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 glyphosate herbicide (*cp4 epsps*, *Glycine max* (L.) Merr.) (40-3-2, OECD UI:MON-04032-6)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	-

Outline of the Biological Diversity Risk Assessment

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

1. Information concerning a recipient organism or the species to which the recipient organism belongs

(1) Taxonomical position and state of distribution in natural environment

i) Soybean is an annual Leguminosae known as the scientific name of *Glycine max* (L.) Merr. which belongs to the subgenus *Soja* under the genus *Glycine*.

ii) The recipient organism is a soybean (*Glycine max* (L.) Merr.) which belongs to the subgenus *Soja* under the genus *Glycine* in Leguminosae.

iii) The subgenus *Soja* includes the wild species *G. soja* and *G. gracilis* in addition to the soybean cultivars. Based on the cytological, morphological and molecular biological knowledge, the cultivar soybean (*G. max*) is considered to originate from the wild species *G. soja*. On the other hand, *G. gracilis* is considered as intermediate species in the process of differentiation from *G. soja* to *G. max* or as crossbreed between *G. soja* and *G. max*. Among these wild species, only the *G. soja* is distributed in Japan and the distribution of *G. gracilis* is not observed. *G. soja* is distributed in China, Korea, Japan, Taiwan and former Soviet Union, and domestically in Hokkaido, Honshu, Shikoku and Kyushu, where it natrually grows primary in the riverbeds, the sites of demolished factory or fields and their neighbors in which the previous vegetation was destructed, and other unshaded fields and roadsides.

(2) History and present state of Use

i) Soybean is considered to originate in the north-eastern part of China and become cultivated there around BC 1100 then spread to the southern part of China, Southeast Asia, Korea and Japan. It is estimated to be introduced into Japan in the Jomon era and become cultivated for use as side dish.

ii) According to the statistical information by the Food and Agriculture Organization of the United Nations (FAO), the worldwide total cultivation area of soybean is about 83.7 million hectares as of 2003, and the breakdown of major countries is as

follows: about 29.27 million hectares in the US, about 18.47 million hectares in Brazil, about 12.47 million hectares in Argentina, and about 9.5 million hectares in China. The cultivation area in Japan in 2003 based on the same statistical information is about 150,000 hectares.

The amount of soybean imported in 2003 into Japan was about 5.17 million ton, about 76% of which came from the US. The supply of soybean in 2002 was 5.41 million ton in total, which was made up by the import of 5.14 million ton and the domestic production of 270,000 ton. The consumption of soybean by use is about 3.81 million ton for oil manufacture, about 1.12 million ton for food and about 280,000 ton for the others. The soybean for oil manufacture was mostly covered by those imported from the US.

In Japan, there are many utilizations of soybean, ranging from food such as miso paste, soy sauce, tofu or bean curd, fermented soybean called natto, sheets of dried tofu, soy flour, cooked beans and sprouts to food additive in the form of separated protein or concentrated protein and cooking vegetable oil of extracted oil and livestock feed of defat soybean.

The Japanese customary cultivation method of soybean is as follows: Soybean cultivars are classified as summer type, autumn type and intermediate type, according to the number of days from seeding to flowering and the number of days after flowering to ripening. The optimum seeding time is early June in the southern part of Tohoku District and Higashi-yama area of Hokuriku District (intermediate type soybean), mid-June in the Kanto District (intermediate type soybean), late June in the districts west of Tokai District to western part of Chugoku District (intermediate type soybean or autumn type soybean), and early July to early August in Kyushu District (autumn type soybean) or early April to late April (summer type soybean). Density of seeding depends on the type of soybean and the cultivation condition, though the dense planting is adopted for the early-maturing varieties, cultivation in cold districts, or late seeds sowing in the season. Early weedkilling during the growing period to get rid of weeds in the bud contributes to vigorous growing of stems and leaves of soybean and resultant easy control of weeds in the subsequent stages. In addition, pest control is one of the most important work for soybean cultivation and then, chemicals are sprayed to insect pests at the early stage of growth. Harveting is accomplished by pulling out or cutting off at the ground level. The harvests are placed on the ground or hung

on for drying and then threshed by a threshing machine, otherwise they are reaped and threshed by a combine harvester.

(3) Physiological and ecological properties

i) Basic properties

Soybean is an annual dicotyledon propagating by seeds. The seed leaves grow opposite to each other, then the egg-shaped primary leaves grow opposite to the seed leaves at right angle, and then compound leaves having three pieces of leaflet grow. The stem is composed of main stem and branches. The branches grow from the leaf axils of compound leaves on the main stem node, and the roots have the adherent root nodules due to the symbiotic association of root nodule bacterium (*Bradyrhizobium japonicum*) having an atomospheric nitrogen-fixing capability. The flower has one pod which contains one to five ovules in the ovary at the base, and the ovary swells after pollination to form [seeds or pods?]. The flower initiation of soybean greatly depends on day length and temperature. For the flower initiation, a dark period of a certain legnth of time is indispensable. For the temperature, a value of 15 or more is required and the higher the temperaure up to around 25 , the more the flower initiation is accelerated. The acceleration is faster under short-day and high-temperature conditions, whereas accleartion may be interrupted or even deceleration can occur under long-day and high-temperature conditions.

ii) Environmental conditions allowing inhabiting or growth

The optimum germination temperatures of soybean seed are 30 to 35 and the minimum germination and minimum growing temperatues are 2 to 4 . At 10 or less, germination is very poor. For soybean cultivation, temperatures of 18 to 28 , longer hours of sunshine and proper amount of rainfall in the growing period are repertedly preferable. However, currently available soybean cultivars feature minutely controlled sensitivities to day length and resultant higher adaptability to a variety of weather conditions and then, cultivation is feasible in the areas from Indonesia right on the equator to Sweden at N60°latitude.

