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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Soybean tolerant to glufosinate herbicide (*pat*, *Glycine max* (L.) Merr.) (A5547-127, OECD UI: ACS-GM006-4)
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 Effect on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of the donor nucleic acid that was used for the production of the soybean tolerant to glufosinate herbicide (*pat, Glycine max* (L.) Merr.) (A5547-127, OECD UI: ACS-GM006-4) (hereinafter referred to as "the recombinant soybean A5547-127") and origins of component elements are shown in Table 1.

The *pat* gene transferred in the recombinant soybean A5547-127 is a modified type of the native *pat* gene obtained from *Streptomyces viridochromogenes* whose sequence is modified to fit codons used in the plant. The amino acid sequence of the enzyme which is produced by this modification remains unchanged (References 7, 29, and 44). Structure of nucleotide sequence of the modified *pat* gene is shown in Figure 1.

Table 1 Origin and function of component elements

Component Elements (abbreviation)	Size (kbp)	Position in vector (bp)	Origin and function
pat gene expression cassette			
P35S	0.54	461-1003	35S RNA promoter derived from Cauliflower Mosaic Virus. It expresses modified *pat* genes in plants constitutively (Reference 31).
pat	0.55	1012-1563	It encodes PAT protein and gives tolerance to glufosinate herbicide, derived from *Streptomyces viridochromogenes* (Reference 7).
T35S	0.20	1582-1784	35S RNA terminator derived from Cauliflower Mosaic Virus. It terminates transcription and induces polyadenylation of transcripts (Reference 36).
Others			
bla	0.86	3876-3016	It is an ampicillin resistant gene *(bla)* derived from *E.coli*. It expresses β-lactamase only in bacteria (Reference 42).
ori	0.55	2253-2803	It is the replication origin (ColE1) of pUC19, and initiates replication of plasmid (Reference 47).
RB	0.06	189-243	Right border derived from *Agrobacterium tumefaciens* Ti plasmid pTiAch5 (Reference 11).

Figure 1 Comparison between the native *pat* gene and the *pat* gene transferred in the recombinant soybean A5547-127

2) Function of component elements

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

Functions of individual component elements of donor nucleic acid which were used for the production of the recombinant soybean A5547-127 are shown in Table 1.

(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

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. Glutamate synthase plays an important role in detoxification of the ammonia produced, though the glutamate synthase is inhibited if crops are sprayed with glufosinate herbicide, ammonia accumulates, and the crops wither and die.

On the other hand, in the plant body to which the *pat* gene is transferred, phosphinothricin acetyl transferase (PAT protein) is produced, and this enzyme acetylates the glufosinate to transform it to N-acetylglufosinate. This helps prevent the inhibition of glutamine synthase by the glufosinate, ammonia does not accumulate in the plant body, and the crop does not die even if it is sprayed with glufosinate (Figure 2).

It is reported that the PAT protein is not toxic to humans and other animals, and it shows no significant homology except with PAT proteins derived from various other species as a result of search for any homology with amino acid sequence of all proteins registered in the GENBANK database (Reference 29). In addition, as a result of comparison of physico-chemical and biochemical characteristics of PAT protein with known allergen, it was observed that this protein has no possibility to possess allergenicity (Reference 45).

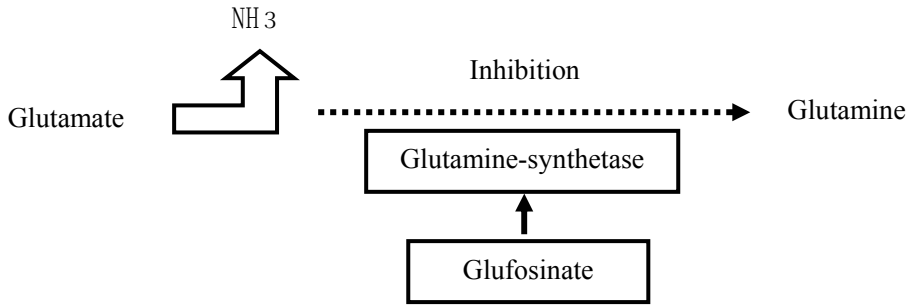
Moreover, based on the nucleotide sequence and amino acid sequence of this protein, overall homology search (EMBL and Swiss Prot) and allergen epitope search were conducted. As a result, this protein did not show any homology with known allergens.

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

The PAT protein encoded by the *pat* gene exhibits a high affinity to glufosinate classified into *L*-amino acid, though it does not cause any acetyl group transfer to the other various amino acids and it has little affinity for the glutamic acid which has specifically high structural similarity and it causes virtually no transfer reaction in any living body (Reference 43). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by PAT protein was never inhibited (References 29 and 46). As a result, it is considered that the PAT protein possesses high substrate specificity and it does not affect the metabolic system of the recipient organism.

A) Normal Plant

Since glufosinate herbicide inhibits glutamine synthetase, ammonia accumulates in the plant body, causing the plant to die.



B) Recombinant Plant

Glufosinate herbicide is acetylated and becomes N-acetylglufosinate by action of the PAT protein, and the inhibition of the glutamine-synthetase does not occur. Therefore, ammonia does not accumulate in the plant body, and the plant can grow.

