-11643	61	A region including the leader sequence of the tapetum-specific E1 gene (*GE1*) from *Oryza sativa* (Michiels *et al*., 1992). It enhances the transcription efficiency.
Ps7s7	11644-12685	1042	A sequence including the tandem promoter of segment 7 from

			the Subterranean clover stunt virus (SCSV) (Boevink *et al*., 1995). It initiates constitutive transcription.
Modified *bar* gene expression cassette			
P35S3	12686-13543	858	The promoter region of Cauliflower Mosaic Virus 35S RNA (Odell *et al*., 1985). It initiates constitutive transcription.
Modified *bar*	13544-14095	552	A sequence including the gene encoding phosphinothricin acetyltransferase (PAT protein) derived from *Streptomyces hygroscopicus* (Thompson *et al*., 1987). It confers tolerance to glufosinate herbicide. Two codons at the N-terminal of the wild *bar* gene have been replaced with ATG and GAC, respectively.
3'nos	14096-14393 1-12	309	A sequence including the 3′ untranslated region of nopaline synthase gene derived from pTiT37 of *Agrobacterium tumefaciens* (Depicker *et al*., 1982). It terminates the transcription and causes the 3′ polyadenylation.
Others			
LB	13-37	25	A left border repetitive sequence derived from *A. tumefaciens* T-DNA (Zambryski, 1988).
—	38-342	305	A fragment of plasmid pTiAch5 in the left border repetitive sequence (Zhu *et al*., 2000).
aadA	343-1965	1623	A sequence including *E. coli*-derived gene that confers the tolerance to aminoglycoside antibiotics (Fling *et al*., 1985).
*npt*I-fragment	1966-3486	1521	A fragment of transposon Tn903-derived *nptI* gene that encodes neomycin phosphotransferase (Oka *et al*., 1981). This sequence does not function because it is a fragment.
—	3487-3632	146	A fragment including sequences upstream of *aadA* (Fling *et al*., 1985).
ORI pVS1	3633-7403	3771	A sequence including the replication origin (Hajdukiewicz *et al*., 1994) of plasmid pVS1 derived from *Pseudomonas aeruginosa* (Itoh *et al*., 1984).
ORI ColE1	7404-8576	1173	A sequence including the replication origin of plasmid pBR322 derived from *Escherichia coli* (Bolivar *et al*., 1977).
—	8577-8766	190	A fragment of plasmid pTiAch5 in the right border repetitive sequence (Zhu *et al*., 2000).
RB	8767-8791	25	A right border repetitive sequence derived from *A. tumefaciens* T-DNA (Zambryski, 1988).

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

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 component elements of the donor nucleic acid are shown in Table 1 (p. 2-3).

(b) Functions of proteins produced by the expression of target gene and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity.

Modified PAT protein

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. In detoxification of the produced ammonia, glutamine synthase plays a pivotal role, although the enzyme is inhibited in plants sprayed with glufosinate herbicide, resulting in ammonia accumulation and death of the plants.

The modified PAT protein expressed by the modified *bar* gene can acetylate glufosinate to produce *N*-acetyl-glufosinate, which is not toxic to plants, thereby inactivating the inhibitory action of glufosinate on glutamine synthase (OECD, 1999). This mechanism prevents ammonia accumulation, resulting in survival of corps, even if they were sprayed with glufosinate herbicide.

The modified PAT protein expressed by the modified *bar* gene exhibits a high affinity to glufosinate. Glufosinate is classified into L-amino acid, though it does not cause any acetyl group transfer to other various amino acids. It has little affinity to glutamic acid, which has a particularly high structural similarity to glufosinate, and causes virtually no transfer reaction *in vivo* (Thompson *et al.*, 1987). In addition, even in the presence of excessive amounts of various amino acids, the acetyl group transfer reaction to glufosinate by the modified PAT protein was not inhibited (Wehrmann *et al.*, 1996). Consequently, it is considered that the modified PAT protein possesses a high substrate specificity to glufosinate.

In 2011, amino acid sequence homology of the modified PAT protein was explored in known allergens registered in the database (AllergenOnline). As a result, no homology was

found.

The modified *bar* gene is identical to the gene introduced into cotton tolerant to glufosinate herbicide (LLCotton25) (OECD UI: ACS-GHØØ1-3), which has already been granted an approval for Type 1 Use in Japan in February 2006.

Modified Cry1Ab protein

The modified Cry1Ab protein encoded by the modified *cry1Ab* gene introduced into T304-40 is an insecticidal protein (Bt protein) comprising 617 amino acids and having a molecular weight of 69 kDa.

The modified Cry1Ab protein exhibits insecticidal activity against cotton bollworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*), Old world bollworm (*Helicoverpa armigera*), Pink bollworm (*Pectinophora gossypiella*), and other major insect pests of the order Lepidoptera for cotton cultivation (Annex 6: Confidential: Not made available or disclosed to unauthorized persons). When ingested by a target insect, the modified Cry1Ab protein, like any other Bt protein, is digested by a specific protease in the midgut to become an active polypeptide (core protein). The core protein binds to specific receptors on the brush border membrane vesicles (BBMV) on the midgut epithelium, causing formation of ion channels on the midgut columnar cells (Chen *et al*., 1995), loss of homeostasis, induction of sepsis, and, ultimately, death of the insect (Knowles and Dow, 1993; Broderick *et al*., 2006).

