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

Name: Suntory Ltd.
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Applicant: 2-1-40, Dojimahama,
Kita-ku, Osaka-City, Osaka

Approved Type 1 Use Regulation

Name of the type of Living Modified Organism	Purple-violet carnation (*F3'5'H, DFR, sur*B, *Dianthus caryophyllus* L.) (123.8.12, OECD UI : FLO-40689-6)
Content of the Type 1 Use of Living Modified Organism	Appreciation, cultivation, storage, transportation and disposal of cut flowers, and other acts incidental to them
Method of the Type 1 Use of Living Modified Organism	–

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Composition of donor nucleic acids and origins of component elements are shown below, the relative positions between them are presented in Figure 1 (p.3), and the nucleotide sequence of the vector is provided in Annex 1-Figure 1 (p.1).

(a) Expression cassette for selectable marker *sur*B

35S:	35S promoter derived from cauliflower mosaic virus	0.2kb
*sur*B:	*Acetolactate synthase* (*ALS*) gene derived from tobacco	2.0kb
*sur*B 3':	3' untranslated region of *acetolactate synthase* (*ALS*) gene derived from tobacco	1.8kb

(b) Dihydroflavonol 4-reductase (DFR) expression cassette

DFR genomic DNA:	*Dihydroflavonol 4-reductase* gene derived from Petunia (including promoter, translated region and 3' untranslated region)	5.0kb

(c) Expression cassette for flavonoid 3', 5'-hydroxylase (F3'5'H)

CHS:	*Chalcone synthase* gene promoter derived from common snapdragon (*Antirrhinum majus*)	1.2kb
F3'5'H cDNA:	Flavonoid 3'',5'-hydroxylase cDNA derived from pansy (*Viola wittrockiana*)	1.5kb
D8 3':	3' untranslated region of lipids transfer protein derived from Petunia	0.8kb

(d) Others

*lac*Z:	*β-galactosidase* gene derived from *E. coli*	0.3kb

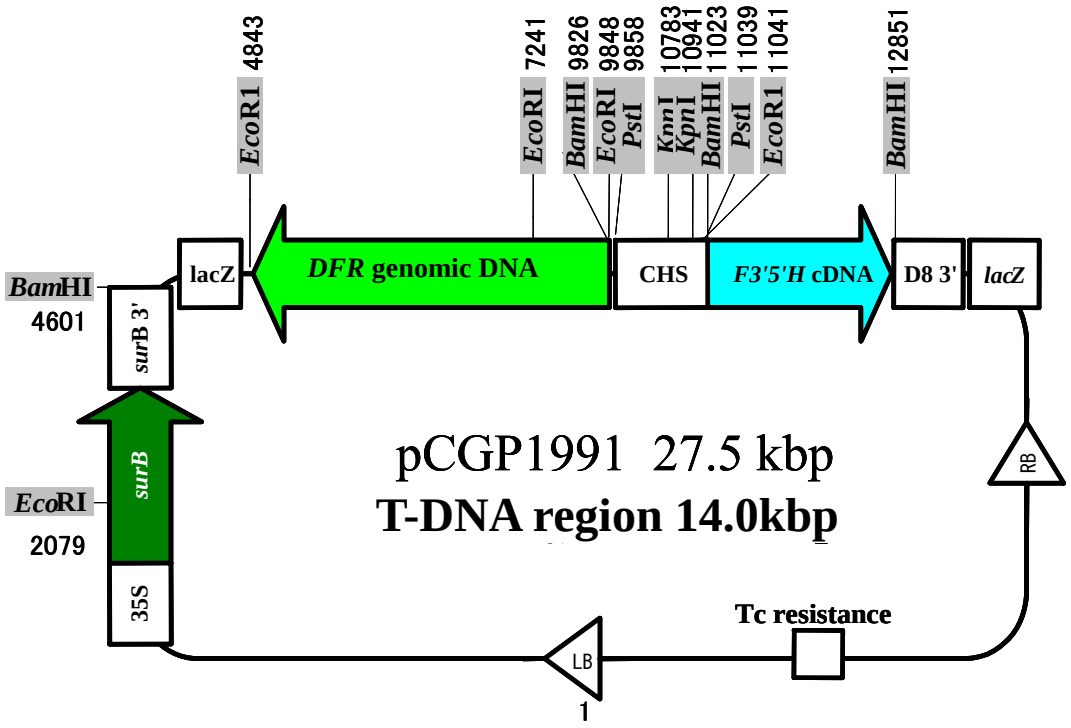


Figure 1 Structure of pCGP1991

Two genes have been transferred into the binary vector pWTT2132.

