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	Alfalfa tolerant to glyphosate herbicide (*cp4 epsps*, *Medicago sativa* L.) (J163, OECD UI: MON-ØØ163-7)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

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

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

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

i) The common name: Alfalfa, Lucerne
The scientific name: *Medicago sativa* L.

ii) The recipient organism is the R2336 line of the breeding maternal line group of Alfalfa (*Medicago sativa* L.*)* which belongs to the genus *Medicago*, a perennial legume, and it belongs to *M. sativa* subsp. *sativa*.

iii) The origin of Alfalfa (*Medicago sativa* L.) is considered to be the areas, Asia Minor, Transcaucasus, Trukmenistan and Iran. Then it spread out to the Mediterranian area, North Africa, Middle East, Europe, Siberia, Northern India and China, where it became rooted.

Alfalfa (*Medicago sativa* L.) is a species composed of several subspecies which can easily cross with each other and have the same karyotype. The subspecies are classified according to the color of flower and the shape of seedpod. Most of Alfalfa cultivated worldwide is classified as tetraploid *M. sativa* L. subsp. *sativa* (violet-flower Alfalfa). The diploid and tetraploid *M. sativa* L. subsp. *falcata* L. (yellow-flower Alfalfa), yellow-flower varieties of Alfalfa, are used for selective breeding of *M. sativa* L. subsp. *sativa* to provide cold-tolerant and drought-tolerant genetic resources, and also cultivated on a small scale primarily in grazing lands in some cold climate areas (Canada and Siberia). Another subspecies which belongs to *M. sativa* L. includes subsp. *glutinosa*. In addtition, the crossbreds produced by crossing of the above mentioned three subspecies to various extents (including *M. sativa* L. subsp. x *varia*, popularly known as Alfalfa of variegated colors, a cross between subsp. *sativa* and subsp. *falcata* L.) are also classifed into *Medicago sativa* L.

In some classification methods for Alfalfa, *M. sativa* L. *falcata L.* and *M. sativa* L. x *varia* (also known as *M. media* Pers.) are defined as in a different species from that of *M. sativa* L., though they are treated as subspecies in recent years due to the lack of definite difference in the reproductive isolation function between them and *M. sativa* L. *sativa*.

Alfalfa is known as one of the oldest grazing crops which has been cultivated from prehistoric times. The cultivars of Alfalfa originated from several lines. One of the two major lines spread out from the highlands in Asia Minor and Transcaucasus to Europe and North Africa to become the origin of Modern European type *M. sativa* L. subsp. *sativa*. The other line is the native Central Asian *M. sativa* L. subsp. *sativa* which is used as gene resources resistant to clavibacter wilt disease and stem Nematoda, and the others. As such, the two lines of *M. sativa* L. subsp. *sativa* in a

limited range of areas are reportedly the basic origin of Alfalfa.

M. sativa L. subsp. *falcata* L. is also considered to hail from Central Asia, and it is spread from the northmost part of Siberia to Europe, offering the cold-tolerant feature. This has been playing an important role as a key cold-tolerant genetic resouce in forming the modern Alfalfa. In the US, since the introduction in 1850 as grazing crop, Alfalfa has been cultivated in much larger areas than in Japan, though it is not included in the Problem Weeds List.

In Japan, Alfalfa was introduced as grazing crop in the early years of Meiji era and then it was reportedly spread and became wild in the flat lands and the roadsides and grasslands in low-mountainous regions across the country. The growth has been actually observed along the roadsides, in vacant lands, and/or orchards in Asahikawa, Akita, Shizuoka, Mie, Kobe, Tokushima, Saga, and Oita. A typical route of penetration of the wild Alfalfa includes mixing in the agricultural materials (the seeds of flowering plants and herbs) imported for roadside and recreation ground maintenance and improvement and tree-planting programs, and the others.

In contrast to this, however, as a result of aggregation of information about the wild-growing genus *Medicago* plants carreid out through cooperation between Gifu Universtity, National Grassland Research Institute of the Ministry of Agriculture, Forestry and Fisheries of Japan, and Aichi-ken Agricultural Research Center, only four sepcies were found growing wild in Japan, *M.polymorpha*, *M.lupulina*, *M.arabica* and *M.minima*, which are all annual, inbreeding plants except the species found growing near the trade port, and no self-seeding Alfalfa was observed.

Based on the above understanding, it is considered that the Alfalfa growing areas in Japan are scattered all over the country and the size of individual populations is not so large, since some reports negate possible self-seeding Alfalfa depending on the investigaitional criteria, though the other reports present self-seeding Alfalfa in various parts of Japan.

(2) History and present state of Use

i) Alfalfa has the longest history of cultivation among the grazing crops. It is rich in protein , calcium and other minerals, and highly plantable to cattle and then it is also referred to as "Queen of Forage Crops." It was discovered in archeological remains in Turkey that Alfalfa was used for livestock feed in BC 1400 to 1200. It was brought into Greece in BC 400, Rome in BC 200, and China in BC 126 through the Turkestan district in Russia. Then, Afalfa became widespread in Europe and North Africa by 18th Century, and then it was introduced form Europe into South and North Americas, Aurstralia and New Zealand from 18th Century. To Japan, it was reportedly introduced in the years from 1716 to 1861 from China, though the practical cultivation started in 1874 in response to the introduction into Hokkaido from US and the widespread use was started after the Second World War (1945-).

