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	Sugar beet tolerant to glyphosate herbicide (modified *cp4 epsps*, *Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) (H7-1, OECD UI: KM-ØØØH71-4)
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 Effect 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

1) The common name: Sugar beet The scientific name: *Beta vulgaris* L. ssp. *vulgaris* var. *altissima*.

2) The recipient organism is a commercially available cultivar [Confidential], which belongs to the sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*), a biennial plant in the goosefoot family (Chenopodiaceae). The [Confidential] refers to the multigerm variety in diploid form.

3) The sugar beet has the formal scientific name of *Beta vulgaris* L. ssp. *vulgaris* var. *altissima*, and it is one of the varieties belonging to the genus *Beta vulgaris* similarly as spinach beet (*Beta vulgaris* L. ssp. *vulgaris* var. *cicla*, *Beta vulgaris* L. ssp. *vulgaris* var. *flavescens*), beetroot for food (*Beta vulgaris* L. ssp. *vulgaris* var. *vulgaris*), fodder beet (*Beta vulgaris* L. ssp. *vulgaris* var. *rapacea*) and other beet varieties (Reference 1).

Cultivated forms of beet including the sugar beet are said to originate in the eastern Mediterranean district (Reference 1). The progenitor species is considered to be the wild sea beet (*Beta vulgaris* ssp. *maritima*) currently seen popularly on the coasts in Europe to West Asia regions. It is generally considered that the ancient sea beet became cultivated in prehistoric times as vegetable for use of leaves as food and the species of swollen roots became cultivated forms around the first century to use the roots for practical application.

The sea beet is considered to have spread from the eastern Mediterranean coast, the center of the motherland, to the Canary Islands and Azores archipelago along the Mediterranean sea and then reached Ireland and the southern Scotland along the coast of the Atlantic. It is now distributed from Egypt to northern Europe (Reference 1; Reference 2). The sea beet is a sea-coast plant and it is growing voluntarily only on the coasts in the inlands 10 to 20 m from the high tide. Also the sugar beet, which has adapted to a natural environment, is not growing in any area distant from the coasts similarly as the sea beet (Reference 1).

There has been no report that the sugar beet and the plants of the genus *Beta* are growing voluntarily in Japan (Reference 3; Reference 4; Reference 5).

(2) History and present state of use

1) Among the sugar beet, spinach beet is closest to wild species in the form and its history of cultivation dates back to prehistoric times. The beet leaves were domesticated in the sixth century B.C. to fourth century B.C. for use as vegetable.

Then, the spinach beet is said to be the first cultivated species specialized from wild species, followed by the beetroot lines (beetroot for food, fodder beet, etc.) and finally the sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*). It was in the 15th century when the fodder beet with swollen roots was actually domesticated, and the sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) was first cultivated in the end of 18th century (Reference 6).

In 1747, Markgraf discovered that the beet contains sucrose (cane sugar) and in 1790, Achard succeeded in extracting sugar from beet and expanded into breeding of beet. Their successors created the White Silesian variety, the origin of currently available cultivars. Since the blocus continental (Blockage of Continental ports) in 1806 by Napoleon I, a trend grew in European countries for self-supporting of sugar and protection of domestic industry, and the sugar production became prosperous (Reference 2).

At present, sugar beet is cultivated in the temperate regions of the world as a primary raw material crop for the production of sugar. Production of sugar beet in 2005 was approx. 242.62 million ton in total worldwide, and the major producing countries were France (approx. 29.30 million ton), Germany (approx. 25.43 million ton), US (approx. 24.72 million ton), Russia (approx. 21.52 million ton) and Ukraine (approx. 15.62 million ton) (Reference 7).

2) In around 1870, sugar beet was introduced to Hokkaido in Japan as a raw material for sugar. In 1950s, introduction of sugar beet to the Tohoku to Kyushu districts was attempted, though it was abandoned due to the low profitability caused by the susceptibility to disease damage in the warmer regions and resultant lower yields; so at present, the cultivation of sugar beet in Japan is limited to Hokkaido (Reference 2). The planted area for sugar beet in 2004 was approx. 68,000 hectares, and the total yield was approx. 4.66 million ton (drawn from Reference 8).

Cultivation of sugar beet is based on the direct sowing in other countries, though in the sugar beet planting areas in Japan (Hokkaido), transplanting culture is primarily adopted to advance the seedtime in spring. Sowing is carried out in late March, approx. one month before settled planting. Seeds are sown in paper pots packed with soil and seedlings are raised in plastic greenhouses. The seedlings are set in fields at the stage of 2 to 4 leaves in late April to late May. For fertilization, manure is applied 2 ton per decare as a rule, and the standard composition of chemical fertilizer in Hokkaido is 12 to 16 kg nitrogen, 18 to 25 kg phosphate, and 14 to 18 kg potassium. During the cultivation, weeding and disease and pest control are conducted as appropriate by chemical spraying and mechanical treatment. In Japan, sugar beet is harvested in October to November and even during the period, the sugar content increases consistently, and the yield may also increase in the years of a mild climate. However, harvesting in these months is typical in Japan since later harvesting can encounter decreased temperatures and resultant freezing and rainfall, and/or difficult harvesting due to the worse soil conditions in the fields caused by autumn rain (Reference 9). Therefore, in the actual cultivation, unless intended for production of seeds, sugar beet plants are not grown in the second year for reproduction and thus, they are least likely to flower and seed setting. However, even during the vegetative growth period, sugar beet may switch to the reproductive growth phase, causing bolting, if exposed to low

temperatures (Reference 9). In practice, however, bolted sugar beet plants result in lower yields and decreased sugar contents, so in the recent breeding, those cultivars are raised that are less likely to bolt in the first year of cultivation.