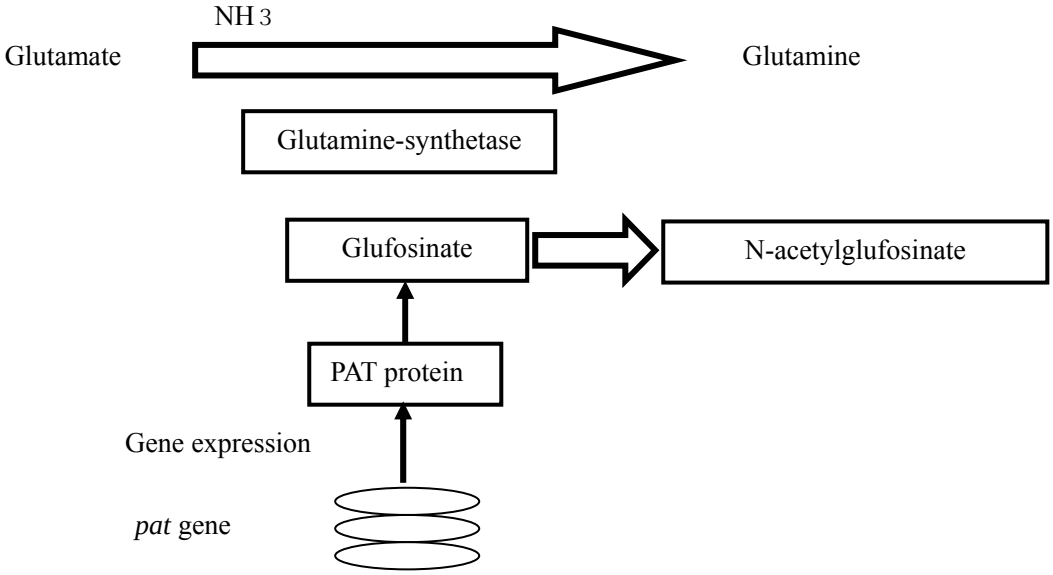


Figure 2 Mechanism of tolerance to glufosinate herbicide by the product of *pat* gene

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

(2) Information concerning vectors

1) Name and origin

The plasmid pB2/35SAck which was constructed based on the plasmid pUC19 was used for the production of the recombinant soybean A5547-127. Figure 3 shows the physical map of plasmid pB2/35SAck.

2) Properties

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

The number of base pairs of the plasmid pB2/35SAck is 4,076 bps. Physical map of the plasmid is shown in Figure 3, and the entire nucleotide sequence is shown in Annex 3.

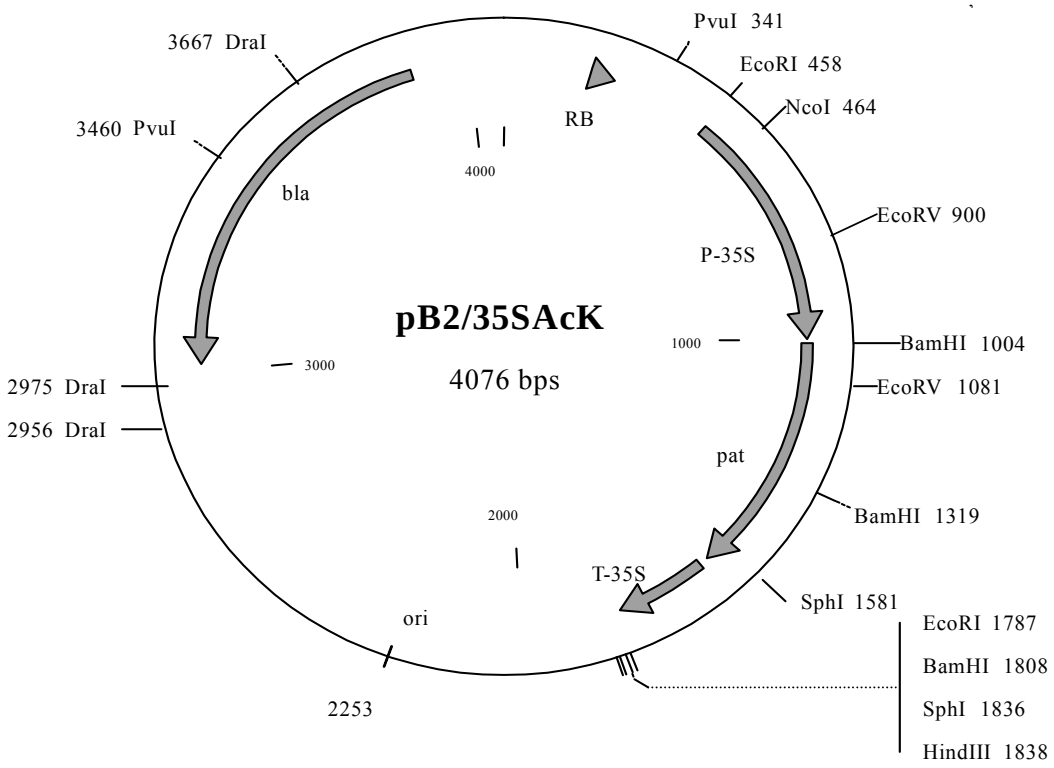


Figure 3 Physical Map of plasmid pB2/35SAck

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

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

The plasmid pB2/35SAck contains the *bla* gene to confer the resistance to ampicillin. The *bla* gene was used as a selective marker for construction of this plasmid, though this gene does not possess any promoter which functions in plants and therefore it does not function in the cells of soybean. Moreover, the plasmid pB2/35SAck has been cut by the restriction enzyme PvuI before the transformation, and the *bla* gene is split to such extent that it cannot function (Figure 4). Northern blotting analysis using the *bla* gene as a probe was conducted regarding RNA extracted from the leaves, stems, roots and seeds of the recombinant soybean A5547-127 (R4 generation). As a result, it was confirmed that the transcript of *bla* gene was not detected in any tissue (limit of detection of 2 pg) and this gene was not expressed in any plant body (Annex 5).