In the US, since the start of independent raising of Alfalfa or combined raising with Poaceae grazing crops in 1997, Alfalfa has been cultivated in a total of 23 million acres (appox. 9.3 million hectares) or more mainly in the 13 States including California, Colorado, Idaho, Iowa, Kansas, Michigan, Minnesota, Montana,

Nebraska, North Dakota, Pennsylvania, South Dakota and Wisconsin.

ii) In Japan, Alfalfa is now cultivated primarily in Hokkaido, though it offers weak competition to weeds and then the growing lands are susceptible to penetration of weeds of various types. For this reason, proper provisions against weeds are needed before seeding and throughout the growing period. However, in practice, it is difficult to completely eliminate the peneration of weeds. In addtion, Alfalfa is not suited for the hot, moist environment in Japan due to the origin in those areas of least precipitation, which provides higher drought tolerance though lower moisture tolerance. Because of such difficuly in cultivation, Alfalfa is not so much spread in Japan and the total cultivation area is limited to around 9,000 hectares.

The stem and leaf of Alfalfa is rich in protein, vitamin and calcium, and the crops are utilized primarily as forage for dairy cattle in the form of hay, cubes, or meal (crushed hay). In addition, Alfalfa sprout is used as food. The seeds of Alfalfa for grazing crop and sprout are significantly different in the unit price, and the seeds for sprout are cultivated under contract and produced, distributed and introduced in different market from that for the seeds for grazing crop.

Japan imports a total of about 85 ton seeds for domestic cultivation and other applications from the US (about 80 ton), France (about 4 ton) and Australia (about 1 ton). In Japan, Alfalfa is in great demand for grazing crop, and about 200,000 ton of Alfalfa meal and pellet was imported in 2000, which corresponds to the cultivation area of 400,000 hectares. Top four exporting countries are Canada (about 150,000 ton), Netherlands (about 27,000 ton), the US (about 15,000 ton), and Italy (about 9,000 ton).

Alfalfa is allogamous and it casues inbreeding depression when genetically fixed through inbreeding. For this reason, commercial cultivars use synthetic varieties obtained from the crossing of several genetically superior lines in the fileds. As a result, genetically fixed culitvars are not available.

The customary method for cultivation of Alfalfa in Japan is as follows: The seeds are typically sown in spring in the cold-weather district Hokkaido or autumn in the other warm-weather districts. The amount of seeding is 1.5 to 2.5 kg/10a for independent planting of Alfalfa, or 1.0 to 1.5 kg/10a for mixed planting with Poaceae garzing crops. As the base manure, 5kg nitrogen, 20 to 30 kg phosphoric acid and 10 kg potassium are applied per 10a. For cultivation of Alfalfa, protection against weeds is a major issue and thus, consideration must be given to use the fields which are less susceptible to weeds and get rid of weeds in the stage of raising the previous crops. Harvesting is available 2 to 3 times in Hokkaido per year and 4 to 8 times in the Kanto District and westward. Harvesting has been carried out conventionally before 10% flowering, though, in recent years, earlier harvesting is recommended.

(3) Physiological and ecological properties

i) Basic properties

Alfalfa is a perennial dicotyledon that reproduces by seed propagation. The leaves are arranged alternately on the stem, having 3 leaflets. It grows to a plant height of 50 to 150 cm, having 5 to 25 stems per individual, and it provides 50 or more stems in any big, vigourously growing plants which stand straight from the crown of the bottom of stem.

ii) Environmental conditions allowing inhabiting or growth

At present, distribution and cultivation of Alfalfa are almost limited to the subtropical to temperate zones in the ragne between latitude 30° to 60° north and latitude 20° to 45° south at an average temperature of -12℃ to +10 in the winter isothermal line and +16 to +27 in the summer. The amount of rainfall in the major cultivation areas of Alfalfa ranges from 250 to 1,000 mm. With respect to the latitudes and the average temperature, Japan falls within the ditribution range of Alfalfa, though the average precipitation in Japan is 1,000 to 2,000 mm, and there is no area worldwide in the cutlivation areas of Alfalfa having such a great deal of rain as in Japan. Alfalfa is a deep-rooted plant and thus, it is not sutied for cultivaltion in wetlands and other dead-water areas but it prefers well-drained lands. In addition, Alfalfa is weak in competition with weeds and then proper lands must be selected that are less susceptible to propagation of weeds. Among the grazing crops, Alfalfa prefers the most fertile soil with the optimum soil pH of 6.5 to 7.0 near the neutral and dislikes any acid soil. The optimum timing for seeding is considered at a ten-days average temperature of 9℃ to 11 for the seeding in spring and around 20 for the seeding in autumn.