Sugar beet primarily consists of above-ground part (stems and leaves) and under-ground part (roots), which can be both utilized. In the processing of sugar beet into sugar, beet pulp is obtained as a by-product, which contains an abundance of digestive nutrients and then applied as livestock feed. Another important by-product is treacle or molasses, which is viscous liquid containing 48% sucrose, the uncrystallizable part of sugar. Molasses is used for production of yeasts, chemical substances, pharmaceutical agents, and mixed feed for bovine (Reference 10). The part of stems and leaves is called beet top and used as livestock feed and in some cases, plowed into fields to serve as good-quality green manure (Reference 9).

In Japan, in 2004, one ton beet sugar (drawn from Reference 8) and approx. 740,000 ton beet pulp (Reference 11) were imported. The beet pulp mainly came from US (372,000 ton), China (284,000 ton), and Chile (57,000 ton). In addition, in 2005, a total of 72.8 ton seeds of sugar beet were imported to Japan, primarily from Germany (approx. 29.9 ton), France (approx. 29.3 ton), US (approx. 9.2 ton), and Belgium (approx. 4.2 ton) (drawn from Reference 12).

(3) Physiological and ecological properties

1) Basic properties

Sugar beet is a seed-propagating, biennial *Chenopodiaceae* family crop with the basic chromosome number of x=9, and it exists in diploid, triploid and tetraploid forms. The plant height typically ranges from 30 cm to 120 cm, though it can reach 200 cm in some plants. The leaves are stalked and they develop in the form of rosette from the crown exposed above the ground. The length of leafstalk, and the shape, size and color of leaf blade vary according to the species and the stage of development. The angle of leafstalk against the ground surface also varies and classified as upright, flat-open, intermediate and other types. The roots become significantly swollen in the first year of growth (Reference 1; Reference 9).

Sugar beet seeds are normally sown in spring and in the first growing season, the roots become massive through the vegetative growth. The growth behavior is generally divided into three stages, seedling, thick growing and ripening. In the seedling stage, leaves emerge as the temperature increases and in early July, larger leaves are extracted. The thick-growing stage refers to mid July to early September, during which the photosynthesis takes place actively, accelerating the swelling of roots. The ripening stage is September and later months, during which the roots are additionally swollen, and leaf yellowing and wilting generally begins above the ground and sugar content is accumulated (Reference 13).

Sugar beet is vernalized when exposed to low temperatures (4 to 7℃) in winter and then switched to the reproductive growth phase (Reference 14). Then, under the long-day condition from the next spring to summer, flower buds are differentiated and a flowering stalk is elongated (known as "bolting"), and flowers become open

and seeds are produced. The flowering time is June to July, and the seeds are formed in around August. The duration of low temperatures required for vernalization is 90 to 110 days, though depending on the variety. When sugar beet is exposed to low temperatures even in the first growing season, it may be switched to reproductive growth, causing bolting and subsequent formation of flower buds. However, bolting in the first year of growth can result in significant decrease in both the yield and the sugar content, so in recent years, varieties with a high bolting resistance are produced and cultivated. Inflorescences are formed at the tip of main stem and usually large, and the flowers are arranged in cymes. The flowers are hermaphrodite; three pistils surrounded by five stamens and five narrow sepals without petal. The flowers initiate at the tip of main stem and on the lateral shoots of inflorescences (Reference 1; Reference 9).

Originally sugar beet has seed pods or seed bulbs composed of multiple flowers to produce the multigerm seeds from aggregated seeds. However, germination of many individuals from one grain of seed requires enormous efforts for thinning during the cultivation and thus makes the mechanization of the operation difficult. For this reason, the recessive gene (*m*) homozygous type, which produces the single-embryo seeds, was selected as a special type among a large scale of seed growing populations (Reference 15). Since the discovery in 1948, the gene *m* has been transferred to all varieties (Reference 1; Reference 9).

Most of the currently raised sugar beet varieties are hybrids using the cytoplasmic male sterility (CMS) (Reference 1). The CMS of sugar beet refers to the male sterility of cytoplasm-nuclear gene type, and it takes place by the interaction between the plasmagene (mitochondrial genome) and the nuclear gene (Reference 16; Reference 17). Cytosolic factor is available in two types, the cytoplasm to express the male sterility and the cytoplasm not to express the male sterility, and nuclear gene is also available in two types, the gene to cause sterility and the gene to restore the fertility. Combinations of these cytosolic factors and nuclear genes govern the fertility of pollen (male fertility). Crossing the CMS line as seed parent (mother plant) with any excellent line provides F1 hybrids.

Moreover, most of the sugar beet varieties, which have been cultivated since 1970, are in the triploid form (Reference 1). The triploid sugar beet is produced by crossing the tetraploid pollen parent line with the seed parent line possessing the diploid male sterility. Consequently, the seeds from this crossing fail to successfully complete the meiosis, thereby resulting in greatly reduced fertility.

2) Environmental conditions allowing inhabiting or growth

Cultivation of sugar beet is widely distributed in the temperate to subarctic regions of the world. In the growing period of 180 to 200 days, an accumulated temperature of 2,400 to 3,000°C and a mean temperature of 12.3 to 16.4°C are essential, and an annual precipitation of approx. 600 mm is said optimum. In Hokkaido, lingering snow and low temperatures in early spring and frost and snow in October caused shorter growth duration, and autumn rain brought about lower sugar content, leading to the problems of lower yields and lower sugar contents. Sugar beet prefers the deep plowed soil rich in organic substances with proper drainage and the soil pH of neutral or alkaline (Reference 2).