There is no report that soybean becomes weeds in Japan.

iii) Mode of propagation or reproduction

a) The soybean seeds fall to the ground when the pods become split. The soybean cultivars adopted in Japan offer difference in the ease of splitting of pods between soybean types. In the US where soybean is cultivated on a large scale and harvesting is mechanized, almost all cultivars feature hard bolls for splitting, thus leading to low shedding habit. In addition, dormancy of the seed is not identified. The germinating ability of the seed will be normally lost in about 3 years when stored at room temperature.

b) Soybean propagates by seeds without any vegetative propagation by tuber or underground stem. There has been no report so far that the tissues or organs capable of regenerating the plant body in the natural conditions offer the germinating ability.

c) Wild relative that can be crossed with soybean in Japan is limited to *Glycine soja*. *G. soja* is an annual climbing plant spreading in Hokkaido, Honshu, Shikoku and Kyushu, and it grows voluntarily primarily in the riverbeds, sites of demolished factory or field where previous vegetation was disturbed, and other unshaded fields and roadsides.

In the 1950s, large-leaf *G. soja* was discovered in Japan and it was considered to be a morphologically intermediate type individual of soybean and *G. soja*. It was estimated to be crossed with soybean rather than the typical *G. soja* because of the closer morphology to soybean. However, there is some reports that large-leaf *G. soja* and other morphologically intermediate type individuals have not been discovered in the long-standing collection progject of *G. soja* from around 800 populations throughout the country over 10 years. Therefore, even if such morphologically intermediate indivdual grows wild in Japan, the extent of growth may be limited.

With regard to the degree of autogamy and allogamy of soybean and *G. soja*, soybean and *G. soja* feature the cleistogamy in which the anthers open before flowering for pollination and in the latter half of flowering peirod, pollination is completed while almost all flowers do not bloom. Therefore, both are considered as typical autogamy plants. In addition, their rate of cross pollination is reported in some literature as 3% at maximum for soybean and 2.3% at maximum for *G. soja*.

However, there is a report that the rate of cross pollination increased to 14% when a group of honey bees was raised together with soybean in a greenhouse [literature citation?]. Also with regard to *G. soja*, there is a report that such a group was discovered in the catchment area of Omono River in Akita Prefecture that exhibited a high rate of cross pollination of about 13% [literature citation?]. It is not shown whether the high rate of cross pollination resulted from the environmental condition specific to the catchment area of Omono River or the genetic characteristics of the group. In actuality, however, the catchment area of Omono River was free from bank protection works and other environmental disturbance, and the size of *G. soja* group was large enough. Then, this area was a very attractive food supply source for flower-visiting insects and in fact, honey bees and carpenter bees were frequently observed in the area and neighbors of the group of *G. soja*. In addition, the number of pollens of *G. soja* per 1ovule collected from the group was 600 to 700 grains on average, which lies between the average number of pollens per lovule of typical self-fertilizing and cross pollinating plants.

With regard to the crossability between soybean and *G. soja*, there is a report on the investigation for the natural crossing between the Japanese endemic cultivar species Tanba Black and *G. soja* (Gls/93-J-01) in which 30 individuals for each were planted alternately [literature citation?]. As a result of investigation on the progeny of 686 individuals obtained from the ripened *G. soja* after experiment of natural crossing, 5 individuals in the progeny were judged as the crossing between soybean and *G. soja*, which corespond to the rate of crossing of 0.73%.

d) The amount of pollen production by soybean is very small, and the fertility is lost in 2 to 4 hours. The diameter of a pollen is 15 to 25μm. Regarding the dispersion distance of pollen, crossing test was conducted at the National Institute for Agro-Environmental Sciences in 2001 using this recombinant soybean [should this refer specifically to RR soybean rather than just “this recombinant soybean”]. As a result, it was found that the rate of hybridization is 0.19% at a distance of 0.7m from the pollen parent, 0.025% at 3.5m, and 0% at 10.5m. In addition, as a result of the similar test conducted in 2002 at the National Institute for Agro-Environmental Sciences, the rate of crossing was found 0.16% at a distance of 0.7m from the pollen parent, 0.08% at 2.8m, and

0% at 3.5m.

iii) Productivity of harmful substances

It is not known that soybean produces any allelochemicals and other harmful substances that affect habitation and growth of wild animals and wild plants. [would it be appropriate to include a mention of the antinutrients in mature soybean seed? These antinutrients could be consumerd by wild animals but would not be toxic to these animals since they tend to just restrict consumption or reduce growth]

2. Information concerning the preparation of the living modified organism

(1). Information concerning the donor nucleic acid

i) Composition and origins of component elements

The composition of the donor nucleic acid that was used for the development of the soybean tolerant to glyphosate herbicide (*cp4 epsps*, *Glycine max* (L.) Merr.) (40-3-2, OECD UI:MON-04032-6) (hereinafter referred to as "this recombinant soybean") and the origins of component elements are shown in Table 1.

ii) Functions of component elements

The functions of the donor nucleic acid used for the development of this recombinant soybean are shown in Table 1.

[*cp4 epsps* gene]

a) Glyphosate herbicide is the active ingredient of Roundup, a nonselective herbicide, and inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19), one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis, by specifically binding to the enzyme. Therefore, plants treated with glyphosate cannot synthesize aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, and die. The target gene of this recombinant soybean, *cp4 epsps* gene, expresses the CP4 EPSPS protein which has high tolerance to the herbicide glyphosate. The activity of the CP4 EPSPS protein that is produced by *cp4 epsps* gene is not inhibited even under the presence of glyphosate. So, in the recombinant plants that express this protein, the shikimate pathway normally functions, allowing the plants to grow.