Based on the above understanding, it is considered that there is little likelihood of that the individuals growing and becoming weeds under the natural conditions in Japan increase and the distribution expand. In fact, there has been no report showing Alfalfa became a problem weed in Japan.

iii) Mode of propagation or reproduction

a) The seedpod is less likely to split open. Ripe seeds often form an impermeable seed coat which prevents absorption of water content, in which case they can survive in the soil for several years.

b) Alfalfa hardly forms any creeping stem or underground stem, by which, thus, the roots cannot grow. A plant body forms a crown after it is reaped or the above ground part withers and dies to survive the winter, and in the next year, a new shoot regrows from the crown. The highly cold-tolerant cultivars show higher dormancy in the autumn season and tend to cease growing immdiately in the shorter-day and lower-temperature conditions in autumn to prepare for overwintering.

c) Alfalfa is an allogamous plant having a higher self-incompatibility, and the seeds are formed by insect pollination with the flower bee, leaf-cutting bee and

honey bee primarily serving for transmission of pollens. The time of flower initiation is May to June. By the insect visiting and nectar-sucking, the style in the carina turns inside out and becomes exposed, allowing the stigma to hit against the vexillum and the insect body. This stimulus causes the stigma to induce the capability of pollination. This is a mechanism to ensure the cross fertilization, known as tripping. In Japan, however, Alfalfa has a limited number of visiting insects, and the varieties of insect serving the tripping at a high efficiency are limited even they visit Alfalfa, this resulting in lower efficiency of pollination. In the sites in Japan for breeding and seed production of Alfalfa, Alfalfa leaf-cutting bees and other insects ensuring higher efficiency of pollination are intentionally set free in the fields to produce the seeds.

Relatives considered to be able to cross with Alfalfa (*M. sativa* L.) under natural conditions include two species, *M. prostrata* and *M. glomerata*, which are not present in Japan.

For reference, there are eight wild relatives of Alfalfa growing naturally in Japan; *M. polymorpha*, growing along the roadsides on the coasts or in the flatlands across the country, *M. laciniata* L. (also known as *M. polymorpha* L. var. *laciniata*) and *M. ciliaris* L. (also known as *M. polymorpha* L. var. *ciliaris* L.), growing in any vacant lots in the flatlands, *M. orbicularis* (also known as *M. polymorpha* L. var. *orbicularis* L.), growing in any vacant lots near the coasts, *M. lupulina*, growing along the roadsides or in the lawn on the coasts or in the flatlands across the country, *M. arabica*, discovered infrequently in the western Japan, *M. minima*, growing relatively infrequently in Honshu, and *M. truncatula*, discovered first in 1995 in Japan, which are all annual plants. Among these, two species *M. polymorpha* and *M. lupulina* were introduced before the Meiji era (1868).

However, between the annual and perennial specieis of the genus *Medicago*, artificial cross-fertilization cannot be attained. In addition, also in nature, no crossing between the both species has been observed. As a result, Alfalfa and the annual genus *Medicago* are considered genetic imcompatible. Then questions were asked to the experts of Alfalfa in the fields of inheritance, classification and breeding of the genus *Medicago* as to the possibility of crossability between the annual genus *Medicago* and Alfalfa under natural conditions. According to the answers from the experts, there is little likelihood of crossability between the perennial genus *Medicago* and the annual genus *Medicago* under natural conditions and there is no successful crossing despite of the efforts by a number of researchers over a long period of time.

In fact, for crossability between Alfalfa and *M. polymorpha* L., it has been observed that the pollen tube fails to grow even if the stigma of *M. polmorpha* L. is pollinated with the pollens of Alfalfa.

In addition, for crossability between Alfalfa (*M. sativa* L.) and *M. lupulina*, there are some reports in the past literature addressing the individuals considered the crossing of these species, though there is no follow-up report published by the same researchers because the plant body is completely sterile and it fails to produce any seed. The experts suspect that the plant body

addressed in the literature really came from crossing since the plant body had not been tested for crossing using the molecular marker. According to the follow-up tests by the experts, crossing could not be successfully repeated.

Morever, according to the reports, the stigma of Alfalfa was pollinated with eight species (incldluing *M. lupulina* and *M. scutellata*) which are considered highly genetic incompatible with the genus *Medicago*, after which the process of development of embryo was examined. For *M. lupulina*, the initiation of development of embryo was only observed, though normal embryo could not be formed in all crossing cases including *M. lupulina*. As a result, no evidence was obtained for fertilization with *M. lupulina*.