It has been confirmed that the modified Cry1Ab protein has low risk to the growth or survival of non-target insects such as honeybee (*Apis mellifera*) and ladybug (*Coleomegilla maculate*), which are potential visitors to cotton (Annex 7: Confidential: Not made available or disclosed to unauthorized persons). In addition, in the U.S. studies conducted in 2007 and 2008, it has been confirmed that the modified Cry1Ab protein has lowrisk to other non-target insects including green lacewing (*Chrysoperia rufilabris*), springtail (*Folsomia candida*), and water flea (*Daphnia magna*) (Table 2, p.6). Moreover, the modified Cry1Ab protein and other Bt proteins are unlikely to affect humans and other mammals, because mammalian digestive organs have proteases and acidic digestive fluids that can digest the Bt proteins or their core proteins and lack the receptors that can bind to these core proteins.

Table 2 Evaluation of effects of the modified Cry1Ab protein on non-target insects

Species	Growth stage	Evaluated items	Results
Green Lacewing (*Chrysoperia rufilabris*) (Neuroptera)	Larva	Mortality	NOEC[1] 29 μg/g[2]
Springtail (*Folsomia candida*) (Collembola)	Larva	Mortality, reproduction	NOEC 4.5 μg/g[2,3]
Water flea (*Daphnia magna*) (Diplostraca)	Immature	Fatality, development, reproduction	NOEC 48 μg/L

[1]: NOEC: No effect concentration

[2]: Mean value of the amount of modified Cry1Ab protein in feed determined by ELISA.

[3]: Due to the non-uniform amount of modified Cry1Ab protein in feed, the NOEC value estimated from the results of ELISA was low. The actual exposure is presumably larger than this.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

In 2011, amino acid sequence homology of the modified Cry1Ab protein was explored in known allergens registered in the database (AllergenOnline). As a result, no homology was found.

The *cry1Ab* gene derived from *B. thuringiensis krustaki* HD-1 strain has been introduced into recombinant maize lines Bt176 (OECD UI: SYN-EV176-9), Bt11 (OECD UI: SYN-BTØ11-1), and MON-810 (OECD UI: MON-ØØ81Ø-6), which have already been granted approval for Type 1 Use in Japan.

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

Modified PAT protein

The modified PAT exhibits a high substrate specificity to glufosinate and is unlikely to cause any acetyl group transfer to compounds other than glufosinate. Moreover, *N*-acetyl-glufosinate, which is the metabolite of glufosinate, does not inhibit glutamine synthase (OECD, 2002) and is therefore unlikely to affect the metabolic system of the recipient organism.

Modified Cry1Ab protein

There is no report that Bt proteins possess any enzyme activities, and the Cry1Ab protein is likely to work independently from the metabolic system of the recipient organism. Thus, it is considered that these proteins unlikely affect the metabolic system of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The vector used for the development of T304-40 was pTDL008 derived from pGSV20 (Figure 1, p. 8; Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

2) Properties

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

The total number of base pairs in the plasmid pTDL008 is 14,393bp.

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

The plasmid pTDL008 possesses the following sequences, and these sequences are located outside the T-DNA region and are not introduced into T304-40 (BC3F1: Figure 2, c, p. 10), which has been confirmed by Southern blot analysis (Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

- The replication origin derived from the plasmid pBR322 (Bolivar *et al*., 1977) of *E. coli* (ORI ColE1), and the replication origin of the plasmid vector pVS1 of *P. aeruginosa* (ORI pVS1) (Hajdukiewicz *et al.*, 1994), each of which functions to cause autonomous replication in *E. coli* and *A. tumefaciens*, respectively
- A gene conferring tolerance to aminoglycoside antibiotics (*aadA*) derived from *E. coli* (Fling *et al.*, 1985), which was used as a selective marker in *E. coli* and *A. tumefaciens*.

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

The plasmid pTDL008 does not have any transmissible element and thus has no infectivity.