EPSPS is one of the enzymes that catalyze the shikimate pathway for biosynthesizing the aromatic amino acids specific for plants and microbes, and exists in chloroplasts or plastids in plants. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway, but it has been

clarified to be extremely unlikely that the stages from DAHP through the production of 5-enol-pyruvylshikimate-3-phosphate (EPSP) to the synthesis of chorismic acid are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesize excessive aromatic amino acids. In addition, Monsanto Co. examined amino acid contents in the seeds of the recombinant crops in the process of food/feed safety assessment of crop plants (soybean, coleseed, cotton, and maize) that are tolerant to the glyphosate herbicide, and confirmed that there is no difference in the aromatic amino acid content as the final product of the shikimate pathway between the original non-recombinant plants and recombinant plants. These facts support that EPSPS is not the rate-determining enzyme in this pathway. Besides, EPSPS is the enzyme that catalyzes a reversible reaction to produce EPSP and inorganic phosphates (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P), and is known to specifically react with these substrates. The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P, but the reactivity with shikimate is only one two millionth of the reactivity with S3P, and it is unlikely that shikimate reacts as the substrate of EPSPS in the living plant.

b) In order to investigate whether the CP4 EPSPS protein shares functionally important amino acid sequences with known allergens, the CP4 EPSPS protein was compared with allergens in the databases (GenBank, EMBL, PIR, NRL3D, SwissProt). As a result, the CP4 EPSPS protein did not have any sequence structurally similar to those of known allergens.

Table 1 Origins and functions of the component elements of plasmid vector PV-GMGT04 used for the development of the soybean tolerant to glyphosate herbicide, 40-3-2

Component element	Origin and Function
cp4 epsps gene expression cassette controlled by P-CMoVa (E35S)	
CMoVa (E35S)	35S promoter of cauliflower mosaic virus (CaMV). Has a duplication enhancer region.
CTP	A nucleotide sequence in the *epsps* gene of *Arabidopsis thaliana*, which encodes the chloroplast transit peptide portion located at N-terminal region of PSPS protein. Transports the target protein to chloroplast.
cp4 epsps	5-enol-pyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* CP4 strain. The detail of the function is described in pages 1-2.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. Terminates transcription of mRNA and induces polyadenylation.

uidA gene expression cassette controlled by P-MAS (not inserted into this recombnant soybean)	
P-MAS	Promoter region of *mannopine synthase 2'* derived from *Agrobacterium tumefaciens*. Involved in the constant expression of the target gene.
uidA(GUS)	*uidA* gene derived from *Escherichia coli*. Encodes GUS (β-D-glucuronidase) protein.
7S3'	3' terminal untranslated region of 7S seed storage protein α subunit of soybean
cp4 epsps gene expression cassette controlled by P-CMoVb (FMV) (not inserted in this recombinant soybean)	
CMoVb (FMV)	35S promoter of figwort mosaic virus. Involved in the constant expression of the target gene in all tissues.
CTP	A nucleotide sequence in the *epsps* gene of *Arabidopsis thaliana*, which encodes the chloroplast transit peptide portion located at N-terminal region of PSPS protein. Transports the target protein from cytoplasm to chloroplast.
cp4 epsps	5-enol-pyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* CP4 strain. The detail of the function is described in pages 1-2.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene derived from T-DNA of *Agrobacterium tumefaciens*. Terminates transcription of mRNA and induces polyadenylation.
Other component elements	
LAC	Consists of a partial sequence encoding a lac repressor, lac promoter, and a partial sequence encoding β galactosidase. Used as a selectable marker for cloning in *E. coli*.
ori-pUC	Replicator region derived from *E. coli* plasmid pUC119.
KAN(*nptII*)	A gene isolated from Tn5 transposon of *E. coli*. Encodes neomycin phosphotransferase type II (PPTII) enzyme protein.

(2). Information concerning the vector

i) Name and origin

The vector used for the development of this recombinant soybean was constructed based on the plasmid pUC 119 derived from *Escherichia coli*, etc.

ii) Properties

The vector consists of kanamycin-tolerant gene (*nptII* gene) as a selectable marker gene of a construction vector in *E. coli*, *ori*-PUC as a replicator region allowing replication in *E coli*, and LAC used as a selectable marker for cloning in *E. coli*. The details of the respective component elements are as shown in Table 1.

The total number of base pairs of PV-GMGT04 used for the development of this recombinant

soybean is 10,505 bp.

The infectivity of this vector is not known.

(3). Method of preparing the living modified organism

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

For developing this recombinant soybean, based on the vector derived from pUC119 having the aforesaid *nptII* gene, (a) CP4 EPSPS gene expression cassette ([E35S]-[CTP]-[*CP4 EPSPS*]-[NOS3']), (b) *GUS* gene expression cassette ([P-MAS]-[*GUS*]-[7S3']), and (c) *CP4 EPSPS* gene expression cassette regulated by a different promoter ([CMoVb]-[CTP]-[*P4 EPSPS*]-[NOS3']) were connected to construct plasmid PV-GMGT04, and this plasmid was used as the vector (see Table 1 and Figure 1).

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

Plasmid PV-GMGT04 was introduced into the shoot apex tissue cells of the non-recombinant soybean cultivar by the particle gun method.