Based on the above understanding, it is considered that there is no possibility of crossing with *M. polymorpha* and *M. lupulina* and there exists no wild relative in Japan which can cross with Alfalfa under evaluation.

d) The pollen is spherical in shape, having a diameter of about 32μm, and about 2,500 pollens are produced per flower. The longevity of the pollen is about one hour. For the dispersion distance of pollen, test was carried out in the US on the traditional Alfalfa, using the the glutamine synthase (GS) gene marker and the RAPD marker which can distinguish between autogamy and allogamy. As a result of the measurement using the GS marker in a small-scale experimental field containing the Alfalfa as the pollen source of 1m in diameter (<0.1 m^2), the rate of natural crossing was found 0.2% at a distance of 4m and 0% at 6m or more. On the other hand, as a result of measurement using the RAPD marker for the rate of natural crossing at a total of 12 crossing checking zones (1m diameter) installed at distances (0, 20, 40, 60, 80, 100, 200, 300, 400, 500, 750, 1000m) from a pollen source of typical field scale (around 10,000 m^2), a higher rate of crossing of 25 to 35% was observed even at the most distant point of 1000m. This higher rate of crossing may be explained by the findings that (1) the strain used in the individual crossing checking zones is a genetic clone obtained by vegetation propagation and thus it is difficult to form any self-fertilization seeds due to the strong self-incompatibility of Alfalfa by nature, (2) as a result, rate of allogamous seeds increases among a small number of fertile seeds (the number of obtained seeds is not reported in the literature), and (3) there is a possibility of crossing due to any other pollens than those from the selected pollen source, and the others. Based on the above understanding, it is judged that this test allows examination of maximum dispersion distance of pollen of Alfalfa under natural conditions, though it can overestimate the rate of crossing compared to that under natural conditions.

In the US, using this recombinant Alfalfa, investigation was made to determine the rate of natural crossing with the traditional Alfalfa. In this test, the rate of crossing between this recombinat Alfalfa and traditional Alfalfa was determined at a total of four crossing checking zones (0.03 acres each) with traditional Alfalafa selected at proper distances from one acre (4,047 m^2) of cultivation zone of this recombinant Alfalfa taken as pollen source. As a result, the rate of natural crossing between this recombinant Alfalfa and traditional Alfalfa was found 1.39% at a distance of 500 ft. (about 174 m) from the pollen source, 0.32% at 1,000 ft. (about 348 m), 0.07% at 1,500 ft. (about 522 m), and 0% at

2,000 ft. (about 610 m).

iv) Productivity of harmful substances

It is known that Alfalfa releases water-soluble allelochemicals which can inhibit the growth of Alfalfa itself and other plants from the stems and leaves, roots and other plant tissues. Ramish *et al.* has reported that, in the soil plowed with only the roots of Alfalfa or with the roots and above ground part, the germinating rate, plant height and plant weight of Alfalfa and Sorghum are decreased and also that the water-soluble substances from young seedlings inhibit the germination and root growth of Alfalfa and Sorghum. In addition, Kawada *et al.* fractionated the hydrophobic or semi-hydrophobic organic substances collected from the exudate from the roots of Alfalfa cultivated in the water culture into acid, neutral and base. As a result, it was reported that the neutral fraction inhibits the germination of lettuces greatest among the fractions and the neutral fraction also inhibits the germination and the growth of young roots of 4 kinds of field crops and 6 kinds of grazing crops though the degree of inhibition varies between different plants. However, no allelochemical for Alfalfa has been identified so far.

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of Alfalfa tolerant to glyphosate herbicide (*cp4 epsps*, *Medicago sativa* L.) (J163, OECD UI No : MON-ØØ163-7) (hereafter referred to as "this recombinant Alfalfa") are shown in Table 1.

ii) Functions of component elements

Functions of component elements of donor nucleic acid that was used for the development of this recombinant Alfalfa are shown in Table 1.

(Modified *cp4 epsps* gene)

a) Glyphosate is the active ingredient in 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. As a result, 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 Alfalfa, modified *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 modified *cp4 epsps* gene is not inhibited even under the presence of glyphosate, thus, the recombinant plants that express this protein have normal functions of shikimate systhesis and grow normally.

EPSPS is one of the enzymes that catalyze the shikimate pathway for

biosynthesizing the aromatic amino acids specific for plants and microbes, and is located 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. 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 in this pathway, 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 end 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 specfically react with these substances. 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 share structually related homologous sequences with any of the known allergens examined.

Table 1 Origins and functions of the component elements of plasmid PV-MSHT4 used for the development of this recombinant Alfalfa

Component DNA	Origin and function
Right Border (RB)	A DNA fragment containing right border sequence (24bp) of nopaline type T-DNA derived from Ti plasmid pTiT37. The right border sequence is used as the starting point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome.
P-eFMV	35S promoter of duplication enhancer, derived from *Figwort mosaic virus* (FMV). Involved in the constant expression of the target gene in the entire tissue of plant body. FMV is a virus not developed in Japan so far, though there is no report indicating any virus closely related to FMV makes the genus *Medicago* to which Alfalfa belongs as the recipient organism. As a result, it is unlikely that the recombination could cause development of any new virus.
HSP70-Leader	5' untranslated leader sequence of petunia hsp70 (heat shock protein) gene. Used to enhance the expression of introduced genes in plants.
CTP2	A chloroplast transit peptide derived from *Arabidopsis* EPSPS to transport the CP4EPSPS protein to the chloroplast which synthesizes the aromatic amino acid. Transports the target protein to chloroplast from cytoplasm.
Modified *cp4 epsps*	*epsps* gene of *Agrobacterium* sp. CP4 strain
E9 3’	3' untranslated region of ribulose-1, 5-bisphosphate carboxylase E9 gene of pea (*Pisum sativum*). Terminates transcription of mRNA and induces polyadenylation.
Left Border (LB)	A DNA fragment containing left border sequence (25bp) derived from Ti plasmid pTiA6. The left border sequence is used as the end point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome.
(Component elements outside T-DNA)	
ori-V	The replication origin region derived from broad-recipient organism range RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens* ABI strain.
ori-322/ROP	The replication origin region isolated from pBR322, a plasmid derived form *E. coli*. Permits autonomous replication of vectors in *E. coli*. This region contains not only replication origin but also *rop* region that is involved in the regulation of the replication, and *oriT* sequence that is necessary for conjugal transfer from *E. coli* to *Agrobacterium tumefaciens*.
aad	A gene encoding 3”(9)-0-aminoglycoside adenyltransferase (AAD) derived from *Staphylococcus aureus*. Confers resistance to spectinomycin and streptomycin.