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

The plasmid pB2/35SAck does not possess a transferring ability, and therefore noninfectious. In addition, it is known that the range of recipient organisms for the autonomous replication of plasmid pUC19, the basis for construction of this plasmid, is limited to *Escherichia coli* and a few gram-negative bacteria.

(3) Method of preparing living modified organisms

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

The plasmid pB2/35SAck was cut at the two (2) PvuI-sites existing in the middle of the *bla* gene and upstream of the P35S (Figure 3), and the plasmid pB2/35SAck was cut into two (2), large and small, fragments. Structure of the entire nucleic acid transferred in the recipient organism is shown in Figure 4.

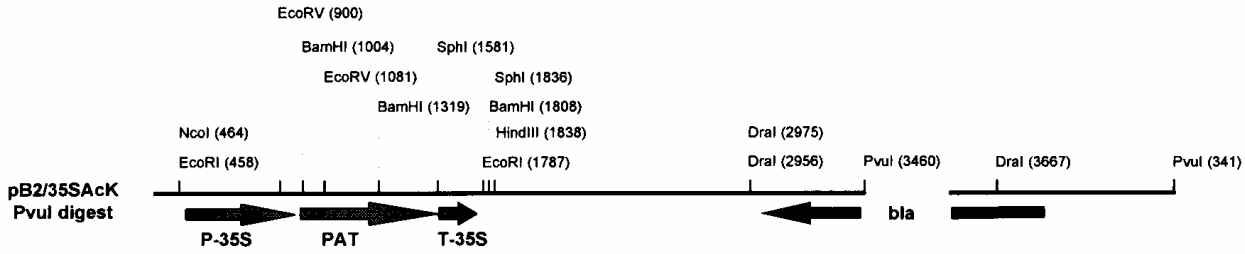


Figure 4 Structure of the transferred nucleic acid
The "PAT" refers to the *pat* gene, and the "bla" refers to the *bla* gene in the figure.

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

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

The two, large and small, fragments of plasmid pB2/35SAck, which was cut at two sites by the restriction enzyme PvuI (Figure 4), were transferred into the tissue cells of 4 to 8 mm long shoot apex of the soybean by the particle gun bombardment.

3) Processes of rearing of living modified organisms

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

After transformation, the nucleic acid transferred cells were formed into protoplasts, which were incubated in the Kao's medium of 10 mL (Reference 17) for eight (8) days in the darkness at room temperature. Then, Kao's medium of 5 mL was added by dropping method and the cells were additionally incubated at room temperature under a low level of lighting of 2,000 lux. Addition of Kao's medium was repeated a total of eight (8) days by dropping the volume equivalent to a half of the culture solution every day. Then the cells were moved to a solid medium containing glufosinate. Two (2) to three (3) weeks later, the glufosinate-tolerant soybean callus that survived among the transformed cells was selected. The selected callus was moved to the MS medium containing plant hormone and regenerated to the young plant bodies. In addition, the regenerated young plant bodies were screened by glufosinate herbicide and the glufosinate-tolerant individuals were acclimatized, planted in pots, and grown in a greenhouse.

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

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

The pedigree tree of the recombinant soybean A5547-127 was shown in Figure 5. The recipient species A5547 (hereinafter referred to as “the non-recombinant soybean”) was transformed by the plasmid pB2/35SAck to obtain the recombinant soybean A5547-127. Then, self-pollination was repeated to obtain the plant bodies in individual generations used in the tests.

In addition, the approvals received from organizations in Japan regarding the recombinant soybean A5547-127 are listed below.

[Environmental safety]

In 1999, based on the “Guideline for the use of recombinant in agriculture, forestry and fisheries”, conducting isolated field test was approved by the

Ministry of Agriculture, Forestry and Fisheries. In addition, in November 2001, based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries", the compatibility to the guideline regarding recombinant being imported to Japan (used for processing and feed) was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

[Feed safety]

Based on the "Procedures for feed safety Assessment of feed and feed additives derived from Recombinant-DNA technology", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in March 27, 2003.

[Food safety]

Based on the "Procedures for food safety Assessment of food and food additives derived from Recombinant-DNA technology", safety of use for food was approved by the Ministry of Health, Labour and Welfare in July 8, 2002.

Confidential: Not made available or disclosed to unauthorized person

Figure 5　Pedigree tree of the recombinant soybean A5547-127

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

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

In 1996 in the US, glufosinate-tolerant individuals were selected by glufosinate herbicide-spraying test using the individuals (R2 generation) grown by self-crossing of the R1 generation of the recombinant soybean A5547-127, a heterozygote with regard to the *pat* gene locus. Then, the seeds obtained by self-crossing of the glufosinate-tolerant individuals were sown to the rows by pedigree and the germinated seedlings were sprayed with glufosinate herbicide to examine the segregation ratio between glufosinate-tolerant and glufosinate-sensitive plants. As a result, a segregation ratio of 1:2 was obtained between the strains exhibiting tolerance to glufosinate in all seedlings (homozygote) and the strains containing glufosinate-tolerant seedlings and glufosinate-sensitive seedlings in proportions of 3:1 (heterozygote) (Annex 4, Table 3). This result corresponds to the segregation ratio expected in single-gene dominant inheritance, therefore it is considered that the transferred gene exists in one chromosome.