3) Predacity or parasitism

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4) Mode of propagation or reproduction

(a) The shedding habit of sugar beet is low, though the seeds may shed when left to stand for a prolonged period of time after ripening. What is generally known as a seed of sugar beet corresponds to fruit from the botanical viewpoint and properly speaking, it is called strobilus or seed bulb, inside which one to four true seeds are contained. The reason why several true seeds are contained is because individual flowers of inflorescence adhering to the leaf axil on the flower stalk are brought into close contact with each other and then become united as the seeds ripen. The seed bulb containing one true seed is called single-embryo seed, and the seed bulb containing two or more true seeds is called multigerm seed, which, though, does not mean a seed contains many germs. The sugar beet seeds are long-life and able to survive 10 or more years in soil. In the laboratory conditions, the germination rate is found 70% for the seeds in the 6th year after seed production and 59% for the seeds in the 8th year after seed production (Reference 1).

(b) Sugar beet normally propagates by seeds. On the other hand, plant body may be regenerated from the roots or crown left in the fields after harvesting (Reference 1).

(c) Sugar beet (Beta vulgaris L. ssp. vulgaris var. altissima) is an allogamous biennial plant having high self-incompatibility. Sugar beet propagates primarily based on the wind-pollination. The flowering period is approx. 4 weeks and the flowers normally open in the morning (Reference 18). The pistils remain fertilizable two or more weeks after flower initiation (Reference 19).

The self-incompatibility of sugar beet is gametophytic, involving at least 4 gene loci, and the gene loci possess several allelic genes respectively. It is also considered that several other genes are involved (Reference 20; Reference 21). The gametophytic self-incompatibility means that when there is any common matter between the S gene in the pollen and the S gene in the pistil, elongation of pollen tube is inhibited and then fertilization is disturbed. In the

event if the genetic mechanism is destructed, "false self-fertilization" or "false self-pollination" could take place. This phenomenon is more or less observed between different genotypes and susceptible to environmental factors, especially temperature (Reference 22). In addition, apart from above, those individuals which possess the S^f gene, a special self-reproduction gene, set the self-propagating seeds at a rate of 90 to 95% even under the open-pollinated condition (Reference 15).

Currently used sugar beet varieties have reduced fertility due to the triploid character (Reference 1), so there is least possibility that these cultivars would produce seeds independently from whether the self-incompatibility is high or low.

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) is equivalent to those varieties such as spinach beet, beetroot for food and fodder beet, which belong to the genus *vulgaris* and thus, it is crossable with any of the varieties without any reproductive isolation mechanism.

In addition, the genus *Beta*, to which the sugar beet belongs, is classified as four sections, *Beta*, *Corollinae*, *Procumbentes* and *Nanae*, and the sugar beet belongs to the section *Beta* together with the wild species, *B. maritime* [1], *B. macrocarpa*, *B. patula*, and *B. vulgaris* ssp. *adanensis* (Reference 1). All these species are cross compatible (Reference 18; Reference 23), and the hybrids produced are all fertile and do not show any incompatibility at the chromosome level (Reference 24). However, the hybrids between sugar beet and *B. macrocarpa* are rare due to differing flowering times of the parental species (Reference 25). In addition, the hybrids between *B. macrocarpa* and *B. vulgaris* exhibit some sterile pollens and extinct germs (Reference 26).

Some varieties among those belonging to the genus *Beta*, section *Corollinae* allow artificial crossing with sugar beet, though involving considerable difficulties. In practice, however, the hybrids obtained are highly sterile and least likely to produce seeds by a backcross with sugar beet. The F1 hybrid obtained by artificial crossing of sugar beet with the section *Procumbentes* dies out normally at the sprouting stage, though it can survive and grow vigorously by grafting to the sugar beet. The above-mentioned hybrids are almost completely sterile and they hardly produce seeds by backcrossing. Between cultivated forms of sugar beet and the variety *B. nana* in the section *Nanae*, no crossing has been reported (Reference 27).

Based on the above understanding, sugar beet is only crossable with the wild species which belong to the genus *Beta*, the section *Beta*. However, in Japan, there is no plant of the genus *Beta* growing voluntarily (Reference 3; Reference 4; Reference 5), therefore, it is considered that there is no possibility of natural crossing between sugar beet and wild relatives in Japan.

(d) Sugar beet forms wind-pollinated flowers. Dispersion of pollens by insects may occur in some cases, though at a low frequency, making a limited

[1] In this Biological Diversity Risk Assessment Report, the designation "*B. maritima*" is used in accordance with Reference (Reference 1), the source of citation, though *B. maritima* refers to *Beta vulgaris* ssp. *maritima*.

contribution to pollination (Reference 1). Pollen grains are spherical in shape and have numerous irregularities on the surface. The number of pollen grains per anther is approx. 17,000. This suggests that the pollen grains of approx. 85,000 per flower and approx. one billion per individual would be produced (Reference 28). The pollen ability of survival is limited to maximum 24 hours, depending on the environmental condition, especially ambient moisture (Reference 1). The size of monoploid pollens produced in the diploid plants is approx. 20 μm in diameter, and the size of diploid pollens produced in the triploid plants is approx. 30 μm in diameter.

According to the program for seed production of sugar beet by OECD, only those locations are approved as producing area of sugar beet seeds which are confirmed that there is no genus *Beta* growing voluntarily. The separation for crossbreeding or commercial seed production is specified in the program for seed production of sugar beet by OECD at 1,000 m from other genus *Beta* for typical seed production and 300 m to 1,000 m for production of certificated seeds, depending on the chromosome number of the intended pollen parent and the chromosome number of sugar beet of the neighboring pollen source (Reference 27).