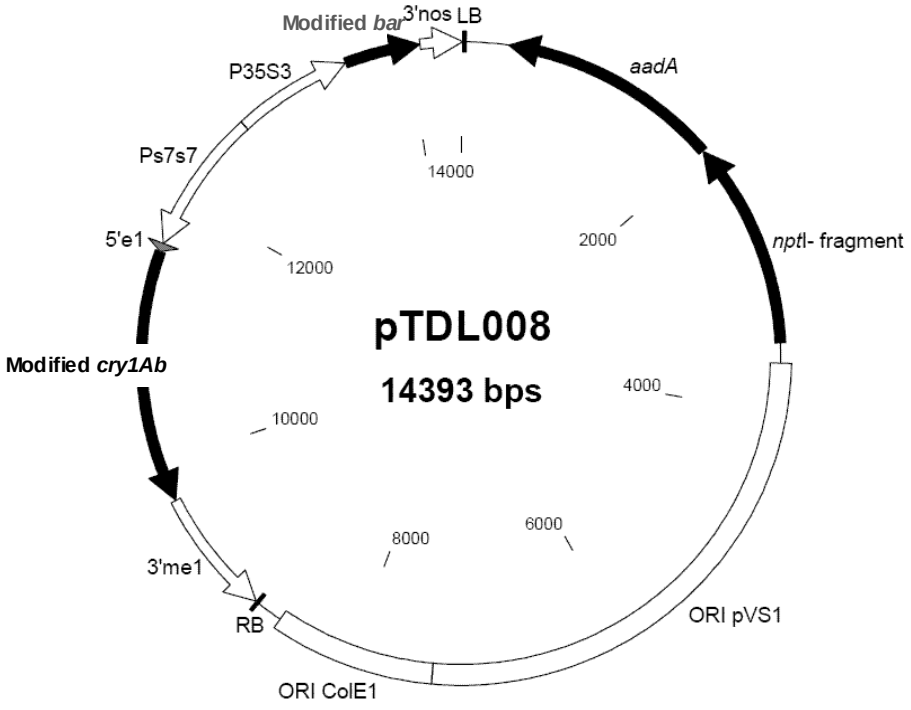

Figure 1 pTDL008 vector map

(Note: All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon the applicant.)

(3) Method of preparing living modified organisms

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

The modified *cry1Ab* gene expression cassette and the modified *bar* gene expression cassette [3′me1]-[modified *cry1Ab*]-[5′e1]-[Ps7s7]-[P35S3]-[modified *bar*]-[3′nos] between LB and RB on the plasmid pTDL008 were transferred into the recipient organism (Figure 1, p. 8). The positions of the cleaved sites by restriction enzymes in the transferred region into the recipient organism are shown in Annex 1 (Confidential: Not made available or disclosed to unauthorized persons).

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

The *Agrobacterium* method (Murray *et al*., 1999) was used for transferring the nucleic acid into the recipient organism. Transformation was conducted through co-cultivation, by exposing the callus induced from cotyledons of the recipient cotton cultivar Coker315 to a liquid culture of non-oncogenic *A. tumefaciens* EHA101 strain carrying the helper Ti plasmid pEHA101 and pTDL008.

3) Processes of breeding of living modified organisms

(a) Mode of selection the cell in which nucleic acid is transferred

Tissue segments in which the nucleic acid was transferred were cultured on the regeneration medium containing glufosinate and 250 mg/L claforan. The regenerated plants were selected based on glufosinate tolerance.

(b) Presence or absence of remaining *Agrobacterium* when the method of transferring nucleic acid is based on *Agrobacterium* method

After transferring the nucleic acid, the transformants were cultured on a medium containing 250 mg/L claforan to remove any residual *Agrobacterium* used for transformation. Then, the regenerated plants were cultured on claforan-free medium to

confirm the absence of residual *Agrobacterium*.

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

The selected plants were transplanted into pots and cultivated in a greenhouse. Then, the plants were selected based on glufosinate tolerance to obtain the original generation (T0) of T304-40. Elite lines were selected based on glufosinate tolerance, agronomic traits, etc. The process of rearing of T304-40 is shown in Figure 2. This application includes the T5 and F1 generations and the progenies of the T5 and F1 generations.

In February 2011, an application for the safety evaluation as food was submitted to the Ministry of Health, Labour and Welfare, and an application for safety evaluation as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries.

[Confidential: Not made available or disclosed to unauthorized persons]

Figure 2 Process of breeding of T304-40

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

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

Examination was done on the segregation ratio between glufosinate herbicide-tolerant and -sensitive individuals in the T1, F1, and BC1F1 generations of T304-40 (Figure 2, a, p. 10). The result fitted the expected segregation ratio for single-locus control with regard to the transferred gene (Table 3). Consequently, it is considered that the inserted DNA transferred into T304-40 resides at a single locus on the cotton genome.

Table 3 Confirmation of segregation ratios

Generation	Number of tested plants	Expected segregation ratio	Observed values		χ^2 Value[1]
			Tolerant	Sensitive	
T1	36	3：1	27	9	0.00
F1	20	1：1	10	10	0.00
BC1F1	15	1：1	7	8	0.07

[1]: Assumes a single-locus model. To reject the null hypothesis, the χ^2 value must be equal to or greater than 3.84, with one degree of freedom and p = 0.05.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

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

As a result of Southern blot analysis using the genomic DNA extracted from leaves of T304-40 (BC1F3: Figure 2, b, p. 10), it was confirmed that almost complete one copy of the T-DNA region, one copy of the modified *cry1Ab* gene expression cassette, and a truncated 3′me1 had been transferred (Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

The result of the sequence analysis of the transferred DNA into T304-40 demonstrated that, as shown in Figure 3 (p. 12), almost complete one copy of the T-DNA region has been transferred in correct orientation. Moreover, a truncated 3′me1 (complete size: 937 bp)

lacking 73 bp at its 5′ end is inserted at the 5′ end, followed by a truncated Ps7s7 (complete size: 1042 bp) lacking 623 bp at its 5′ end, the modified *cry1Ab* gene, 3′me1 and a 3-bp fragment of RB (complete size: 25 bp) in inverted orientations, and 3′me1 (complete size: 937 bp) and 3′nos (complete size: 310) at both ends of the T-DNA region are truncated, lacking 617 bp and 48 bp, respectively, at their 3′ ends. Moreover, the 670th nucleotide, which is located within the truncated 3′me1 at the 5′ end of the DNA sequence transferred into T304-40, was replaced by an adenine, although it was originally a cytosine in the vector pTDL008. Except for that, it has been confirmed that the nucleotide sequences in the inserted DNA are identical to those of the component elements composing the T-DNA region on the vector pTDL008 (Annex 2: Confidential: Not made available or disclosed to unauthorized persons).