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

The genome DNA of the recombinant soybean A5547-127（R4 generation）was cut by eight (8) restriction enzymes (EcoRI, SphI, NcoI, NcoI/HindIII, HindIII, BamHI, EcoRV and DraI), and Southern blotting analysis using three (3) types of probes

(*pat*, 3'*bla*, and 5'*bla*＋vector). As a result, the expected size of the band was detected in each analysis, and it was suggested that the one copy of *pat* gene expression cassette was transferred in the recombinant soybean A5547-127 (Table 2 and Annex 4).

Table 2　Size of the band as a result of Southern blotting analysis

Treatment by restriction enzyme	*pat* probe		3'*bla* prove		"5'*bla*+vector" prove	
	Detected band	Expected band	Detected band	Expected band	Detected band	Expected band
EcoRI	1329	1329	2400	>1673	2800	>957
SphI	1850	>1240	>15000	>1624	1850	>957
NcoI	10000	>2996	10000	>2996	2400	>957
NcoI/HindIII	1374	1374	6000	>1622	1500	>957
HindIII	2900	>1497	6000	>1622	2900	>957
BamHI	484 315	484 315	2650	>1657	6000	>957
EcoRV	8000	>2379 181	8000	>2379	4850	>957
DraI	2500	>2615	550	>485	750	>750 >207

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

In addition, in order to confirm the stability of transferred gene, Southern blotting analysis using 1329 bp probe cut out by EcoRI including *pat* gene expression cassette was conducted for the individual genome DNAs obtained from R3, R4 and R5 generations of the recombinant soybean A5547-127, which were cut by the restriction enzymes HindIII and NcoI.　As a result, the identical band was detected in each generation.　Therefore, it was confirmed that transferred DNA is stably inherited in the multiple generations (Annex 4, Figure 9).

3)　The position relationship in the case of multiple copies existing in chromosome

As a result of Southern blotting analysis and sequence analysis (Annex 6) described in "2) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations", it was confirmed that the two (2) *bla* gene fragments which were separated not to have function were disposed to take one copy of *pat* gene cassette between them, and the sequence of 1 to 28 bp of the 5'-terminal region of *bla* gene was disappeared. The position relationship is shown in Figure 6.

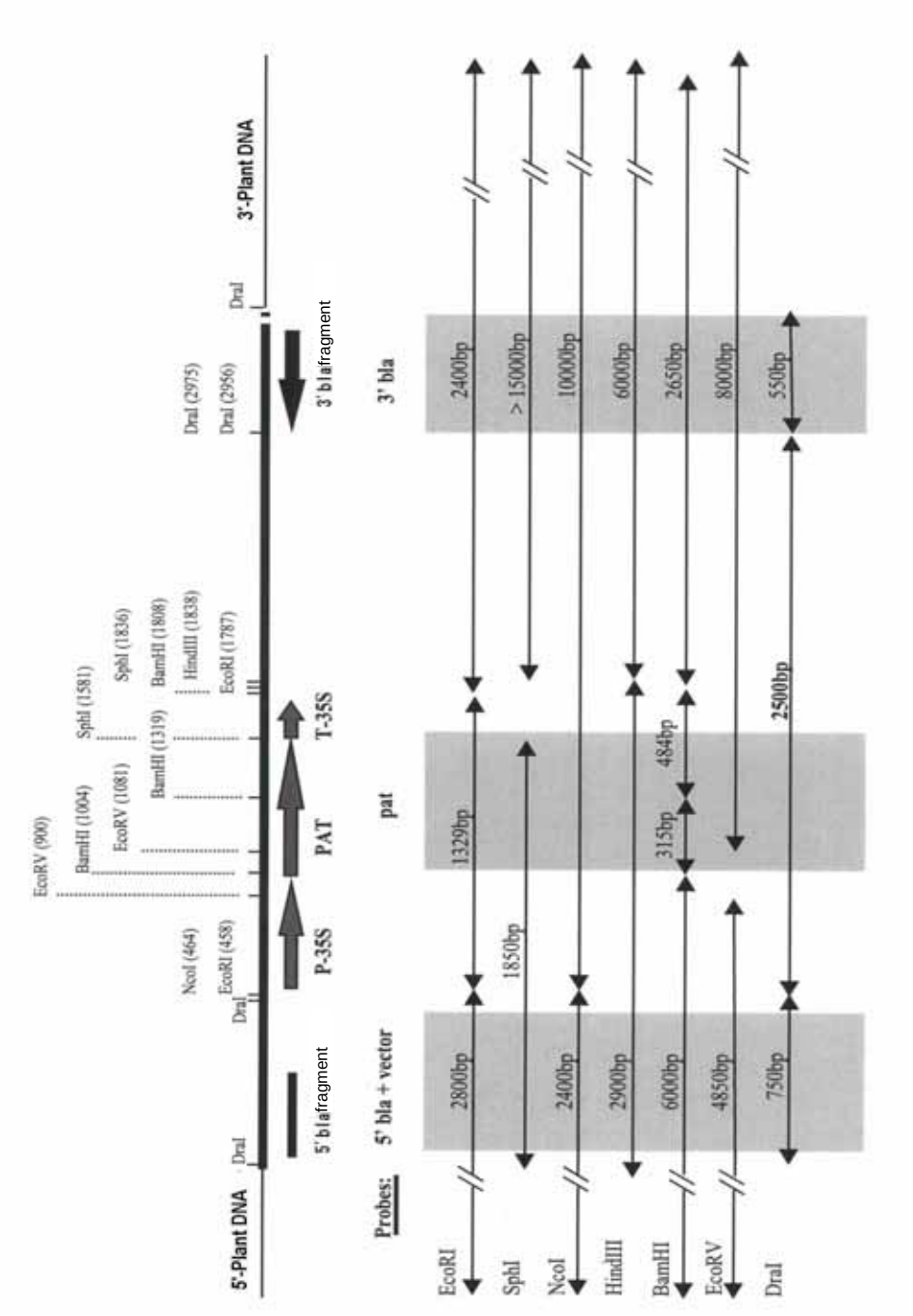

Figure 6 The position relationship of the transferred DNA existing in soybean chromosome and the size of restriction enzyme fragments

The position relationship of the DNA transferred in the recombinant soybean A5447-127 is shown. A schematic view shows the size of restriction enzyme fragments detected by Southern blotting analysis using the *pat*, 3' *bla* and 5' *bla*+vector as probes. The "PAT" refers to the *pat* gene in the figure.