(e) Pathogenicity

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(f) Productivity of harmful substances

It is known that sugar beet does not produce any harmful substances including allelochemicals which can affect the habiting or growth of wild animals and wild plants.

(g) Other information

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2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the production of sugar beet tolerant to glyphosate herbicide (modified *cp4 epsps*, *Beta vulgaris* L. ssp. *vulgaris* var. *altissima*)(H7-1, OECD UI: KM-ØØØH71-4) (hereinafter referred to as "this recombinant sugar beet") are shown in Table 1.

The *cp4 epsps* gene transferred into this recombinant sugar beet has the base sequence at the 3'-terminal modified to provide the restriction site. As a result, the second amino acid from the N-terminal is modified to leucine, instead of serine found in the original amino acid sequence derived from *Agrobacterium* sp. CP4

strain. Therefore, the *cp4 epsps* gene transferred into this recombinant sugar beet is referred to as "modified *cp4 epsps* gene", and the CP4 EPSPS protein encoded by the gene as "modified CP4 EPSPS protein." The estimated amino acid sequence of the modified CP4 EPSPS protein is shown in Figure 3 of Annex 1.

2) Functions of component elements

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

(a) The modified *cp4 epsps* gene, the target gene, produces the modified CP4 EPSPS protein which has high tolerance to the glyphosate herbicide. 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 synthesis pathway for aromatic amino acid biosynthesis, by specifically binding to the enzyme (Reference29, Reference 30). As a result, plants treated with glyphosate cannot synthesize enough amounts of the aromatic amino acids essential for protein synthesis due to the inhibition of EPSPS, resulting in the death of the plant. The activity of the modified CP4 EPSPS protein produced by the 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 synthesis pathway and can grow.

EPSPS is one of the enzymes that catalyze the shikimate pathway for aromatic amino acid biosynthesis that is specific to plants and microorganisms, and is located in chloroplasts and plastids in plants (Reference 31). The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation in plants (Reference 32; Reference 30). 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 steps from DAHP to the synthesis of chorismic acid are inhibited or suppressed by metabolic intermediates or end products of this pathway (Reference 33; Reference 34). This suggests that it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acid, the end products of this pathway. In fact, it is reported that plant cells that produce 40 times as much EPSPS as compared to average do not synthesize excessive aromatic amino acids (Reference 35). 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 in Japan (soybean, oilseed rape, cotton, and maize) that are tolerant to the glyphosate herbicides, and confirmed that there is no difference in the aromatic amino acid content between the non-recombinant control plants and the 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 phosphate (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Reference 36), and is known to specifically react with these substrates (Reference 37). The only substance that is known to react with EPSPS other than these is shikimate, an analogue of S3P. However, the reactivity with shikimate is only

one two millionth of the reactivity with S3P, and it is unlikely that shikimate acts as the substrate of EPSPS in the living body.

Consequently, it is considered to be extremely unlikely that the production of the modified CP4 EPSPS protein, which is functionally parallel to plant EPSPS protein, has an effect in someway on the metabolic pathways of plants.

(b) In order to investigate whether the modified CP4 EPSPS protein shares functionally important amino acid sequences with known allergens, the modified CP4 EPSPS protein was compared with allergens in the databases (AD4, TOXIN5, ALLPEPTIDE). As a result, modified 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 PV-BVGT08 used for the production of this recombinant sugar beet[2]

Component elements	Size (kbp)	Origin and function
T-DNA region		
Right Border (RB)	0.36	A DNA sequence of right border sequence of nopaline type T-DNA derived from Ti plasmid pTiT37 . Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome (Reference 38).
P-FMV	0.67	Figwort mosaic virus (hereinafter referred to as "FMV") 35S promoter (Reference 39; Reference 40; Reference 41; Reference 42). Involved in the constant expression of the target gene in the entire tissue of plant body. In the downstream of FMV promoter, the untranslated region of 67bp exists, derived from *epsps* gene, which encodes the EPSPS protein in *Arabidopsis thaliana*.
ctp2	0.23	N-terminal of chloroplast transit peptide sequence in the *epsps* gene of *Arabidopsis thaliana* (Reference 43). Transfers the target protein to the chloroplast.
modified *cp4 epsps*	1.37	*epsps* gene of *Agrobacterium* sp. CP4 strain (Reference 44). Functions are detailed in pages 8 to 9.
E9 3'	0.64	3' untranslated region of pea ribulose-1, 5-bisphosphate carboxylase E9 gene. Terminates transcription of mRNA and induces polyadenylation. (Reference 45; Reference 46).
Left Border (LB)	0.44	A DNA fragment in the left border sequence, derived from Ti plasmid pTiA6. It is the termination point of T-DNA transfer from *Agrobacterium tumefaciens* to plant genome (Reference 47).
Outside of T-DNA region		
ori-V	0.40	The replication origin isolated from the broad-recipient range plasmid RK2. Permits autonomous replication of vectors in *Agrobacterium tumefaciens.* (Reference 48).
rop	0.19	The replication origin regulating region derived from pBR322, suppressing the formation of RNA primer to maintain the number of plasmids in the recipient organism such as *E. coli* (Reference 49; Reference 50).
ori-322	0.59	The replication origin derived from *E. coli* plasmid pBR322, conferring the ability of autonomous replication in the *E. coli* to the vector (Reference 51).
aadA	0.89	Bacteria promoter, code region and terminator for the 3'(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin/streptomycin (Reference 52). (GenBank accession X03043)

[2] All the rights pertinent to the information in the above table and the responsibility for the content rest upon Monsanto Co. and KWS SAAT AG.