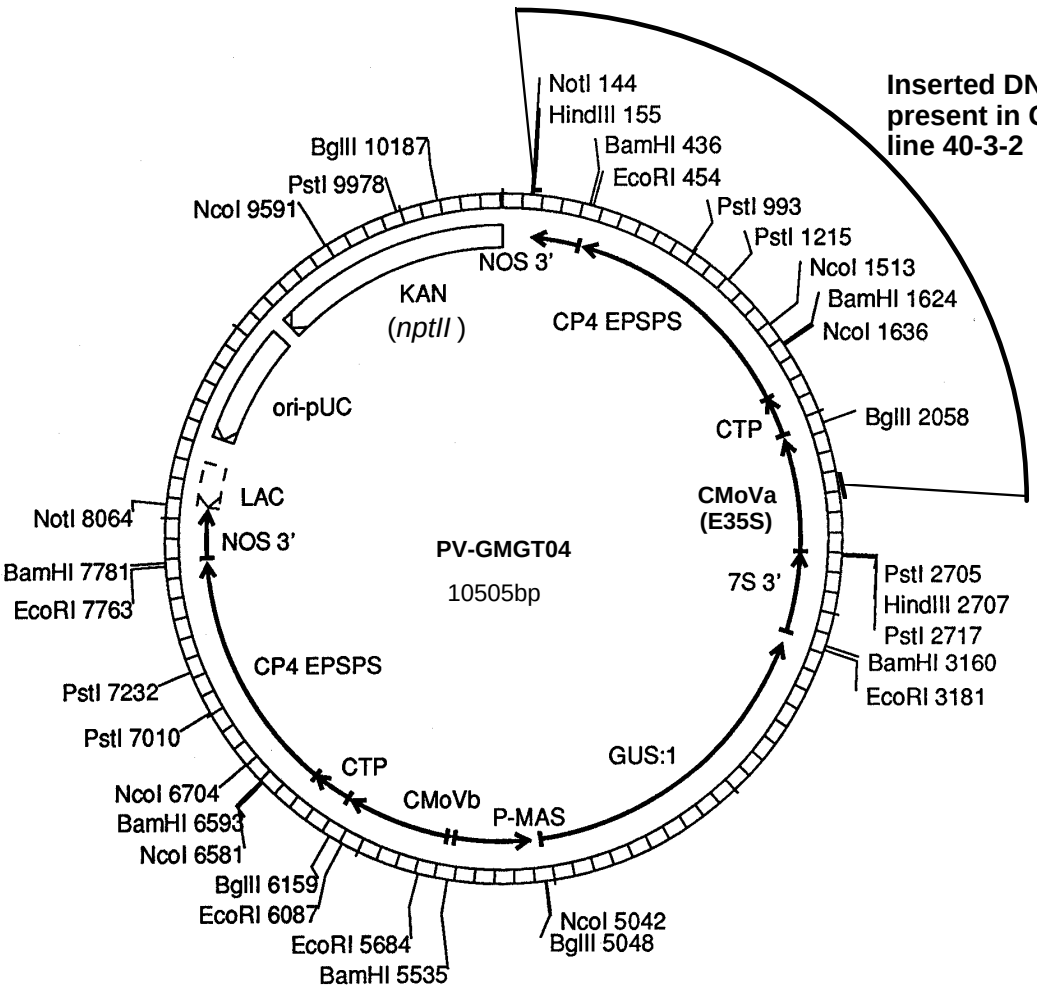

Figure 1 PV-GMGT04 plasmid map

iii) Processes of rearing of the living modified organism

a) The shoot apex cells having the plasmid DNA introduced by the particle gun method were grown for a while on a medium containing cytokinin and auxin, to induce adventitious buds.

b) One hundred and fifty individuals showing positivity to the GUS reaction were selected as individual transformants (= R0 generation) from among redifferentiated individuals and were grown in a greenhouse (1990). After the R0 generation, the estimation of tolerance to glyphosate, the estimation of hereditary mode of tolerance, the estimation of agricultural traits and the analysis of inserted gene were conducted in greenhouses and fields. Finally based on the results of field tests (R2, R3, R4 and R5 generations) conducted in 1991 to 1993 in USA and Puerto Rico, this recombinant soybean (40-3-2 pedigree) was selected as a commercial pedigree. Based on these results, necessary approvals were granted and general commercial cultivation started from 1996 in USA.

The following shows the approvals received from organizations in Japan.

March 1996: Based on the "Guideline for the use of recombinants in agriculture, forestry and fisheries," the compatibility to the guideline regarding the recombinant to be imported to Japan and to be cultivated in Japan (used for processing and feed) was certified by the Ministry of Agriculture, Forestry and Fisheries.

September 1996: Based on the "Guideline for the Conduct of Food Safety Assessment of Food and Additives Derived from Recombinant-DNA Plants, Chapter 4," the safety of use for food was approved by the Ministry of Health, Labour and Welfare (the then Ministry of Health and Welfare).

September 1996: The safety of use of the cultivar for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in accordance with "Guideline for the safety evaluation of feed derived from recombinant-DNA plants, 6-(2)."

March 2001: The Ministry of Health, Labour and Welfare ensured the safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives Derived from Recombinant-DNA Plants."

March 2003: The Ministry of Agriculture, Forestry and Fisheries ensured the safety of the use of the cultivar as feed, following "Procedure to Check the Safety of Feed and Additives Produced by Recombinant-DNA Techniques."