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

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics that were accompanied by the expression of the transferred nucleic acid

ELISA analysis was conducted on the PAT protein in the roots, stems and leaves of five (5) individuals of each of the recombinant soybean A5547-127 and the non-recombinant soybean. As a result, PAT protein was detected at all sites in the entire plant body in the recombinant soybean A5547-127, though PAT protein was not detected from the non-recombinant soybean (Table 3-1). In addition, as a result of ELISA analysis of PAT protein in the seeds of the recombinant soybean A5547-127 cultivated in US and Canada, PAT protein was detected in all the tests conducted (Table 3-2).

Table 3-1 Measurement of PAT protein in the roots, stems and leaves of the recombinant soybean A5547-127 based on the of ELISA method

Plant body	Site	Mean PAT protein content (μg/g fresh weight)±SD	Crude protein/Fresh weight (%)	PAT protein/Crude protein (%)
Recombinant soybean A5547-127	Root	3.73±0.98	2.15	0.017
	Stem	11.5±1.8	3.62	0.032
	Leaf	19.0±5.0	6.70	0.028
Non-recombinant soybean	Root	<LOD	2.39	-
	Stem	<LOD	4.30	-
	Leaf	<LOD	7.13	-

2002, US
(n=5)
LOD : Limit of Detection, Root 2.72 ng/g, Stem 3.72 ng/g, and Leaf 9.76 ng/g

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

Table 3-2 Measurement of PAT protein in the harvested seeds of the recombinant soybean A5547-127 based on the ELISA method

Sample No.	PAT (ng/g sample) Mean±SD	Crude protein content (%)	PAT/Crude protein (%)
1	6341	-	-
2	10800±1210	-	-
3	9971±846	35.26	0.00282
4	20202±359	40.4	0.0050

－No data available

Sample No. 1: A mean of two (2) measurements of the samples obtained from individual plots in the field tests at four (4) locations and extracted once per each for three (3) repeats (a mean of a total of 24 measured values)

Sample No. 2: A mean of three (3)-repeat measurements of the samples obtained in the field tests and extracted twice (a mean of a total of six (6) measured values, LOD 4.0 ng/g sample)

Sample No. 3: A mean of two (2) measurements of the samples obtained from individual plots in the field tests at four (4) locations and extracted twice per each for three (3) repeats (a mean of a total of 48 measured values, LOD 4.0 ng/g sample)

Sample No. 4: A mean of two (2) measurements of the samples obtained from the field tests extracted twice (a mean of a total of four (4) measured values, LOD 4.0 ng/g sample)

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

In the isolated field tests conducted at the National Institute for Agro-Environmental Sciences,the Ministry of Agriculture, Forestry and Fisheries of Japan (the present National Institute for Agro-Environmental Sciences, an independent administrative institution) in 1999, the recombinant soybean A5547-127 (R5 generation) and the non-recombinant soybean were cultivated and sprayed with glufosinate herbicide diluted to a factor of 300. As a result of observation for the herbicide effect hundred (100) hours after spraying test, it was confirmed that the non-recombinant soybean all showed yellowing, then withered and died, though the individuals of the recombinant soybean A5547-127 all exhibited tolerance to the herbicide (Annex 1, Table 1 and Figure 3). In addition, as a result of glufosinate herbicide spraying test, it was confirmed that the seedlings (200 strains) germinated from the seeds of the recombinant soybean A5547-127 harvested in this isolated field all exhibited tolerance to the herbicide (Annex 1, Table 6).

Moreover, as a result of spraying of glufosinate herbicide to the seedlings germinated from the seeds of the recombinant soybean A5547-127 (R6 generation) and the non-recombinant soybean subjected to the special screened greenhouse test, it was confirmed that the seedlings of the non-recombinant soybean all withered and died, though the seedlings of the recombinant soybean A5547-127 all exhibited tolerance to glufosinate and survived. In addition, as a result of the same examination using the seeds of the next generation recombinant soybean A5547-127 and the non-recombinant soybean cultivated and harvested in the same special screened greenhouse, it was also confirmed that the seedlings of the

non-recombinant soybean all withered and died and the seedlings of the recombinant soybean A5547-127 all survived (Annex 2).

Based on the results discussed above, it was confirmed that the *pat* gene in the recombinant is stably expressed among individuals and generations.

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

The recombinant soybean A5547-127 contains no DNA sequence which possesses transferring factor and therefore, there is no possibility of transmission of nucleic acid transferred to wild animals and wild plants under a natural environment.

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

Specific detection method for this recombinant soybean is available by PCR method using the transferred DNA in the recombinant soybean A5547-127 and its surroundings as 20-mer and 21-mer primers. The PCR method has been typically used as an effective means for control of cultivation of the recombinant soybean A5547-127 (Annex 8).