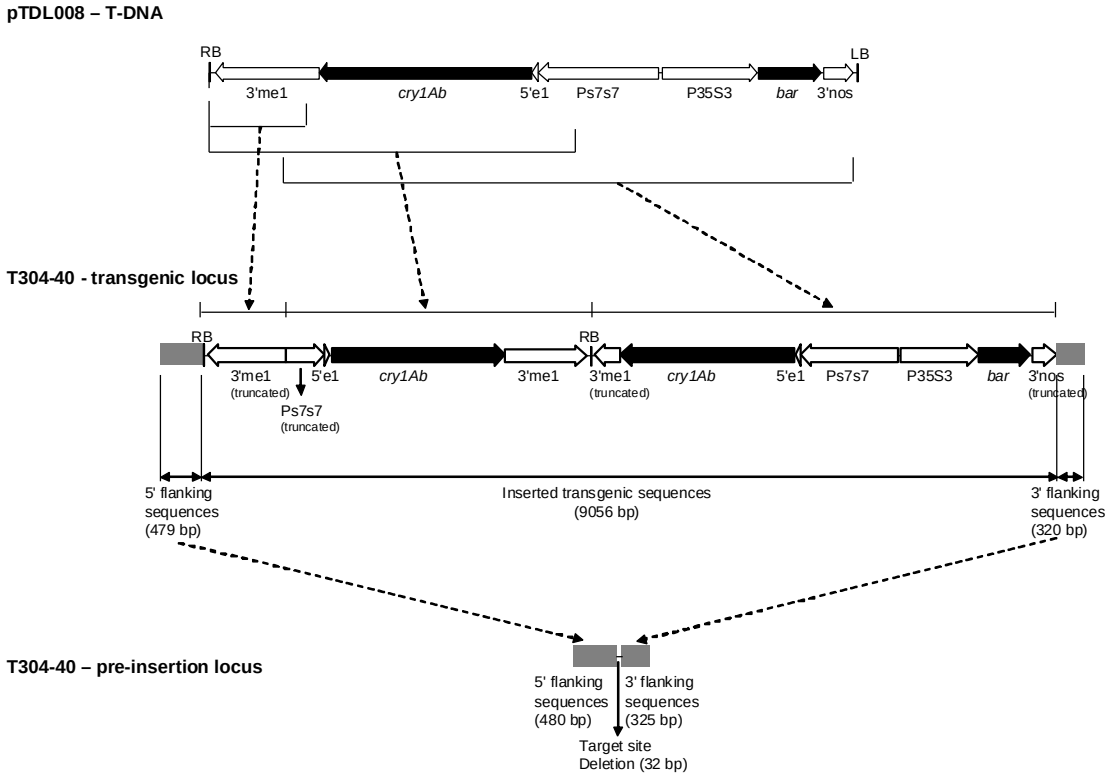

Figure 3 Map of the transferred DNA into T304-40

In the figure, "*bar*" and "*cry1Ab*" indicate the "modified *bar*" and the "modified *cry1Ab*," respectively.

(Note: All the rights pertinent to the information in the figure above and the responsibility for the contents rest upon the applicant.)

In order to confirm stable inheritance of the inserted gene, genomic DNAs extracted from leaves of five T304-40 generations (F1, BC1F1, BC2F1, BC2F2, and T7: Figure 2, d, p. 10) were subjected to Southern blot analysis using the modified *cry1Ab* gene as a probe. As a result, all five generations exhibited the same band pattern of expected size, demonstrating that the transferred gene is stably inherited in offspring (Annex 1: Confidential: Not made available or disclosed to unauthorized persons).

(c) Nearby or separate location of multiple copies, if present, on chromosome

As mentioned above, almost complete one copy of the T-DNA region has been transferred into T304-40 in correct orientation, in addition, a truncated 3′me1, a truncated Ps7s7, the modified *cry1Ab* gene, 3′me1, and a RB fragment, which are all inserted in inverted orientations at the 5′ end in this order, and truncated forms of 3′me1 and 3′nos at both ends of the T-DNA region. The results of the sequence analysis demonstrated that these sequences have been transferred adjacent to each other (Annex 2: Confidential: Not made available or disclosed to unauthorized persons).

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

In 2007, five T304-40 plants (T5: Figure 2, e, p. 10) cultivated in a greenhouse in the U.S. were subjected to ELISA for measuring the expression levels of the modified PAT protein and the modified Cry1Ab protein in the root, stem, leaf, flower bud, apex, boll, whole aerial parts, pollen, nectar, flower, and seed at various growth stages (vegetative period, just prior to flowering period, flowering period, and harvest period). As a result, the modified PAT protein and the modified Cry1Ab protein were detected from all tissues and nectar. Consequently, it has been confirmed that the modified PAT protein and the modified Cry1Ab protein are stably expressed across T304-40 individuals (Annex 3: Confidential: Not made available or disclosed to unauthorized persons).