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

The Southern blotting analysis for this recombinant soybean using its R5 generation was conducted, and as a result, it was confirmed that one copy of *cp4 epsps* gene expression cassette ([E35S]-[CTP]-[*cp4 epsps*]-[NOS3']) region was inserted in the genome, and that *uidA* gene expression cassette, *cp4 epsps*

gene expression cassette regulated by a different promoter ([CMoVb]-[CTP]-[*cp4 epsps*]-[NOS3']), and a region derived from pUC119 and *nptII* gene did not exist in the genome. Meanwhile, this recombinant soybean was selected as one of the redifferentiated individuals of the R0 generation positive in the GUS assay, however, the *uidA* gene expression cassette was not detected as a result of the Southern blotting analysis. It was suggested that the *uidA* gene and the *cp4 epsps* gene were inserted at different chromosomes and then the *uidA* gene was eliminated from this recombinant soybean by ordinary genetic separation in the following breeding process where the *cp4 epsps* gene as the target gene was fixed homozygously in the R1 to R2 generations.

Since the analytical methods progressed thereafter, Southern blotting analysis with higher sensitivity, cosmid cloning, genome walking, etc. were used in combination to re-analyze the inserted gene using R5 generation. As a result, it was confirmed that a 250 bp *cp4 epsps* gene fragment existed adjacently to the NOS3' terminus of the inserted *cp4 epsps* gene expression cassette, and further that a 937 bp DNA fragment containing a 72 bp fragment of the *cp4 epsps* gene was obtained by digestion with the restriction enzyme, *Hin*dIII. Moreover, it was clarified that low activity of producing the transcript from the inserted *cp4 epsps* gene expression cassette beyond the NOS3' terminator .

The newly detected 250 bp and 72 bp fragment of the *cp4 epsps* gene were stably inherited in the progeny, and furthermore, it was confirmed by Northern blotting analysis and Western blotting analysis that these gene fragments did not function. On the other hand, Western blotting analysis confirmed that the possibility that any protein other than the CP4 EPSPS protein might be produced from the transcript transcribed beyond the NOS3' terminator was also very low. Moreover, it was demonstrated that even if an open leading frame should be formed, the estimated polypeptide that may be translated from the transcript does not have any similarity to or correlativity with known harmful substances such as toxins.

It was demonstrated by Southern blotting analysis in the two generations (R3 and R6 generations were used) that the inserted gene was stably inherited in the progeny. Furthermore, it was also confirmed by conducting glyphosate sprinkling tests in the process of selection and in isolated field tests that the CP4 EPSPS protein capable of conferring the tolerance to glyphosate herbicide was also stably expressed in more than three generations.

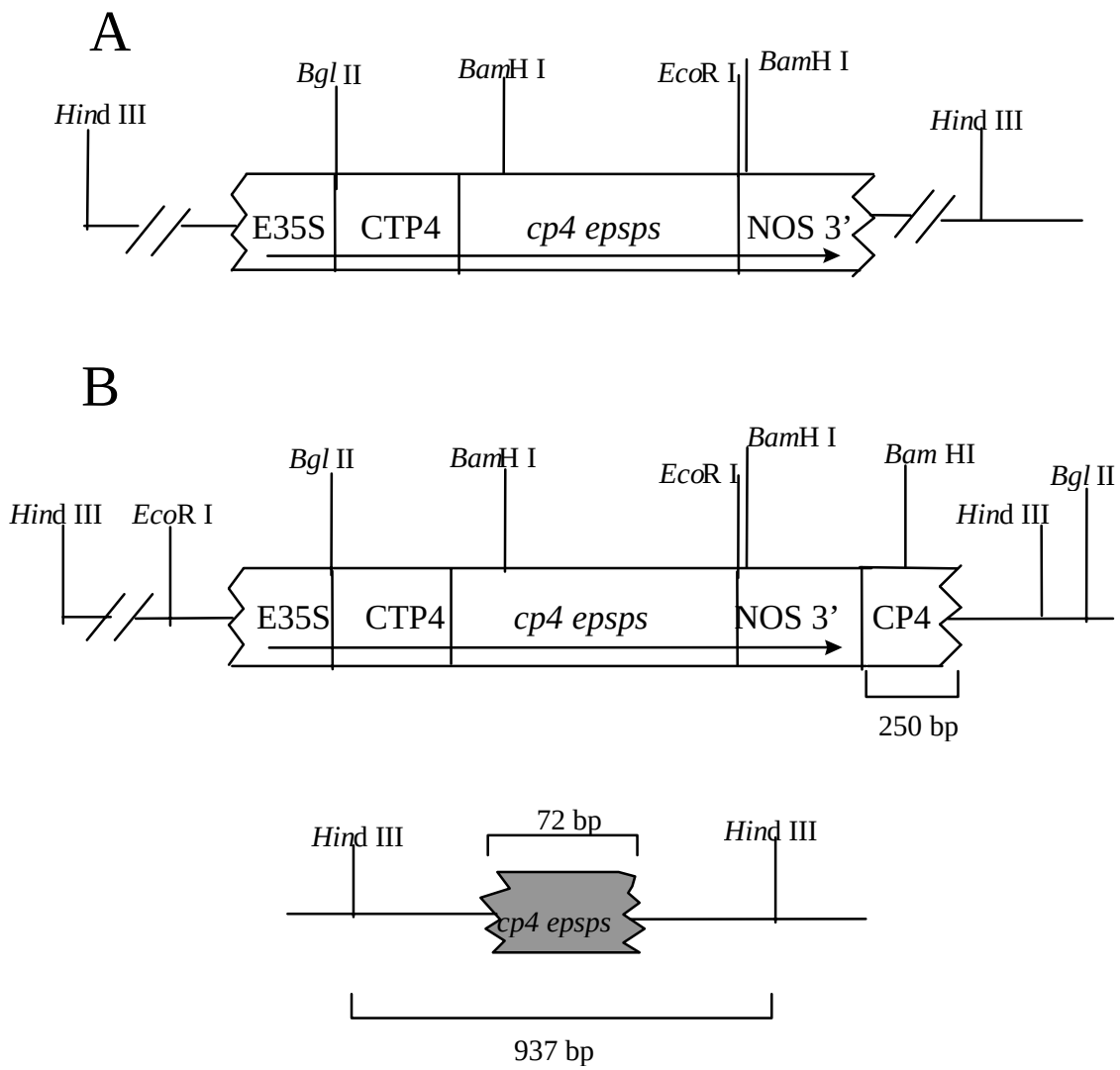

Figure 3 Inserted gene map of the soybean tolerant to glyphosate herbicide, 40-3-2