(2) Information concerning vectors

1) Name and origin

The plasmid vector used to produce this recombinant sugar beet was constructed based on the plasmid pBR322 derived from *Escherichia coli*.

2) Properties

The total number of base pairs of PV-BVGT08 used to produce this recombinant sugar beet is [Confidential]. As a selectable marker gene for construction vector in *E. coli*, the *aadA* gene derived from *E. coli* transposon Tn7 is present outside of the T-DNA region, which confers resistance to spectinomycin and streptomycin. The entire base sequences of this plasmid vector are provided in Annex 3.

The infectivity of this vector is not known.

(3) Method of preparing living modified organisms

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

For the production of this recombinant sugar beet, based on the vector derived from pBR322 which possesses the above-mentioned *aadA* gene, the plasmid PV-BVGT08 was constructed by joining the modified *cp4 epsps* gene expression cassette ([P-FMV]-[*ctp2*]-[modified *cp4 epsps*]-[E9 3’]) and the plasmid was used as the vector (see Table 1 and Figure 1).

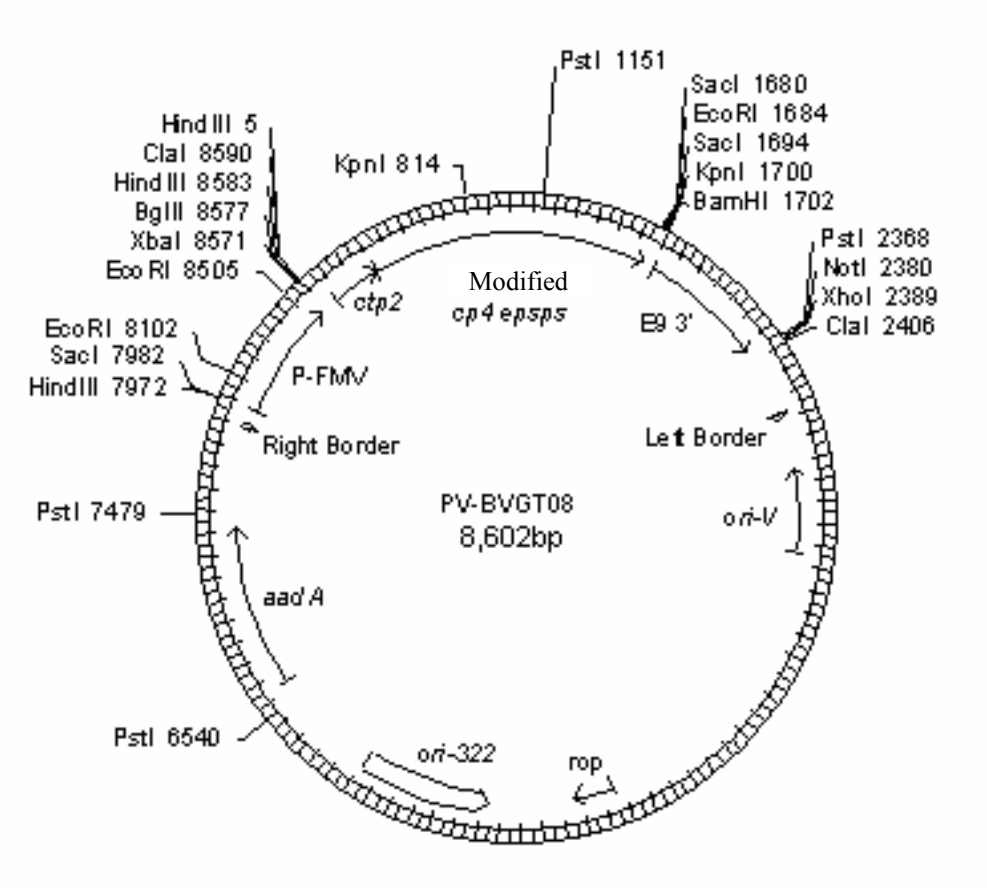

Figure 1 Map of plasmid PV-BVGT08[3]

The intended expression region in the plasmid vector PV-BVGT08 used for transformation of this recombinant sugar beet is from the Right Border to the Left Border in the above map in the clockwise direction.

[3] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Co. and KWS SAAT AG.

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

The T-DNA region of the plasmid vector PV-BVGT08 was transferred into the cotyledon tissue of seedling of breeding line [Confidential] of sugar beet by the *Agrobacterium* method. The [Confidential] used as the introduction mother plant is the third generation of diploid inbred line and does not possess any self-incompatibility.

3) Processes of rearing of living modified organisms

The *Agrobacterium tumefaciens*, to which the plasmid PV-BVGT08 was transferred, was inoculated to the sterilized seedling cotyledons of sugar beet and incubated, then co-cultivated with *Agrobacterium* for 2 to 4 days. Then, *Agrobacterium* was removed on the sterilized medium containing 500 mg/L carbenicillin. Callus was then selected after cultivation on the selective medium containing glyphosate, and the obtained callus was re-differentiated and transplanted to pots to evaluate the re-differentiated individuals for tolerance to glyphosate. The re-differentiated generation (R0) was analyzed for transferred genes. The seeds (R1:[Confidential]) obtained from re-differentiated generation (R0:[Confidential]) which has undergone vernalization were used for later rearing of H7-1 line. F1 hybrid varieties were produced and subjected to field tests from 1995, where H7-1 was selected eventually as the commercial event. In this Biological Diversity Risk Assessment Report, this recombinant sugar beet line H7-1 refers to the re-differentiated generation obtained by introduction of gene (=R0 generation) and all its posterities.

This recombinant sugar beet has been produced jointly by Monsanto Co. and KWS SAAT AG in Germany.