In the isolated field test conducted in Japan in 2011, the expression of the modified PAT protein and the Cry1Ab protein was studied using the same generations used in the isolated

field test (BC1F5: Figure 2, g, p. 10) and the subsequent generation derived from the harvested seeds. The expression of the modified PAT protein and the Cry1Ab protein was checked by the glufosinate herbicide-spraying test and the immunochromatography method, respectively. As a result, all T304-40 individuals in both tested generations exhibited glufosinate tolerance and expressed the Cry1Ab protein. Consequently, it has been confirmed that both proteins are stably expressed across T304-40 generations (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

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

T304-40 does not contain any DNA sequence related to transmission, and therefore, it is considered that the DNA is unlikely transmitted to wild animals and wild plants in a natural environment.

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

T304-40 can be identified by the PCR method employing primers corresponding to the neighboring sequences of the transferred DNA. The quantification limit of this detection method is 0.08%, and the reliability of the method has been verified as a result of tests performed within the company and at two other laboratories (Annex 4: Confidential: Not made available or disclosed to unauthorized persons).

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

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

T304-40 shows tolerance to glufosinate herbicide due to the expression of the modified *bar* gene. The modified PAT protein metabolizes glufosinate to produce *N*-acetyl-glufosinate, which does not inhibit glutamine synthase (OECD, 2002), and it is

therefore considered that the metabolite unlikely affect the metabolic system of the recipient organism. Moreover, this metabolite is designated as a compound subject to the maximum residue limit for glufosinate in cottonseed (4 ppm) (The Japan Food Chemical Research Foundation), and toxicity studies were conducted with mammals including rats, mice, and dogs demonstrated that its toxicity is lower than that of glufosinate, which is classified as an ordinary substance* (Bayer CropScience K.K., 2009).

In addition, T304-40 shows resistance to cotton bollworm (*H. zea*), tobacco budworm (*H. virescens*), Old world bollworm (*H. armigera*), Pink bollworm (*P. gossypiella*), and other Lepidoptera pests in cotton cultivation due to the modified Cry1Ab protein encoded by the modified *cry1Ab* gene.

(b) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified crops and taxonomical species to which the recipient organism belongs, and the degree of difference, if present

In 2010, an isolated field test was conducted in an open field at the Frontier Science Research Center, University of Miyazaki (hereinafter referred to as “isolated field”); however, sufficient amount of data for assessing the Adverse Effects on Biological Diversity could not be obtained due to the delayed initiation of the study and insectfeeding damage by Lepidopter. Therefore, another isolated field test was conducted in 2011 to compare T304-40 (BC1F5 generation: Figure 2, g, p. 10) with the commercial cultivar FM966 (hereinafter referred to as “non-recombinant cotton”) (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). T304-40 used in this study was developed using FM966 as its genetic background. In addition, a study was conducted in a P1P laboratory at Bayer CropScience K.K. Yuki Research Center in 2008 to compare the tolerance to low temperature at the early stage of growth of T304-40 (T6: Figure 2, f, p. 10) and the recipient cultivar Coker315 (hereinafter referred to as “recipient cultivar”) (Annex 6: Confidential: Not made available or disclosed to unauthorized persons).

a. Morphological and growth characteristics

In an isolated field test, we compared T304-40 with the non-recombinant cotton concerning about following items: the uniformity of germination, flowering time, boll

opening time, leaf shape, plant type, flower color, boll shape, lint color, seed shape, seed color, leaf length, leaf width, plant height, number of flower buds, number of nodes, total number of branches, number of harvested bolls per plant, weight of aerial parts, weight of under-ground parts, boll length, boll width, boll weight, number of segments of a boll, number of seeds per boll, and 100-seed weight.

As a result, both the flowering time and the boll opening time were one day earlier in T304-40 than in the non-recombinant cotton. The total number of branches was statistically significantly smaller in T304-40 than in the non-recombinant cotton (Annex 5: Confidential: Not made available or disclosed to unauthorized persons). In other items, difference or statistically significant difference was not observed between the two cotton lines (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

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

In the P1P laboratory in Japan, young plants of T304-40 and the recipient cultivar were cultivated at 5°C under 12-h day length for 1 month, and degree of withering was evaluated every week. As a result, no statistically significant difference was observed between the two lines at any of the evaluation time points, and all individuals of both lines were dead four weeks after they had been transferred to the low-temperature condition (Annex 6: Confidential: Not made available or disclosed to unauthorized persons).

c. Overwintering ability and summer survival of matured plant

In the isolated field, T304-40 and the non-recombinant cotton were continuously cultivated after the harvest time in winter; as a result, all plants were dead in February 2012 (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

d. Fertility and size of pollen

No statistically significant difference was observed between the size of pollens collected from T304-40 and the non-recombinant cotton cultivated in an isolated field. In addition, the pollens of both lines showed high fertility (99% or higher) without any statistically significant difference between the two lines (Annex 5: Confidential: Not made available or

disclosed to unauthorized persons).