(2) Information concerning vector

i) Name and origin

The plasmid vector PV-MSHT4 used to generate this recombinant Alfalfa is assembled from plasmids inclduing pBR322, which is a synthetic plasmid vector derived from *Escherichia coli* (*E. coli*).

ii) Properties

The total number of base pairs of PV-MSHT4 used to generate this recombinant Alfalfa is 9,023bp. As a selective marker gene of the vector assemled in *E. coli*, the *aad* gene derived from *E. coli* transposon Tn7 is present outside the T-DNA region, which confers resistance to spectinomycin and streptomycin.

The infectivity of this vector is not known.

(3) Method of preparing living modified organisms

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

The plasmid vector PV-MSHT4 used to generate this recombinant Alfalfa is assembled from plasmids including pBR322, which is a synthetic plasmid vector derived from *E. coli*, containing the modified *cp4 epsps* gene expression cassette ([P-eFMV]-[HSP70-Leader]-[CTP2]-[modified *cp4 epsps*]-[E9 3']) (see Table 1 and Figure 1).

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

The T-DNA region of the plasmid PV-MSHT4 was introduced into the breeding maternal group, R2336 line, by the Agrobacterium method.

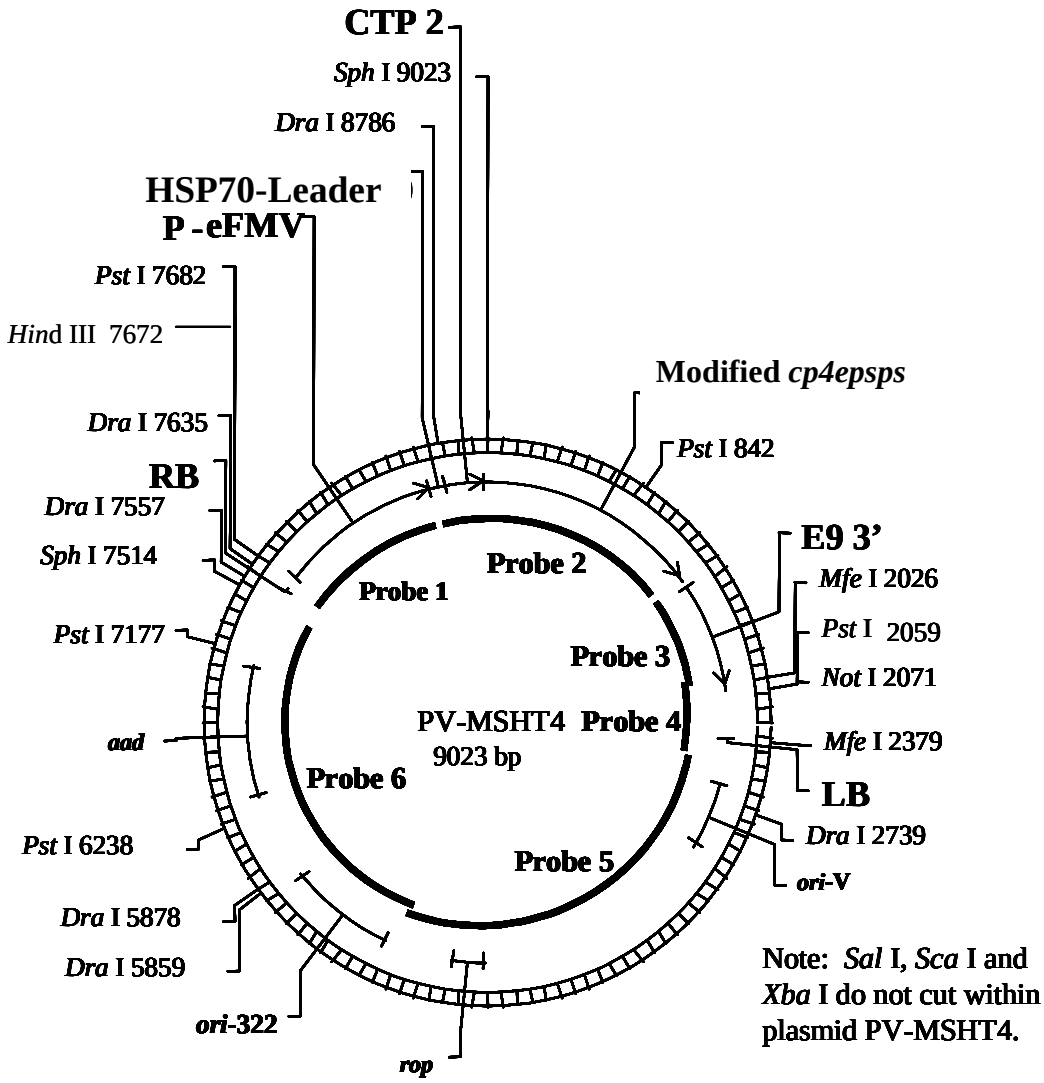

Figure 1 Plasmid map of PV-MSHT4

The T-DNA region introduced into this recombinant Alfalfa is from RB to LB in the above map in the clockwise direction.

iii) Processes of rearing of living modified organisms

T-DNA region in the plasmid vector PV-MSHT4 was introducted into tissue section of R2336 line by the Agrobacterium method, then transferred to the tissue culture medium including carbenicillin and cefotaxime to dezymotize *A. tumefaciens* ABI strain, and finally placed onto the medium added with glyphosate to regenerate the plant body from growing callus tissue. In this process, it was confirmed that there remains no residual Agrobacterium. From the regenerated individuals (referred to as T_0 generation), the glyphosate-tolerant 52 lines were selected by confirming the introduced gene based on the glyphosate tolerance testing and Southern blotting analysis. Then field tests were carried out from 1999 at a total of 70 sites in US, Canada and Argentine. Based on the test results, pedigree selection was started to rear excellent maternal line individual group, and finally this recombinant Alfalfa J101 line was selected as the excellent line for production of commercial cultivars. In practice, only the progeny raised through cross-breeding between J101 line and glyphosate-tolerant Alfalfa J163 line is to be commercialized, and the J101 line alone is never commercialized.