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

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

With the production of the PAT protein due to the expression of the transferred *pat* gene, tolerance to glufosinate herbicide is conferred to the recombinant soybean A5547-127.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between recombinant crop and the taxonomic species to which the recipient organism belongs, and the degree of difference, if any

In the isolated field tests conducted in 1999 at the National Institute for Agro-Environmental Sciences, comparison was made for differences between the recombinant soybean A5547-127 (R5 generation) and the non-recombinant soybean (Annex 1). In addition, with regard to wintering ability of the matured plant, shedding habits of the seeds, germination rate of the seeds and production of harmful substances, examination was conducted using R6 generation in FY 2005 in a special screened greenhouse in Japan (Annex 2). Regarding the fertility and size of the pollen, comparison was made in 2000 at Aventis CropScience (the present Bayer CropScience) in the US between the recombinant soybean A5547-127 and the non-recombinant soybean (Annex 7). In all of the tests mentioned above, the A5547 line was used as the control non-recombinant soybean.

(a) Morphological and growth characteristics

In the isolated field tests, comparison between the recombinant soybean A5547-127 and the non-recombinant soybean was made for main stem length; the number of branches; the number of nodes; the number of stems; total number of flowers; leaf length × leaf width; color of flowers; leaf color; trichome color and trichome quantity in the investigation during intermediate period of growth (Annex 1, Tables 3-1 and 3-2), the weight of the aerial part; total weight of pod seeds; the weight of ripe pods; grain weight per plant; the weight of perfect grains per plant; the number of perfect grains per plant, the number of grains per pod and 100-grain weigh in the investigation at harvesting time (Annex 1, Tables 4-1 and 4-2), and plant type; seed hull color; hilum color and pod bursting for harvest (Annex 1, Table 5). As a result, in the investigation during intermediate period of growth, the recombinant soybean A5547-127 was short regarding main stem length and significant difference was observed (Annex 1, Table 3-1). In the investigation at harvesting time, significant difference was observed as follows. Regarding the number of grains per pod, the recombinant soybean A5547-127 represented slightly large value, as the recombinant soybean A5547-127 and the non-recombinant soybean showed 2.22 and 2.04 respectively. Regarding 100-grain weigh, the recombinant soybean A5547-127 represented slightly large value such as the recombinant soybean A5547-127 and the non-recombinant soybean showed 23.2 g and 20.6 g respectively (Annex 1, Table 4-2). None of the other items showed significant difference between the recombinant soybean A5547-127 and the non-recombinant soybean.

In the special screened greenhouse tests, comparison between the recombinant soybean A5547-127 and the non-recombinant soybean was made for the fresh weight of the aerial part; total weight of a plant; weight of pod seeds per plant; the number of ripe pods per plant; the weight of ripe pods per plant; the number of grains per plant; the number of perfect grains per plant; the number of grains per pod and 100-grain weight in the morphological investigation at harvesting time. As a result, none of the items showed significant difference between the recombinant soybean A5547-127 and the non-recombinant soybean (Annex 2, Tables 2 and 3).

For the significant difference found in the main stem length in the isolated field tests, the possibility could not be denied that the difference is caused by unintended effect of gene transfer. However, the main stem length of the recombinant soybean A5547-127 is shorter than that of the non-recombinant soybean. Therefore, even if the difference is caused by unintended effect of gene transfer and the difference would happen on a constant basis, it would be disadvantage in competitiveness, and it is considered there is no risk of Adverse Effect on Biological Diversity. In addition, significant difference was observed in the items of the number of grains per pod and 100-grain weight in the isolated field tests. However, there was no significant difference found in the items of the number of grains per pod and 100-grain weight in the special screened greenhouse tests. Therefore, it is considered that the significant difference observed does not occur on a constant basis.

(b) Cold-tolerance at the early stage of growth

Cold-tolerance test at the early stage of growth of the plant body was not conducted. Instead, in order to examine the wintering ability of the seeds, the seeds were taken from the recombinant soybean A5547-127 and the non-recombinant soybean grown in an isolated field, dried up and then placed in the earth to a depth of 10 cm in the isolated field in December 10, 1999, then observed in February 10 of the next year. As a result, it was confirmed that the seeds were all decayed and they lost the germinating ability (Annex 1). Therefore, it is considered that the recombinant soybean A5547-127 and the non-recombinant soybean can not germinate and grow and survive over the winter under natural condition in the winter season.

(c) Wintering ability of the matured plant

For the recombinant soybean A5547-127 and the non-recombinant soybean which were cultivated in the isolated field tests, the matured plants were left to stand in the field in the winter season even after maturation and they were observed after 60 days. As a result, it was confirmed that all the individuals withered and died (Annex 1). For the recombinant soybean A5547-127 and the non-recombinant soybean sown in 2005 and grown in a special screened greenhouse, the matured plants were left to stand in the special screened greenhouse in the winter season even after maturation and they were observed in February of the next year. As a result, it was also confirmed that all the individuals withered and died (Annex 2). Based on the above, it is considered that the matured plants of the recombinant soybean A5547-127 and the non-recombinant soybean both show no wintering ability.

(d) Fertility and size of the pollen

In the tests conducted in the US in 2000, pollens of the recombinant soybean A5547-127 and the non-recombinant soybean were obtained to examine the fertility of the pollen (Annex 7, Table 1), germination rate (Annex 7, Table 2), the shape of the pollen (Annex 7, Figures 1 and 2), and the shape of the pollen of the germinated plant (Annex 7, Figures 3 and 4). As a result of comparison between the recombinant soybean A5547-127 and the non-recombinant soybean, no difference was observed in all items between them.