A: Typical view of the inserted gene based on the results of initial analysis

B: Typical view of the inserted gene based on newly obtained findings

(5). Methods of detection and identification of the living modified organism and their sensitivity and reliability

For the methods of detecting and identifying of this recombinant soybean, standard analytical methods are presently published in http://www.maff.go.jp/sogo_shokuryo/jas/manual00.htm.

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

i) It was confirmed by sprinkling glyphosate herbicide that the CP4 EPSPS protein encoded by *cp4 epsps* gene was expressed in this recombinant soybean.

ii) The differences between this recombinant soybean and the control non-recombinant soybean as the

recipient organism were examined mainly based on the results of the isolated field tests conducted by National Institute for Agro-Environmental Sciences from May 1995 to March 1996, but were comprehensively discussed also using the results of non-containment greenhouse tests conducted by Monsanto Japan Limited from March to December 1994.

a) Morphological and growth characteristics

The differences between this recombinant soybean and the control non-recombinant soybean in morphological and growth characteristics were investigated in isolated field tests in reference to 44 items (germination time, full germination time, germination rate, beginning of flowering, flowering time, full flowering time, end of flowering, leaf yellowing time, leaf falling time, pod yellowing time, maturing time, number of flowers, color of flowers, main stem length, number of main stem nodes, thickness of stem, number of branches, leaf color, leaf size, trichome color, trichome quantity, trichome length, shape of trichome, stiffness of trichome, total weight of subterranean part, elongation type, plant type, growth habit, difficulty in pod bursting, the lowest main stem node position of podding, the lowest main stem node height of podding, total weight of plant, weight of pod seeds per plant, number of ripe pods per plant, weight of ripe pods per plant, pod color, seed weight per plant, number of perfect seeds per plant, weight of perfect seeds per plant, number of seeds per pod, weight of 100 seeds, seed hull color, uniformity of seeds, hilum color). As a result, none of the items showed any difference between this recombinant soybean and the control non-recombinant soybean.

b) Chilling-tolerance and heat-tolerance at the early stage of growth

The seedlings of this recombinant soybean and the control non-recombinant soybean were grown in incubators respectively set at 10°C and 1°C, to examine thermal effect. As a result, at both the temperatures, this recombinant soybean and the control non-recombinant soybean were browned and died without forming new leaves.

c) Overwintering ability and summer survival of the matured plant

For this recombinant soybean and the control non-recombinant soybean, at harvesting time, the subterranean parts (including the first nodes of the aerial parts) of 14 individuals each of three replications were left as they were, and on December 11 corresponding to the 46^{th} day after harvesting, whether or not new buds were regenerated was examined. Furthermore, in plots (7 individuals each of three replications) in which they were not harvested at the harvesting time and were allowed to stand, the growth of their aerial parts was observed on December 11. As a result, the formation of new buds from the aerial parts after harvesting was not observed in either this recombinant soybean or the control non-recombinant soybean.

d) Fertility and size of the pollen

Pollen was sampled from this recombinant soybean and the control non-recombinant soybean, and the samples were stained with the Lugol solution (3% potassium iodide aqueous solution + 1% iodine), to observe their fertility and sizes under a microscope for investigation. As a result, the

fertility values of this recombinant soybean and the control non-recombinant soybean were 99%, showing no significant difference. Furthermore, no difference was observed either in size.

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

For the production of seeds, investigated were the differences between this recombinant soybean and the control non-recombinant soybean in the number of ripe pods per plant, weight of ripe pods per strain, weight of seeds per plant, number of perfect seeds per plant, weight of perfect seeds per plant, number of seeds per pod, and weight of 100 seeds as described in (i) Morphological and growth characteristics. As a result, in none of the items, any difference was observed between this recombinant soybean and the control non-recombinant soybean.

With regard to the shedding habit, two 11 cm x 17 cm and 10 cm deep pots were placed in each (three replications) of a plot for cultivating this recombinant soybean and a plot for cultivating the control non-recombinant soybean in such a manner that each pot was placed directly under each plant, in the period from about the bean thickening time (September 25) to one month (November 29) after the maturing time, to examine whether or seeds dropped. As a result, during the investigation period, no pod or seed was observed at all in either the pots of this recombinant soybean or the pots of the control non-recombinant soybean.

With regard to the dormancy and germination rate, the seeds harvested from this recombinant soybean and the control non-recombinant soybean were sown in greenhouses by 25 seeds per each of three replications, and on the 18^{th} day after sowing, the termination rates were investigated. As a result, between this recombinant soybean and the control non-recombinant soybean, no significant difference was observed in germination rate. The germination rates were more than 95%.

f) Crossability

Whether or not the natural crossing rate of this recombinant soybean with *Glycine soja* Sieb. and Zucc. is significantly higher than that with the control non-recombinant soybean was investigated.