The following shows the approvals received from organizations in Japan.

June, 2003: The safety of use of the cultivar for food, in accordance with "Safety Evaluation Criteria for Food and Additives derived from Recombinant-DNA Techniques" was certified by the Ministry of Health, Labour and Welfare.

September, 2005: 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" was certified by the Ministry of Agriculture, Forestry and Fisheries.

The process of rearing this recombinant sugar beet is shown in Figure 2.

Figure 2 The process of rearing this recombinant sugar beet line H7-1

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

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

Based on the findings that the traits acquired by the transferred gene are expressed in the posterity at a segregation ratio expected in accordance with the law of Mendelian inheritance, the transferred nucleic acid is judged to exist on the chromosome (Table 5 of Annex 1).

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

As a result of Southern blotting analysis for existence of the transferred gene, it was confirmed that one copy of the T-DNA region was transferred at a site in the genome of this recombinant sugar beet (Figure 6 of Annex 1, Figure 3). In addition, the plasmid backbone region outside of the T-DNA region was not transferred (Figure 11 of Annex 1), and the modified *cp4 epsps* gene expression cassette inside the T-DNA region was transferred in the intact form (Figures 7 to 9 of Annex 1). Furthermore, Southern blotting analyses on multiple generations indicated that transferred genes were stably inherited in offspring (Figure 15 of Annex 1). However, as a result of comparison between the intended expression region in the plasmid vector (from the RB sequence to the LB sequence) and the actually transferred sequences to this recombinant sugar beet, it was confirmed that up to the 18th base in the RB sequence at the 5' terminal of the transferred gene was transferred to the genome DNA but the RB sequence was not contained in the transferred gene (Figure 13 of Annex 1; Figure 3).

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

This item is not applicable due to the one copy (Figure 6 of Annex 1).

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

The amount of expression of modified CP4 EPSPS protein in this recombinant sugar beet was determined based on the ELISA method. In the test, the leaves at the harvest time and the brei (processed root tissue), which are used as food and feed in the commercial cultivation, were used as samples. As a result, the amount of expression of modified CP4 EPSPS protein was found 161 μg/g of mean fresh weight in the leaves at the harvest time with the range of the amount of expression from 112 to 201 μg/g fresh weight, and 181 μg/g of mean fresh weight in the root (brei) with the range from 145 to 202 μg/g fresh weight (Table 6 of Annex 1, p56). Furthermore, the expression stability of the modified CP4 EPSPS protein was evaluated based on the tolerance to glyphosate herbicide across several generations. As a result, it was confirmed that the modified CP4 EPSPS protein is expressed stably across multiple generations (Figure 2; Figure 15 of Annex 1).

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

For production of this recombinant sugar beet, *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.

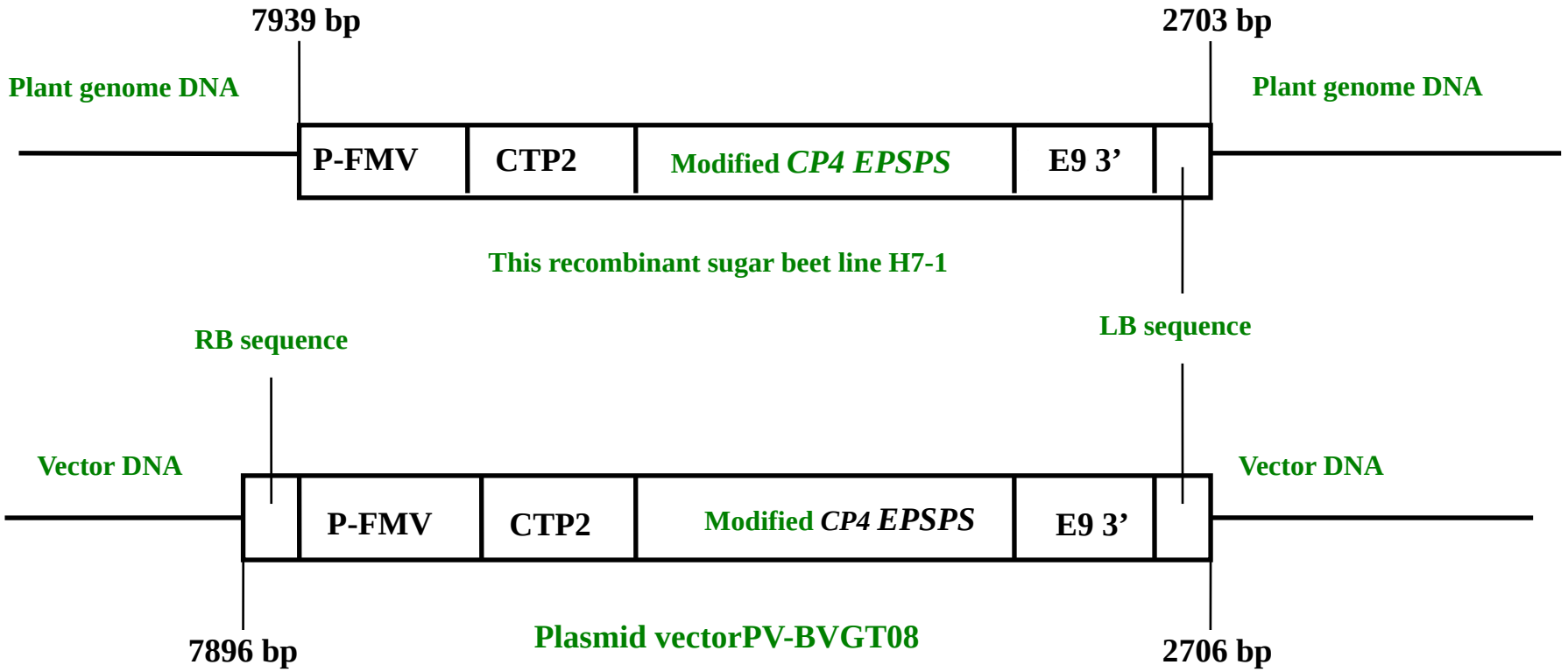

Figure 3 Genetic map of the transferred genes to this recombinant sugar beet H7-1 [4]

In this recombinant sugar beet, the RB sequence of the T-DNA region in the plasmid vector PV-BVGT08 is not transferred.