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

Regarding seed production, T304-40 was compared with the non-recombinant cotton in the number of harvested bolls per plant, the number of seeds per boll and 100-seed weight. As a result, no statistically significant difference was observed between T304-40 and the non-recombinant cotton in any of the items examined (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Regarding the shedding habit, shedding was not observed in both T304-40 and the non-recombinant cotton (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Regarding dormancy and germination rate, seeds of T304-40 and the non-recombinant cotton harvested in the isolated field were sown either immediately after harvest or after 3-month air drying at room temperature to evaluate the germination rate. As a result, the germination rates of the just-harvested seeds of T304-40 and the non-recombinant cotton were 66% and 74%, respectively, while the germination rates of the 3-month air-dried seeds of T304-40 and the non-recombinant cotton were 94% and 88%, respectively. Under either condition, no statistically significant difference was observed between the two cotton lines (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

f. Crossability

Since related species that can cross with cotton are not voluntarily grow in Japan, crossability was not examined.

g. Productivity of harmful substances

In order to check in the isolated fields whether any substances are secreted from the roots to affect other plants, exist in the plant to affect other plants after dying, and are secreted from the roots to affect microorganisms in soil, the succeeding crop test, plow-in test and

soil microflora test were carried out, respectively

Succeeding crop test

After cultivating T304-40 and the non-recombinant cotton in the isolated field, radish seeds were sown in the remaining soil, and comparison was made between the two plots for germination rate, plant height, fresh weight, and dry weight. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Plow-in test

Plants of T304-40 or the non-recombinant cotton harvested from the isolated field were mixed into soil, and radish seeds were sown in the soil to compare the germination rate, plant height, fresh weight, and dry weigh between the two plots. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

Soil microflora test

Soil was sampled after cultivating T304-40 or the non-recombinant cotton in the isolated field, and comparison was made for the number of bacteria, actinomycetes, and fungi with the dilution plate method. As a result, no statistically significant difference was observed between the two plots in any of the items tested (Annex 5: Confidential: Not made available or disclosed to unauthorized persons).

II. Review by persons with specialized knowledge and experience concerning Adverse Effects on Biological Diversity

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

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

This recombinant cotton was developed by introducing the expression vector pTDL008 constructed from the plasmid pGSV20 into the recipient organism by the *Agrobacterium* method.

Based on the segregation ratio of the transferred gene, the Southern blot analysis and the sequence analysis, it has been confirmed that almost complete one copy of the T-DNA region, which contains the modified *bar* gene encoding the modified PAT protein (phosphinothricin acetyltransferase) derived from *S. hygroscopicus* and the modified *cry1Ab* gene encoding the modified Cry1Ab protein derived from *B. thuringiensis*, and one copy each of the modified *cry1Ab* gene expression cassette and a truncated 3′me1 are present adjacent to each other on the chromosome of this recombinant cotton and are stably inherited across multiple generations. In addition, it has been confirmed by ELISA that the target genes are stably expressed across multiple generations.

1) Competitiveness

Cotton, the taxonomical species to which the recipient organism belongs, has been used for a long time in Japan, though there is no report that it has grown voluntarily in the natural environment.

As a result of studies on various characteristics in competitiveness of this recombinant cotton, which were carried out in 2008 in the P1P laboratory in Japan and in 2011 in the isolated field in Japan, differences or statistically significant differences were observed between this recombinant cotton and the non-recombinant control cotton in the flowering

time, boll opening time, and total number of branches.

Both the flowering time and the boll opening time were one day earlier in this recombinant cotton than in the non-recombinant control cotton, but these were merely slight differences, and it is unlikely that they would enhance the competitiveness of this recombinant cotton. In addition, the total number of branches was statistically significantly smaller in this recombinant cotton than in the non-recombinant cotton. Because some plants of this recombinant cotton were additionally planted to replace the individuals that had damaged by insects at early stage of growth, a temporary delay was observed in their growth, particularly in terms of branching, which is likely to have induced the observed difference in the total number of branches examined at the pinching date. Nevertheless, statistically significant difference was not observed in the weight of aerial parts, etc., examined at the harvest time, so the difference was deemed to be a temporary difference.

This recombinant cotton is given the traits to be tolerant to glufosinate herbicide by the modified PAT protein and resistant to Lepidoptera by the Cry1Ab protein. However, since it is difficult for the cultivated cotton to grow voluntarily in the natural environment in Japan, it is unlikely that these traits could increase the competitiveness of this recombinant cotton in the Japanese natural environment.

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

2) Production of harmful substances

Cotton, the taxonomical species to which the recipient organism belongs, contains gossypol, which exhibits toxicity to nonruminant animals, and cyclopropene fatty acid, which inhibits desaturation of saturated fatty acids leading to discoloration or decreased hatchability of hen eggs. However, there is no report that cotton seeds are eaten by wild animals. In addition, it is not known that cotton produces any allelopathic substances that could affect the inhabitation or growth of wild animals and plants.

It has been confirmed that the modified PAT protein and the Cry1Ab protein, which are

expressed in this recombinant cotton by the transferred genes, have no sequence homology with any known allergens.

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

The modified PAT protein has a high substrate specificity, and it is considered that this protein unlikely affect the metabolic pathway of the recipient organism and produce any harmful substances. When this recombinant cotton is exposed to glufosinate, *N*-acetyl-glufosinate is produced by the modified PAT protein. *N*-acetyl-glufosinate is designated as a compound subject to the maximum residue limit for glufosinate in cottonseed, and it has been confirmed that its toxicity is lower than that of glufosinate, which is classified as an ordinary substance.