At the center of each 1.4 m x 3 m crossing test plot (three replications) where 35 plants of either this recombinant soybean or the control non-recombinant soybean were cultivated at an inter-row spacing of 60 cm and an intra-row spacing of 20 cm, two pots each of *Glycine soja* (B01061 pedigree obtained from Faculty of Agriculture, Hokkaido University) regulated in flowering time in an air conditioning room were placed for growing in the period from the soybean flowering time (August 7) to flowering end time (August 18). The distances between *Glycine soja* and the adjacent *Glycine max* were 30 to 60 cm.

After completion of the natural crossing experiment, all the pots were recovered, and the seeds of *Glycine soja* were obtained in a greenhouse. Fifty individual *Glycine soja* seeds (selected at random from two pots) each of three replications were cultivated, and in the two-leaf stage, the incidence of the hybrid individuals with the *Glycine max* was investigated. For judging the hybrids, the results of the non-containment tests conducted for developing hybrids by artificial crossing were referred to, and the hybrid individuals among the *Glycine soja* cultivated in the non-recombinant

soybean plot were identified in reference to the shape of trichome, while those among the *Glycine soja* cultivated in the plot of this recombinant soybean were identified in reference to the susceptibility to glyphosate (Roundup 250 ml/10 a).

As a result, among the individuals germinated from the *Glycine soja* seeds cultivated in the plot of this recombinant soybean, no hybrid showing the tolerance to glyphosate was obtained. Similarly, among the individuals germinated from the *Glycine soja* seeds cultivated in the plot of the non-recombinant soybean, no individual showing crossing in terms of the characteristics of trichome was observed at all.

Meanwhile, the crossability between *Glycine max* and *Glycine soja* by means of wind pollination only was investigated when the non-containing greenhouse tests were conducted. Two individuals of this recombinant soybean or the control non-recombinant soybean were placed as a pollen source at 20 cm in front of a fan, and at points of 50 cm and 1 m on its extension, *Glycine soja* was disposed. For one month, wind with a velocity of 4 m/sec was blown, and seeds were obtained. From the obtained seeds, 100 seeds each were selected at random from this recombinant soybean and the non-recombinant soybean and grown in pots. In the three-leaf stage, crossability was confirmed. As a result, hybrids were not obtained from either the *Glycine soja* seeds grown at 50 cm or those at 1 m using this recombinant soybean or the control non-recombinant soybean as the pollen source.

g) Productivity of harmful substances

To confirm whether or not this recombinant soybean produces any substances affecting other plants and soil microbes, soil microflora tests were conducted in the isolated field tests, and soil microflora tests, succeeding crop tests and plowing-in tests were conducted in the non-containment greenhouse tests. However, in none of the items, any statistically significant difference was observed between this recombinant soybean and the control non-recombinant soybean.

Furthermore, in the isolated field tests, for confirming that this recombinant soybean does not produce any substances affecting insect species or other plants, the inspect species that came flying into the cultivation plot of this recombinant soybean and the cultivation plot of the control non-recombinant soybean and the weeds that were generated in those plots were compared, but the inspect species that came flying to the cultivation plot of this recombinant soybean and the numbers of individuals of the respective inspect species were not different from those found in the cultivation plot of the control non-recombinant soybean. Moreover, the weed species generated in the cultivation plot of this recombinant soybean and their plant lengths were also compared with those found in the cultivation plot of the control non-recombinant soybean, but on the averages of three replications, no difference was observed.

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 applied 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 soybeans (*Glycine max* (L.) Merr.), to which the recipient organism belongs, is cultivated for a long time in Japan, but there is no report that it grows voluntarily in Japan.

This recombinant soybean has the tolerance to glyphosate herbicide because of the transferred *cp4 epsps*, but it is hard to consider that glyphosate becomes a selection pressure in the natural environment. Existing documents also show that a recombinant plant tolerant to a herbicide works more dominantly than its control non-recombinant plant for the selection pressure in the natural environment. Furthermore, various traits relating to the competitiveness of this recombinant soybean were investigated in Japanese isolated fields, and no significant difference from the non-recombinant soybean was observed.

In view of the above, 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 *Glycine max* (L.) Merr. to which the recipient organism belongs, there is not report that it produces a substance harmful to wild animals and wild plants.

This recombinant soybean produces the CP4 EPSPS protein having the tolerance to glyphosate, but it is not reported that the protein is a harmful substance. Furthermore, the EPSPS protein is an enzyme that catalyzes the shikimate pathway where aromatic amino acids are synthesized. However, it is clarified that the EPSPS protein is not a rate-determining enzyme in the pathway, and actually it is confirmed that this recombinant soybean does not change the aromatic amino acid content. In addition, EPSPS is an enzyme that specifically reacts with phosphoenolpyruvate and shikimate-3-phosphate. Therefore, it is unlikely that the CP4 EPSPS protein catalyzes reactions of other substances to produce different substances.

In Japanese isolated field tests, the ability of this recombinant soybean to produce harmful substances (the substances secreted from the roots with a threat to affect other plants, the substances secreted from the roots with a threat to affect soil microbes, and the substances contained in the plant

bodies with a threat to affect other plants after they die) was investigated, and no significant difference between this recombinant soybean and the non-recombinant soybean was observed.

In view of the above, 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 the productivity of harmful substances is reasonable.

(3) Crossability

A. Identification of wildlife likely to be affected

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

B. Evaluation of concrete details of adverse effect

Existing documents do not show any obstacle to the growth and reproduction of the hybrid obtained from this recombinant soybean and *Glycine soja*. So, in the case where this recombinant soybean and *Glycine 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 *Glycine soja* diffuses among the group of *Glycine soja* without remaining at a low level.