Application for approval for use as food was submitted to the Ministry of Health, Labour and Welfare in August 2004 and it is under examination. Application for approval for use as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries in August 2004.

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

i) Place where the replication product of transferred nucleic acid

The replication product of transferred nucleic acid is located on the chromosome of the recombinant plant.

ii) The number of copies of replication product of transferred nucleic acid and the stability of transferring in multiple generations

As a result of Southern blotting analysis on the inserted gene, it was confirmed that one copy of T-DNA region is inserted at one site on the chromosome of this recombinant Alfalfa. It was also confirmed that no other fragment than T-DNA region is inserted and the modified *cp4 epsps* gene expression cassette in T-DNA is inserted in the intact form. Southern blotting analyses on multiple generations revealed that the inserted genes are stably inherited in posterity.

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

This item is not applicable due to the one copy.

iv) The stability of the expression among individuals and generations under natural conditions

The stability of the expression of CP4 EPSPS protein in this recombinant Alfalfa has been evaluated based on the tolerance to glyphosate herbicide in multiple

generations.

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

For generation of this recombinant Alfalfa, Agrobacterium method is used, though it is confirmed that there is no residual Agrobacterium. As a result, there is no risk that any DNA fragment can be transmitted to wild animals and wild plants.

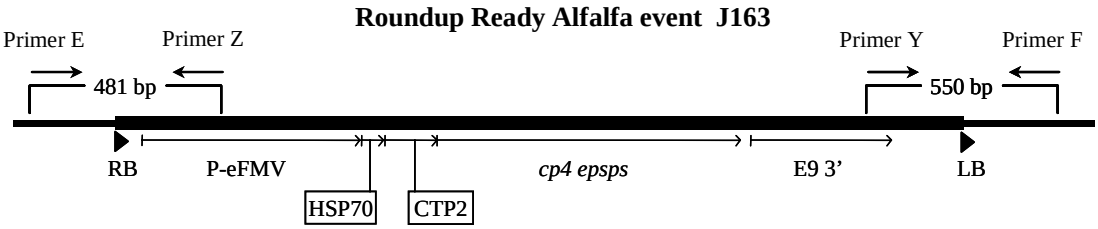

Figure 2 Inserted gene map of this recombinant Alfalfa J163

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

For the methods for detection and identification of this recombinant Alfalfa, a qualitative PCR method has been developed where the DNA sequences of the inserted genes and neighboring areas of plant genome are used as primers (primers E, Z, Y and F in Figure 2). This method makes it possible to sepcifically detect this recombinant Alfalfa.