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

Regarding the characteristics referring to the production of seeds, as mentioned in "(a) Morphological and growth characteristics", for the items of total weight of pod seeds, the number of ripe pods, grain weight per plant, the number of perfect grains per plant, the weight of perfect grains per plant, the number of grains per pod and 100-grain weigh, comparison were made between the recombinant soybean A5547-127 and the non-recombinant soybean in the investigation at harvesting time of the isolated field tests (Annex 1, Tables 4-1 and 4-2). In addition, in the special screened greenhouse tests, the weight of pod seeds per plant, the number of ripe pods per plant, the weight of ripe pods

per plant, the number of grains per plant, the weight of perfect grains per plant, the number of grains per pod and 100-grain weight were examined (Annex 2, Tables 2 and 3). As a result, significant difference was observed in the items of the number of grains per pod and 100-grain weight in the isolated field tests. However, there was no significant difference found in all items in the special screened greenhouse tests. Therefore, it is considered that the significant difference observed in the isolated field tests does not occur on a constant basis. In addition, since no significant difference was observed in the other items, it is considered that there is no difference between the recombinant soybean A5547-127 and the non-recombinant soybean regarding the production of the seed per plant.

With regard to the shedding habit, as a result of examination on the difficulty in pod bursting in the isolated field tests, the recombinant soybean A5547-127 and the non-recombinant soybean were both difficult to burst the pods, and the degree of pod bursting for both were equivalent (Annex 1, Table 5). In addition, in the special screened greenhouse tests, the recombinant soybean A5547-127 and the non-recombinant soybean were planted in six (6) pots for each and kept grown in the period up to one month after the maturing time to examine the rate of pods burst among the total number of pods per plant. As a result, no significant difference was observed between them (Annex 2, Tables 6 and 7).

As a result of examination by sowing the following seeds in boxes in a greenhouse; 200 seeds and 500 seeds harvested from the recombinant soybean A5547-127 and the non-recombinant soybean respectively which were cultivated in an isolated field, the germination rates were 100% for the both plants (Annex 1, Table 6). Since they showed high germination rate right after harvesting, it is considered that the recombinant soybean A5547-127 and the non-recombinant soybean both have lower levels of seed dormancy.

(f) Crossability

In the isolated field, the recombinant soybean A5547-127 and the non-recombinant soybean were cultivated at an inter-row spacing of 80 to 160 cm, then the 200 seeds and 500 seeds were obtained respectively. The seeds were sown and grown, and then sprayed with glufosinate herbicide to observe their tolerance to glufosinate. As a result, all individuals from the seeds of the recombinant soybean A5547-127 were found survived, on the other hand, all individuals from the seed of the non-recombinant soybean withered and died due to the effects of glufosinate herbicide and no individual was found to show transition of herbicide-tolerance to the non-recombinant soybean by natural crossing (Annex 1, Table 6).

(g) Productivity of harmful substances

With regard to the characteristics referring to the productivity of harmful substances, succeeding crop tests were carried using radish, a plant relatively highly sensitive to growth-inhibiting substances.

The soil of the cultivation areas in the isolated field of the recombinant soybean A5547-127 and the non-recombinant soybean were obtained after cultivating them. Then the soil (depth: 20cm) obtained from both areas were moved to wagner pots of 1/5000a, and the 30 seeds of radish were sown per a pot. After germination, they were moved to a greenhouse without heating to observe their growth. As a result, no significant difference was observed between the soil of the recombinant soybean A5547-127 and the soil of the non-recombinant soybean regarding germination rate, plant height, root length, number of true leaves and dry weight of the radish (Annex 1, Table 7).

In addition, with regard to the characteristics referring to the productivity of harmful substances of the recombinant soybean A5547-127 in a special screened greenhouse, the following tests were carried out to compare the recombinant soybean A5547-127 and the non-recombinant soybean: succeeding crop tests using radish for examining the substances secreted from the roots with a threat to affect the growth of other plants; plow-in tests using radish for examining the substances contained in the plant tissues with a threat to affect other plants after they die; and soil microflora tests for examining the substances secreted from the roots with a threat to affect soil microbes.

[Succeeding crop tests]

The recombinant soybean A5547-127 and the non-recombinant soybean grown in a special screened greenhouse for about three (3) months were sieved after harvesting to remove the plant tissues and other residues of plant bodies and obtain the soil. In the soil, the seeds of radish were sown and grown to examine the germination rate, plant height, root length, fresh weight and dry weight. As a result, no significant difference was observed in all items examined between the recombinant soybean A5547-127 and the non-recombinant soybean (Annex 2, Tables 8 to 13).

[Plow-in tests]

The plant bodies including the roots of the recombinant soybean A5547-127 and the non-recombinant soybean harvested after cultivation in a special screened greenhouse for about three (3) months were dried, crushed and mixed with new soil at a rate of about 1% by weight, then packed into nursery pots. Seeds of radish were sown in and grown in the pots to examine the germination rate, plant height, root length, fresh weight and dry weight. As a result, no significant difference was observed in all items examined between the recombinant soybean A5547-127 and the non-recombinant soybean (Annex 2, Tables 14 to 21).

[Soil microflora tests]

The soils shaken off from the roots of plant bodies of the recombinant soybean A5547-127 and the non-recombinant soybean harvested after cultivation in a special screened greenhouse for about three (3) months were compared to examine the number of bacteria, the number of actinomyces and the number of filamentous fungi. As a result, no significant difference was observed in all

items examined between the recombinant soybean A5547-127 and the non-recombinant soybean (Annex 2, Tables 22 to 24).

Based on the above results, it is considered that there is no difference between the recombinant soybean A5547-127 and the non-recombinant soybean in the productivity of harmful substances.