Since this recombinant cotton is given resistance to Lepidopter by the Cry1Ab protein, there was a concern that this recombinant cotton would affect the survival of Lepidopter living in Japan, which eat the plant body or pollen of this recombinant cotton. However, cotton is not reported to grow voluntarily in Japan, so it is extremely unlikely that imported cotton seeds that were spilled during transportation grow or become self-seeding in the natural environment. Even if they grew, the pollens of cotton are relatively heavy and sticky, so they have little chance to be widely dispersed by wind. Therefore, it is considered extremely unlikely that Lepidopter species living in Japan would eat this recombinant cotton or be exposed to its pollens.

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

3) Crossability

In the Japanese natural environment, there are no wild species that can cross with cotton. Therefore, it was judged that there are no specific wild plants or animals that are possibly affected by this recombinant cotton, and that the use of such cotton poses no risk of Adverse Effects on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by the applicant is reasonable.

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

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

References

1. Alvarez, I., Cronn, R., Wendel, J.F. (2005) Phylogeny of the New World diploid cottons (*Gossypium* L., Malvaceae) based on sequences of three low-copy nuclear genes. Plant Syst. Evol. 252: 199-214.

2. Berardi, L.C., Goldblatt, L.A. (1980) Gossypol. *In*: Liener, I.E. (ed.) Toxic Constituents of Plant Foodstuffs, 2nd edn. Academic Press, New York. pp. 184-237.

3. Boevink, P., Chu, P.W.G., Keese, P. (1995) Sequence of subterranean clover stunt virus DNA: Affinities with the geminiviruses. Virology 207: 354-361.

4. Bolivar, F., Rodrigues, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H., Falkow, S. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95-113.

5. Broderick, NA., Raffa, KF., Handelsman, J. (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc. Natl. Acad. Sci. 103: 15196-15199.

6. Brown, A.H.D., Brubaker, C.L. (2000) Genetics and the conservation and use of Australian wild relatives of crops. Aust. J. Bot. 48: 297-303.

7. Chen, X.J., Curtiss, A., Alcantara, E., Dean, D.H. (1995) Mutations in domain I of *Bacillus thuringiensis* δ-endotoxin CryIAb reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. J. Biol. Chem. 270: 6412-6419.

8. Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., Goodman, H.M. (1982) Nopaline synthase: Transcript mapping and DNA sequence. J. Mol. Appl. Genet. 1: 561-573.

9. Duke, J.A. (1983) *Gossypium hirsutum* L. *In*: Handbook of Energy Crops, unpublished. Center for New Crops & Plant Products, Purdue Univ. West Lafayette, Indiana.

10. FAO FAOSTAT ProdSTAT: Crops. Food and Agriculture Organization of the United Nations. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567 (accessed 2012-03-23)

11. Fling, M.E., Kopf, J., Richards, C. (1985) Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase. Nucleic Acids Res. 13: 7095-7106.

12. Fryxell, P.A. (1992) A revision of *Gossypium* sect. *Grandicalys* (Malvaceae), including the description of six new species. Systematic Botany 17: 91-114.

13. Gunstone, F.D. (2007) Cottonseed oil (*Gossypium hirsutum* and G. *barbadense*). *In*:

Gunstone, F.D., Harwood, J.L., Dijkstra, A.J. (eds.) The Lipid Handbook, 3rd edn. CRC Press, Florida. pp. 44-45.

14. Hajdukiewicz, P., Svab, Z., Maliga, P. (1994) The small, versatile *pPZP* family of *Agrobacteium* binary vectors for plant transformation. Plant Mol. Biol. 25: 989-994.

15. Höfte, H., De Greve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vandekerckhove, J., Vanderbruggen, H., Van Montagu, M., Zabeau, M., Vaeck, M. (1986) Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner* 1715. Eur. J. Biochem. 161: 273-280.

16. Itoh, Y., Watson, J.M., Haas, D., Leisinger, T. (1984) Genetic and molecular characterization of the *Pseudomonas* plasmid pVS1. Plasmid 11: 206-220.

17. Kandylis, K., Nikokyris, P.N., Deligiannis, K. (1998) Performance of growing-fattening lambs fed whole cotton seed. J. Sci. Food Agric. 78: 281-289.

18. Kerkhoven, G.J., Mutsaers, H.J.W. (2003) *Gossypium* L. *In*: Brink, M., Escobin, R.P. (eds.) Plant Resources of South-East Asia, No.17, Fiber plants. Backhuys Publishers, Leiden. pp. 139-150.

19. Knowles, B.H., Dow, J.A.T. (1993) The crystal δ-endotoxins of *Bacillus thuringiensis*: Models for their mechanism of action on the insect gut. BioEssays15: 469-476.

20. Marshall, J.S., Stubbs, J.D., Taylor, W.C. (1996) Two genes encode highly similar choroplastic NADP-malic enzymes in *Flaveria*. Plant Physiol. 111: 1251-1261.

21. McGregor, S.E. (1976) Insect pollination of cultivated crop plants. Agriculture Handbook No.496. U.S. Department of Agriculture.

22. Michiels, F., Morioka, S., Scheirlinck, T., Komari, T. (1992) Stamen-specific promoters from rice. Patent Application WO92/13956A1 (20AUG-1992). PLANT GENETIC SYSTEMS N.V. (BE).