C. Evaluation of likelifood of adverse effect

Glycine soja grows voluntarily and widely throughout Japan in sunny fields, on the roadsides and the like. So, in the case where this recombinant soybean is cultivated in Japan, it cannot be denied that there are chances where both grow closely to each other. However:

(i) Both *glycine max* and *Glycine soja* are typical autogamous plants engaged in cleistogamy, and the flowering time of *Glycine soja* is generally later than that of *Glycine max* (though both can also be the same in flowering time as the case may be).

(ii) Crossing by means of insect pollination can also occur. However, according to existing documents, even when *Glycine soja* was grown adjacently to *Glycine max* under such a condition that the *Glycine soja* pedigree and *Glycine max* pedigree flowered at the same time, the crossing rate was less 1%.

(iii) According to the results of isolated field tests in Japan, it can be considered that there is no difference between this recombinant soybean and the non-recombinant soybean in the crossing rate with *Glycine soja*.

(iv) When the hereditary relation between *Glycine max* and *Glycine soja* was analyzed using a chloroplast DNA marker, the *Glycine soja* that showed a pattern mainly seen in cultivated *Glycine max* accounted for 1.8% of the *Glycine soja* collected in Japan.

(v) According to existing documents, a recombinant plant tolerant to a herbicide does not work dominantly for the selection pressure under the natural condition compared with the control

non-recombinant plant. So, the possibility that this recombinant soybean tolerant to glyphosate herbicide works dominantly for the selection pressure under the natural environment is considered to be low.

Even in the case where this recombinant soybean grows near *Glycine soja* as a rare case, it cannot be considered that the probably that they cross with each other become higher than the probably that conventional *Glycine max* and *Glycine soja* cross with each other or that the possibility that the transferred gene diffuses among the group of *Glycine soja* under the Japanese natural environment without remaining at a low level become higher than the possibility that a gene diffuses from *Glycine max* into *Glycine soja*. The occurrences of such events are considered to be probabilistically very low. There was also an opinion that the information concerning the crossability between *Glycine soja* and *Glycine max* is too insufficient to conclude that the occurrences of such events are probabilistically low.

(4) Judgment of existence of Adverse Effect on Biological Diversity

As described above, the probability that this recombinant soybean and *Glycine soja* cross with each other is very low. Even if crossing occurs, it is hard to think that the hybrid produced from this recombinant soybean and *Glycine soja* ousts wild plants. Furthermore, the possibility that the transferred gene diffuses among the group of *Glycine soja* without remaining at a low level is also considered to be very low probabilistically. So, it cannot be considered that the crossability poses any significant risk of Adverse Effect on Biological Diversity. Meanwhile, there is also an opinion that the information concerning the crossability between *Glycine soja* and *Glycine max* is too insufficient to conclude that the possibility that the transferred gene diffuses among the group of *Glycine soja* without remaining at a low level is probabilistically low.

2. Conclusion

Based on the above discussion, it was judged that the conclusion of the Biological Diversity Risk Assessment Report that in the case where this recombinant soybean is used according to the Type 1 Use Regulation, there is no significant risk of Adverse Effect on Biological Diversity is reasonable.

The possibility that the transferred gene spreads among the group of *Glycine soja* due to the crossing between this recombinant soybean and *Glycine soja* is considered to be very low probabilistically. However, from the viewpoint of further enriching scientific findings, it is considered necessary to collect the following information concerning the situation in which the gene transferred in the *Glycine max* diffuses into the *Glycine soja* existing near the places where this recombinant soybean is cultivated.

(i) Whether or not the gene tolerant to the herbicide is transferred from the recombinant soybean into *Glycine soja* due to crossing.

(ii) Behavior of the gene tolerant to the herbicide among the group of *Glycine soja* in the case where the transfer is observed.

There is also an opinion that the information necessary for considering the conservation of *Glycine*

soja such as the geographical genetic variations and distribution pattern of *Glycine soja* is insufficient, and that they could not conclude for the time being that the cultivation of this recombinant soybean does not pose a significant risk of Adverse Effect on Biological Diversity.

Requests from the Committee for Discussing Biological Diversity Risk Assessment

The committee requests that the public organization concerned should collect the information on the following items relating to the crossability between *Glycine max* and *Glycine soja* and the fitness of the progeny of the hybrid produced from *Glycine max* and *Glycine soja*, from the viewpoint of further enriching scientific findings.

(i) Crossing rate between *Glycine max* and *Glycine soja* under the natural environment

(ii) Fitness of the progeny of the hybrid obtained from *Glycine max* and *Glycine soja*

(iii) Natural crossing rate between this recombinant soybean and *Glycine soja* and the fineness of this recombination gene in the progeny of the hybrid

(iv) Geographical genetic variation and the like of *Glycine soja*

(v) Preparation of a model concerning the behavior of the gene based on (i) through (iv)

Note: Based on the above-mentioned opinion of Experts, the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment request that the corporation obtaining the approval should collect the following information, when they cultivate this recombinant soybean, based on the provision of Section 6, Subsection 2 of the CARTAGENA law (Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms).

(i) The name of the place where information is collected, and how *Glycine soja* grows in the place

(ii) Whether or not the herbicide-tolerant gene exists in the seeds of *Glycine soja* confirmed in (i)

(iii) Period of information collection

(iv) Information collection period, frequency and other information collection methods

(v) Other necessary matters

Furthermore, with regard to the fitness of the progeny of the hybrid obtained from *Glycine max* and *Glycine soja*, etc., it is planned to pursue research in the independent administrative institution concerned, etc.