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

i) By the expression of CP4 EPSPS protein encoded by the modified *cp4 epsps* gene in this recombinant Alfalfa, tolerance to glyphosate herbicide is conferred.

ii) Differences between this recombinant Alfalfa and Null type Alfalfa were examined based on the results of isolated field tests conducted from July 2002 to February 2004 at National Agricultural Research Center for Hokkaido Region (NARCH). The Null type Alfalfa refers to a group of glyphosate-sensitive individuals obtained in the process of development of this recombinant Alfalfa by segregating the modified *cp4 epsps* gene from BC2 generation. For the Null type Alfalfa, it was confirmed for every individual based on the ELISA method that CP4 EPSPS protein is not expressed, based on the PCR method that the modified *cp4 epsps* gene is not introduced, and based on the Southern blotting method that any DNA fragment derived from plasmid is not inserted.

Due to the property of inbreeding depression inherent in Alfalfa, cross-breeding with several groups of excellent individuals of conventioal species has been conducted in the process of development and maintenance of this recombinant Alfalfa. As a reulst, there exists no control species which possesses any equivalent genetic background as this recombinant Alfalfa. Then, for the control species for

the test, the Null type Alfalfa in the same generation as this recombinant Alfalfa under the test was selected rather than the recipient organsim species used for the gene introduction.

Since it has not been clarified whether this recombinant Alfalfa can grow under the natural conditions in Japan, two species, Makiwakaba and Rambler, were also tested as reference species. Makiwakaba is one of the Alfalfa cultivars developed by the NARCH to accommodate the environmental condition in Hokkaido. On the other hand, Rambler is a typical cultivar in the US, though it is reportedly less adaptable to the environmental condition in Hokkaido.

a) Morphological and growth characteristics

For the morphological and growth characteristics, evaluaion was made on the following items: 6 items for the first year of cultivation (uniformity of germination, germinating rate, leaf color, plant height at harvesting, weight of above ground part, and plant height at regeneration in autumn), and 13 items for the second year of cultivation {cold-tolerance (rate of lost roots), cold-tolerance (strength of plant in early spring), plant type, number of stems, flowering time, the color of flower, plant height at flowering time, number of effective flower buds, extent of seedpod splitting, weight of above ground part, number of seeds per seedpod, weight of 1,000 seeds}. As a result, a statistically significant difference was observed in the plant height at regeneration in autumn for the first year of cultivation between this recombinant Alfalfa and the control Null type Alfalfa, though no specific difference was found in the other items.

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

Chilling-tolerance and heat-tolerance at the early stage of growth were not evaluated due to the facts that no difference was observed in wintering ability between this recombinant Alfalfa and the control Null type Alfalfa with regard to the mature plants as mentioned in the following section and that Alfalfa is perennial by nature and this recombinant Alfalfa is found to be able to overwinter and survive summer in the field tests in the US and no difference is observed in the perennial property between this recombinant Alfalfa and conventional Alfalfa.

c) Wintering ability and summer survival of the matured plant

For this recombinant Alfalfa and the control Null type Alfalfa, wintering ability was evaluated by investigating the dormancy in autumn of plants regenetated after surviving summer (investigating the plant height of plants regenerated after harveting in autumn) and the rate of lost roots, beginning of germination, and strength of plant in early spring in the second year of cultivation. As a result, a statistically significant difference was observed in the plant height at revegetation in autumn between this recombinant Alfalfa and the control Null type Alfalfa, though no specific difference was found in the other items. Therefore, it is considered that there is no difference in wintering ability between this recombinant Alfalfa and the control Null type Alfalfa, even if this recombinant Alfalfa may be lower cold-tolerance than the control Null type

Alfalfa. In addition, based on the findings that no difference was observed in the weight of above ground part at harvesting time between the recombinant Alfalfa and the control Null type Alfalfa, it is considered that there is no difference in the summer survival between this recombinant Alfalfa and the control Null type Alfalfa.

d) Fertility and size of the pollen

Flowers of this recombinant Alfalfa and the control Null type Alfalfa at flowering time were sampled, and the pollens were stained with the potassium iodide aqueous solution to examine their fertility and sizes. As a result, no statistically significant difference was found in the fertility of pollens between this recombinant Alfalfa and the Null type Alfalfa. In addition, no difference was observed in shape and size of pollens between them.

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

In the isolated field tests, insect screens had to be installed during the flowering period to protect the testing fields against invasion of pollen-transmitting insects for preventing possible crossing with any Alfalfa cultivars outside the isolated field. For this reason, for the production of seeds, evaluation was made by investigating the number of blooming flowers, the number of seeds per seedpod obtained by the artificial cross pollination and artificial self-pollination in a given plot, and the weight of 1,000 seeds. As mentioned in a) Morphological and growth characteristics, no statistically significant difference was observed in the number of blooming flowers, number of seeds per seedpod, and weight of 1,000 seeds between this recombinant Alfalfa and the control Null type Alfalfa. In addition, it is found that the number of seeds per seedpod is smaller in the self-fertilization compared to the cross-fertilization and also there is no difference in the self-incompatibility.

For the shedding habit, seedpods of this recombinant Alfalfa and the Null type Alfalfa were taken hold of with a hand at harvest time after seed set to evaluate the ease of splitting of seedpods. As a result, no split seedpod was found both in this recombinant Alfalfa and the control Null type Alfalfa, and no difference was observed between them.