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

(1) Competitiveness

The plant of soybean (*G. max*) to which the recipient organism belongs, has been cultivated for a long time in Japan, but there is no report that it grows voluntarily in Japan.

This recombinant soybean is given the tolerance to glufosinate herbicide because of the transferred *pat* gene, but it is hard to consider that glufosinate exerts a selective pressure under a natural environment. In addition, various characteristics relating to the competitiveness of this recombinant soybean have been investigated in Japanese isolated fields, and no significant difference from the non-recombinant soybean has been observed.

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

(2) Productivity of harmful substances

Regarding the plant of soybean (*G. max*) to which the recipient organism belongs, there is no report that it produces a harmful substance to wild animals and wild plants.

This recombinant soybean produces the PAT protein having the tolerance to glufosinate, but it is not reported that the protein is a harmful substance. In addition, the PAT protein has high substrate specificity and exhibits little affinity except to glufosinate; therefore it is considered that the PAT protein does not affect the metabolic system of the recipient organism.

In addition, the ability of this recombinant soybean to produce harmful substances (the substances secreted from the roots to affect other plants, the substances secreted from the roots to affect microorganism in soil, and the substances contained in the plant bodies to affect other plants after dying out) was investigated, and no significant difference between this recombinant soybean and the non-recombinant soybean was observed.

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

(3) Crossability

1) Identification of wild animals and wild plants likely to be affected

Since it is known that if the *Glycine soja* Sieb. & Zucc. that grows voluntarily in Japan is crossed with soybean(*G. max*), it produces fertile seeds, the *G. soja* was specified as a wild plant likely to be affected, to perform the following examination.

2) Evaluation of concrete details of adverse effect

Existing documents do not show any obstacle to the growth and reproduction of the hybrid obtained from soybean and *G. soja*. So, in the case where this recombinant soybean and *G. soja* are crossed with each other in the Japanese natural environment, there is possibility that the hybrid grows and that the gene transferred into this recombinant soybean through the back crossing from the hybrid to *G. soja* diffuses among the population of *G. soja* without remaining at a low level.

3) Evaluation of likelihood of adverse effect

G. soja grows voluntarily and widely throughout Japan in sunny fields, on the roadsides and the like. So, in the case where this recombinant soybean grows near *G. soja*, it cannot be denied that there are chances where both plants cross with each other. However:

A. There is no plan of cultivation of this recombinant soybean in the future in Japan. It is considered that any seeds may be spilled during transportation and such spilled seeds germinate and grow, though there is no report so far that soybean becomes self-seeding.

B. Even in the case where this recombinant soybean grows voluntarily near *G. soja* as a rare case:

(a) Both *G. max* and *G. soja* are typical autogamous plants engaged in cleistogamy*.

(b) According to existing documents, even when *G. soja* was grown adjacent to *G. max* under such a condition that the *G. soja* pedigree and *G. max* pedigree flowered at the same time, the crossing rate was less than 1%.

(c) Among the individuals derived from the seeds harvested from the non-recombinant soybean cultivated closely to this recombinant soybean in Japanese isolated fields, there were noindividual that are free from any effects of glufosinate herbicide, Consequently, it is considered that the crossability of this recombinant soybean with *G. soja* is similarly low as

that of conventional soybean.

(d) It is considered to be low that the tolerance to glufosinate conferred with the expression of the *pat* gene works dominantly for the selection pressure under the natural environment.

In view of the above, it is considered to be very low that this recombinant soybean and *G. soja* could cross with each other and that the transferred gene could diffuse among the population of *G. soja* without remaining at a low level.

4) Judgment of existence of Adverse Effect on Biological Diversity

As described above, the probability that this recombinant soybean and *G. soja* cross with each other is very low. Even if crossing occurs, it is hard to consider that the hybrid produced from this recombinant soybean and *G. soja* ousts wild plants. In addition, the possibility that the transferred gene diffuses among the population of *G. soja* without remaining at a low level is also considered to be very low stochastically. Consequently, it was judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability is reasonable.

2. Conclusion based on the Biological Diversity Risk Assessment Report

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

* Cleistogamy means self-pollination within a flower that does not open that occurs in angiosperms. It involves a very low probability of cross-pollination with the pollens of other plants due to the physical obstacles of flower bud (petal/calyx). However, it does not mean any physiological incompatibility, so it may allow cross-pollination with the pollens of other plants transmitted by insects, etc.

Reference

Confidential: Not made available or disclosed to unauthorized person

Annex List

Annex 1： FY 1999 Isolated Field Test Report on Genetically Modified Soybean A5547-127
Confidential: Not made available or disclosed to unauthorized person

Annex 2： Environmental Safety Evaluation Test on Glufosinate Herbicide-Tolerant Soybean A5547-127 in Special screened greenhouse
Confidential: Not made available or disclosed to unauthorized person

Annex 3： The Entire Nucleotide Sequence of Plasmid pB2/35SAck
Confidential: Not made available or disclosed to unauthorized person

Annex 4： Material based on the Result of Safety Test in the US
Confidential: Not made available or disclosed to unauthorized person

Annex 5： Evaluation of *bla* gene expression in the recombinant soybean A5547-127
Confidential: Not made available or disclosed to unauthorized person

Annex 6： Determination of nucleotide sequence of the gene transferred in the recombinant soybean A5547-127
Confidential: Not made available or disclosed to unauthorized person

Annex 7： Investigation for reproductivity of the recombinant soybean A5547-127
Confidential: Not made available or disclosed to unauthorized person

Annex 8： Event Identifying Method
Confidential: Not made available or disclosed to unauthorized person