[4] All the rights pertinent to the information in the above diagram and the responsibility for the content rest upon Monsanto Co. and KWS SAAT AG.

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

For the detection and identification of this recombinant sugar beet, a qualitative PCR method has been established. This method makes it possible to specifically detect this recombinant sugar beet. The detection limit and the determination limit are 10 copies and ≤0.045% respectively. The detection limit was analyzed in 6 repeats with the samples obtained by diluting the genome DNA of this recombinant sugar beet to 7 different concentrations, 0.313 genome copies to 20 genome copies per reaction. As a result, the detection limit was defined as 10 copies of genome which obtained the positive reaction in the analysis of all of the 6 repeats. In addition, the determination limit of ≤0.045% refers to the proportion of the amount of genome DNA in this recombinant sugar beet compared to the amount of genome DNA in the non-recombinant control sugar beet. In the process of development of the detection method, those samples were used that were prepared by mixing the genome DNA of this recombinant sugar beet with the genome DNA of the non-recombinant control sugar beet in six different proportions of 2.0% to 0.045%, and in all the samples, this recombinant sugar beet could be detected. Then, the determination limit was defined as 0.045% or less. Details on the detection method are available in the Web site of EU Community Reference Laboratory (Reference 53).

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

1) The modified *cp4 epsps* gene transferred into this recombinant sugar beet encodes the modified CP4 EPSPS protein which possesses high tolerance to glyphosate herbicide. By the expression of this protein in plant body, this recombinant sugar beet can grow without any effect of glyphosate herbicide (Figure 3 of Annex 4).

2) [5] In 2005, the test of this recombinant sugar beet for specific characteristics was conducted at an isolated field in Kawachi Research Field of Monsanto Co. Japan. In this test, the following hybrids were used: for this recombinant sugar beet, the hybrid [Confidential], 5th generation of this recombinant sugar beet; and for a control hybrid, the non-recombinant sugar beet hybrid [Confidential] which is close to this recombinant sugar beet hybrid [Confidential] in the genetic background. This hybrid [Confidential] was produced by crossing the fertile H7-1 pollinator plant with male-sterile individuals. In addition, Kitasayaka and Nozomi, the existing sugar beet cultivars for commercial cultivation in Hokkaido, were also tested as reference varieties.

(a) Morphological and growth characteristics

Differences in morphological and growth characteristics were examined between this recombinant sugar beet, the non-recombinant control sugar beet and the reference existing sugar beet varieties with respect to the following 17 items: the uniformity of germination; germination rate; leaf type; leaf length; the number of leaves; leaf color; leaf shape; leafstalk length; total weight at the harvest time; root length; root circumference; exposed shoulder; top weight;

[5] All the rights pertinent to the information in (a) through (g) in this section and the responsibility for the contents rest upon Monsanto Co. and KWS SAAT AG.

root weight; T/R ratio; sugar content; and bolting resistance) (Tables 2 to 4 of Annex 4).

As a result, a statistically significant difference was observed between this recombinant sugar beet and the non-recombinant control sugar beet in leaf length and leafstalk length, though not in other items (Tables 3 to 4 of Annex 4). The leaf length was found 71.8 cm in this recombinant sugar beet and 65.6 cm in the non-recombinant control sugar beet. In addition, the leafstalk length was 35.8 cm in this recombinant sugar beet and 30.6 cm in the non-recombinant control sugar beet.

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

This recombinant sugar beet, the non-recombinant control sugar beet and the reference varieties were grown in a closed greenhouse controlled below a maximum temperature of 25°C and then raised in a climate chamber exposed to low temperature conditions (8°C during daytime/5°C during nighttime, 12-hour daytime length) and high temperature conditions (35°C during daytime/3°C during nighttime, 14-hour daytime length) at the 2.5 leaf stage for a total of 30 days to examine the differences in the weight of above- and under-ground parts (Table 5 of Annex 4).

As a result, in all the conditions examined, no statistically significant difference was observed between this recombinant sugar beet and the non-recombinant control sugar beet.

(c) Wintering ability and summer survival of the matured plant

Sugar beet is a biennial plant and it requires low temperature conditions (4 to 7°C) in winter in the first year of growth to switch to the reproductive growth phase and set seeds. However, sugar beet is known as such a cold-sensitive crop that dies out due to the frost at temperatures below -5°C (Reference 14). In fact, at the end of the isolated field test, stems and leaves of this recombinant sugar beet wilted to the similar degree as in the non-recombinant control sugar beet.

(d) Fertility and size of the pollen

The fertility and the size of pollen grains were not examined in this isolated field test based on the facts: this recombinant sugar beet is considered difficult to grow up to flower or ripen in Japan for the reasons described below; and there are no wild relatives in Japan which can be crossed with sugar beet.