23. Murray, F., Llewellyn, D., McFadden, H., Last, D., Dennis, E.S., Peacock, W.J. (1999) Expression of the *Talaromyces flavus* glucose oxidase gene in cotton and tobacco reduces fungal infection, but is also phytotoxic. Molecular Breeding 5: 219-232.

24. Odell, J.T., Nagy, F., Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810-812.

25. OECD (1999) 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. ENV/JM/MONO(99)13.

26. OECD (2004) 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. ENV/JM/MONO(2004)16.

27. OECD (2008) Consensus document on the biology of cotton (*Gossypium* spp.). Series on Harmonization of Regulatory Oversight in Biotechnology, No.45. ENV/JM/MONO(2008)33.

28. Oka, A., Sugisaki, H., Takanami, M. (1981) Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. 147: 217-226.

29. Oosterhuis, D.M., Jernstedt, J. (1999) Morphology and anatomy of the cotton plant. *In*: Smith, W.C., Cothren, J.T. (eds.) Cotton: Origin, History, Technology and Production. Wiley, New York. pp. 175-206.

30. Purseglove, J.W. (1968) Tropical Crop, Dicotyledons. Longmans, London. p. 719.

31. Reddy, K.R., Reddy, V.R., Hodges, H.F. (1992) Temperature effects on early season cotton growth and development. Agron. J. 84: 229-237.

32. Richards, J.S., Stanley, J.N., Gregg, P.C. (2005) Viability of cotton and canola pollen on the proboscis of *Helicoverpa armigera*: Implications for spread of transgenes and pollination ecology. Ecol. Entomol. 30: 327-333.

33. Stanton, M.A., Stewart, J.McD., Percival, A.E., Wendel, J.F. (1994) Morphological diversity and relationships in the A-genome cottons, *Gossypium arboreum* and *G. herbaceum*. Crop Sci. 34: 519-527.

34. Stipanovic, R.D., Puckhaber, L.S., Bell, A.A., Percival, A.E., Jacobs, J. (2005) Occurrence of (+)- and (–)-gossypol in wild species of cotton and in *Gossypium hirsutum* var. *marie-galante* (Watt) Hutchinson. J. Agric. Food Chem. 53: 6266-6271.

35. Thies, S.A. (1953) Agents concerned with natural crossing of cotton in Oklahoma. Agron. J. 45: 481-484.

36. Thompson, C., Van Montagu, M., Leemans, J. (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO Journal 6: 2513-2518.

37. Van Deynze, A.E., Frederick, J.S., Bradford, K.J. (2005) Pollen-mediated gene flow in California cotton depends on pollinator activity. Crop Sci. 45:1565-1570.

38. Vollesen, K. (1987) The native species of *Gossypium* (Malvaceae) in Africa, Arabia and Pakistan. Kew Bull. 42: 337-349.

39. Wehrmann, A., Van Vliet, A., Opsomer, C., Botterman, J., Schulz, A. (1996) The

Similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nat. Biotechnol. 14:1274-1278.

40. Wendel, J.F., Cronn, R.C. (2003) Polyploidy and the evolutionary history of cotton. Adv. Agron. 78: 139-186.

41. Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. 22: 1-30.

42. Zhang, B.-H., Pan, X.-P., Guo, T.-L., Wang, Q.-L, Anderson, T.A. (2005) Measuring gene flow in the cultivation of transgenic cotton (*Gossypium hirsutum* L.). Mol. Biotechnol. 31: 11-20.

43. Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K., Winans, S.C. (2000) The bases of crown gall tumorigenesis. J. Bacteriol. 182: 3885-3895.

44. The Japan Food Chemical Research Foundation. Standards for Agricultural Chemicals, etc. Glufosinate. http://m5.ws001.squarestart.ne.jp/zaidan/agrdtl.php?a_inq=18900 (accessed 2011-06-13)

45. Niya, I. (1989) Shokuhin Yushi no Kagaku (Science of Edible Fats and Oils). Saiwai Shobo, Tokyo. 31-32.

46. Tatsumi, J. (2000) Wata (Cotton). "Sakumotsugaku (II) Kougei/Shiryou Sakumotsu-hen (Crop Science (II) Industrial and Feed Crops" R. Ishii (Representative author), Buneido Publishing, Co., Ltd., Tokyo. 8-15.

47. Bayer CropScience K.K. (2009) Nouyaku Shouroku (Agrochemical Abstract).Glufosinate (Herbicide). Food and Agricultural Materials Inspection Center. http://www.acis.famic.go.jp/syouroku/glufosinate/index.htm (accessed 2011-06-13)

48. Harada, S. (1981) II. Seniryou (Textile plants), Wata (Cotton). "Kougei Sakumotsugaku (Industrial Crop Science)" Edited by H. Kurihara, Rural Culture Association, Tokyo, 26-42.

49. Ministry of Agriculture, Forestry and Fisheries. (2012) Exports and Imports Statistics of Agricultural, Forestry and Fisheries Products. Trade Statistics (Import) 2011 (Issued on March 23, 2012) http://www.e-stat.go.jp/SG1/estat/List.do?lid=000001087653 (accessed 2012-03-23)

50. Ministry of Finance. Trade Statics of Japan. http://www.customs.go.jp/toukei/srch/index.htm?M=01&P=0 (accessed 2012-03-23)

Contents of Annexes