With regard to the dormancy and germinating rate, the seeds harvested from this recombinant Alfalfa and the control Null type Alfalfa were put in a Petri dish containing dampened filter paper, 30 seeds per each of three replications, which is placed in an incubator maintained at around 25 to investigate the germinating rate. In addition, the germinating rate was also investigated in the similar manner for the harvested seeds which had the water-resistant seed coat damaged since the matured seeds of Alfalfa often form impermeable seed coat preventing water absorption. As a result, for both the "as is" seeds and the seeds having the seed coat damaged, no statistically significant difference was observed in germinating rate between this recombinant Alfalfa and the control Null type Alfalfa. It was also confirmed that the seeds which failed to germinate in the germination test for those having the seed coat damaged became all rotten in both this recombinant Alfalfa and the control Null type Alfalfa and left out

from dormancy. Therefore, also for the dormancy of seeds, it is considered that there is no difference between this recombinant Alfalfa and the control Null type Alfalfa.

f) Crossability

As mentioned earlier in (3)-iii) –c), there exists no wild relative in Japan which can cross with Alfalfa. For this reason, crossability test was not conducted.

g) Productivity of harmful substances

As a result of succeeding crop test, plow-in test and soil microflora tests on this recombinant Alfalfa and the control Null type Alfalfa to evaluate the productivity of harmful substances, no statistically significant difference was observed between them.

It is known that Alfalfa releases some water-soluble allelochemicals which can inhibit the growth of Alfalfa itself and other plants from stem and leaf, roots and other plant tissues, though any additional allelochemical has not been identified. As a result of the Sandwich method for the production of allelochemical in this recombinant Alfalfa and the control Null type Alfalfa, no statistically significant difference was observed.

Alfalfa is a legume and the root nodule bacteria coexist in the roots, thereby forming the root nodules. At harvest time of this recombinant Alfalfa and the control Null type Alfalfa, the roots were dug up for 5 individuals in the central furrow in each plot to determine the number of root nodules per individual which was then converted into the value per 1kg of roots for evaluation of possible effects on the root nodule bacteria. As a result, no statistically significant difference was observed between them.

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

It is reported that Alfalfa became self-seeding in Japan after introduction in the early years of Meiji era as grazing crop, though the reports on the habitat are random and infrequent and there has been no finding so far showing it offers the properties that can exterminate other wild animals and wild plants.

This recombinant Alfalfa has been conferred the tolerance to glyphosate herbicide because of the transferred modified *cp4 epsps*, but it is hard to consider that the glyphosate becomes a selection pressure in the natural environment. In addition, various traits relating to the competitiveness of this recombinant Alfalfa were investigated in Japanese isolated fields with the result that the plant height at regeneration in autumn in the first year of cultivation exhibited significantly higher values compared to the test sample. However, these values are found within those for the Alfalfa (*Medicago sativa* L.), the plant species, to which the recipient organism belongs.

In view of the above, it is not generally considered that the ability of propagation and survival of this recombinant Alfalfa is enhanced in the natural environment to such an extent in which this recombinant Alfalfa becomes competitiveness over the recipient organism Alfalfa.

Based on the above understanding, it was judged that the conclusion by the applicant that there are no specific wild animals and wild plants possibly affected by this recombinant Alfalfa and that the use of this recombinant Alfalfa poses no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

(2) Productivity of harmful substances

Alfalfa (*Medicago sativa* L.) to which the recipient organism belongs is known to produce some allelochemicals that inhibit the growth of Alfalfa itself and other plants, though there is no report that it has exerted such effects that can expel wild animals and wild plants.

As a result of plow-in test, succeeding crop test and microflora tests in Japanese isolated fields for productivity of harmful substances of this recombinant Alfalfa to other plants and microorganisms, no significant difference was observed among the test samples.

In addition, this recombinant Alfalfa produces CP4 EPSPS protein that offers the tolerance to glyphosate, though there is no report that this protein is any harmful substance. Furthermore, 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 other gene-modified crops to which *cp4 epsps* is transferred do not change the aromatic amino acid content. Therefore, it is not considered that this recombinant Alfalfa could produce any excessive amount of aromatic amino acid. In addition, based on the fact that EPSPS protein is an enzyme which specifically reacts with phosphoenolpyruvate and shikimate-3-phosphate, it is unlikely that the CP4 EPSPS protein catalyzes reactions of other substances to produce different substances.

Thus, it is hard to consider that this recombinant Alfalfa goes beyond the recipient organism Alfalfa and affects wild animals and wild plants.

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 Alfalfa poses no significant risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is valid.

(3) Crossability

In Japan, some types of the genus *Medicago* to which the recipient organism belongs grow voluntarily, though they are all introduced species and annual. According to the findings in the literature revealing that it has been difficult to create crossbreed even by the artificial crossing between the perennial Alfalfa (*Medicago sativa* L.) and the above annual genus *Medicago* plants, it is hard to consider the possibility of natural pollination.

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 Alfalfa poses no significant risk of Adverse Effect on Biological Diversity attributable to the crossability is valid.

2. Conclusion

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