The reason why sugar beet is considered unlikely to survive in Japan until it flowers or ripens is that the competitiveness with weeds is low. Sugar beet is biennial and requires a period of vernalization in the winter at the first year before it can enter the reproductive growth phase and flower and ripen in the second year. However, sugar beet is very susceptible to damage due to the competitiveness with weeds or diseases in the first growing season (Reference 1), and it is also known less competitive with weeds even in the subsequent

growing period (Reference 54; Reference 55; Reference 56). In England, glyphosate herbicide-tolerant sugar beet and non-recombinant control sugar beet were grown in a natural environment to monitor any changes in individual population. As a result, it was reported that only about 5% of the seeds sown could grow up to adult individuals in the both of glyphosate-tolerant and non-recombinant control sugar beets in the first year and that the surviving individuals all disappeared in two years from start of monitoring (Reference 57). In practice, sugar beets are discovered occasionally outside of the sugar beet cultivating fields in the sugar beet planting areas, though there is no report that those plants are becoming wild (Reference 58). In Japan, sugar beets were once introduced in 1950s to Tohoku to Kyushu districts, though there is no report that they became volunteer in those districts.

Furthermore, as mentioned earlier, sugar beet is crossable with wild species which belong to the genus *Beta*, section *Beta*, though there is no voluntarily growing plant of the genus *Beta* in Japan (Reference 3; Reference 4; Reference 5).

In consideration of a possibility that this recombinant sugar beet would flower and ripen due to any factors other than those discussed above, evaluation was made for the reproduction characteristics of this recombinant sugar beet based on the investigational results obtained in the Germany. In this test, this recombinant sugar beet, the non-recombinant control sugar beet and the existing sugar beet breeding lines were used to examine the reproduction characteristics (the weight of anther, the number of anthers per flower, the number of stamens per flower, pollen diameter, the number of pollens per anther, the germination rate of pollen, the number of embryos per seed, the length of embryos, the width of embryos, the ratio of length to width of embryo). As a result, no significant difference was observed between this recombinant sugar beet and the non-recombinant control sugar beet, and their values were found to fall within or close to the range of the existing sugar beet breeding lines (Annex 2).

The commercialized variety of this fertile recombinant sugar beet is produced by crossing with the breeding line containing the male-sterile cytoplasm component. Subsequently, the resulting hybrid also has the male sterile cytoplasm component.

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

In the isolated field test, examination was not conducted on the production, shedding habit dormancy and germination rate of the seed for the reasons described below.

As discussed above, sugar beet is less competitive, and it has a history of introduction to Tohoku to Kyushu districts in Japan in 1950s, though there is no report that sugar beet has been established in a natural environment in Japan. This suggests that this recombinant sugar beet also lacks the ability of establishment in natural environment in Japan.

According to breeders at KWS, it is reported that from the start of production of this recombinant sugar beet line H7-1 (1993) to date, in the process of maintenance and selection of this recombinant sugar beet line, no difference has been observed in seed yield between this recombinant sugar beet and the existing sugar beet varieties. As a result of the comparison in Germany for the morphological characteristics of reproductive organs involved in the production of seeds (the weight of anther, the number of anthers per flower, the number of stamens per flower, diameter of pollen, the number of pollens per anther, the germination rate of pollen, the number of ovules per ovary, ovule length, ovule width, slenderness ratio of ovule), no significant difference was observed between this recombinant sugar beet and the non-recombinant control sugar beet, and the values were found to fall within or close to those of the existing sugar beet breeding lines (Annex 2). In addition, no statistically significant difference was observed in dormancy and germination rate of seed between this recombinant sugar beet and the non-recombinant control sugar beet (Table 17 of Annex 1).

(f) Crossability

Crossability test was not conducted because no wild relatives exist in Japan that belong to the genus *Beta* which show crossability with this recombinant sugar beet (Reference 3; Reference 4; Reference 5).

(g) Productivity of harmful substances

A microflora test, a succeeding crop test and a plow-in test were carried out to evaluate this recombinant sugar beet for production of any harmful substances that can affect other plants or microorganisms in soil. As a result, in all the items examined, no statistically significant difference was observed between this recombinant sugar beet and the non-recombinant control sugar beet (Tables 6 to 8 of Annex 4).

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

There has been no report that sugar beet is growing voluntarily in Japan.

This recombinant sugar beet is given the tolerance to glyphosate herbicide because of the transferred modified *cp4 epsps* gene, though it is hard to consider that the glyphosate becomes selection pressure in the natural environment. In addition, as a result of investigation on the various traits relating to the competitiveness of this recombinant sugar beet, a significant difference was observed in leaf length and leafstalk length between this recombinant sugar beet and the non-recombinant control variety, but no significant difference observed compared to the other non-recombinant control cultivar Kitasayaka. Consequently, it is considered that the leaf length and leafstalk length in this recombinant sugar beet fall within the variable ranges for the traits of sugar beet.

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 sugar beet poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

There have been no reports that sugar beet, the plant species to which the recipient organism belongs, produces harmful substances that have an effect on wild animals and plants.

This recombinant sugar beet produces the modified CP4 EPSPS protein that confers the tolerance to glyphosate; however, it has not been reported that this protein is harmful.

The modified CP4 EPSPS protein is the enzyme that catalyzes the shikimate pathway to synthesize aromatic amino acids, though it is clarified that it is not a rate-determining enzyme in this pathway. It is therefore confirmed that all the parts of this recombinant sugar beet including stems, leaves and roots do not contain significantly different amount of aromatic amino acid contents compared to the non-recombinant control plant. Besides, CP4 EPSPS protein has high substrate specificity, therefore, it is considered unlikely that CP4 EPSPS protein catalyzes a reaction between other substances and contributes to the production of harmful substances.

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

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

(3) Crossability

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

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

Reference

Confidential: Not made available or disclosed to unauthorized person