35S:	35S promoter derived from cauliflower mosaic virus
*sur*B:	*Acetolactate synthase* (*ALS*) gene derived from tobacco
*sur*B 3':	3' untranslated region of *acetolactate synthase* (*ALS*) gene derived from tobacco
DFR genomic DNA:	*Dihydroflavonol 4-reductase* gene derived from petunia (including promoter, translated region and 3' untranslated region)
CHS:	*Chalcone synthase* gene promoter derived from common snapdragon (*Antirrhinum majus*)
F3'5'H cDNA:	Flavonoid 3',5'-hydroxylase cDNA derived from pansy (*Viola wittrockiana*)
D8 3':	3' untranslated region of lipids transfer protein derived from Petunia
*lac*Z:	*β-galactosidase* gene derived from *E. coli*

* The numbers provided with names of restriction enzyme represent the positions of cleavage (bp) in relation to the end of left border defined as 1.

2) Function of component elements

(a) Anthocyanin biosynthetic pathway and possible effects of transferred genes in carnation

Outline of anthocyanin biosynthetic pathway is presented in Figure 2 (p.5). The anthocyanin biosynthetic pathway is maintained in the plant kingdom and also in the carnation, anthocyanin is synthesized in accordance with the pathway shown in Figure 2 (p.5). For the anthocyanin present in the petals of carnation, it is known that 3' and 5' are glycosylated and the glycosides produced contain malyl groups bonded. The pathway shown in Figure 2 (p.5) also applies to the synthesis of flavonol which is colorless by itself though can indirectly govern the color of flowers by forming a complex with anthocyanin. It is also known that the pH of vacuole of petal cell can affect the flower color.

Outline of anthocyanin biosynthetic pathway in this recombinant carnation is presented in Figure 3 (p.6). The orange-red carnations contain pelargonidin 3,5-(malyl) diglucoside which has a single hydroxyl group (only 4' is hydroxylated) in the B ring of anthocyanin, and the purple-red carnations contain cyanidin 3,5-(malyl) diglucoside which has two hydroxyl groups (3' and 4' are hydroxylated) in the B ring of anthocyanin. Under natural condition, there never exists such carnation that contains delphinidin 3,5-(malyl) diglucoside which has three hydroxyl groups (3', 4', and 5' are hydroxylated) in the B ring of anthocyanin.

Pattern of hydroxylation of B ring is governed by the flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H). Their hydroxylation reactions take place in the state of dihydroflavonol and the enzymes hydroxylate dihydrokaempferol. The dihydroflavonol is a precursor of the flavonol and therefore, without the presence of both hydroxylases, pelargonidin 3,5-(malyl) diglucoside and kaempferol accumulate. In the presence of F3'H, cyanidin 3,5-(malyl) diglucoside and quercetin exist. Carnation does not contain F3'5'H and thus, delphinidin 3,5-(malyl) diglucoside does not exist.

Therefore, by transferring the *DFR* gene derived from petunia and the *F3'5'H* gene derived from pansy into carnation in white color in which anthocyanidin synthesis never take place due to the lack of dihydroflavonol 4-reductase (DFR) activity, delphinidin is produced in the petals and the flower color changes to purple-violet. In addition, the produced delphinidin is transformed to delphinidin 3,5-(malyl) diglucoside by the flavonoid 3-glycosyl-transferase (3GT) contained in the delphinidin produced. In some carnations that contain methyltransferase (MT), petunidin is produced.

Where genes are transferred in plants, the positions of the transferred genes on the chromosome vary according to the transformed plant line, and the extent of the functions of the transformed genes is considered to depend on the transferred positions. In addition, available functions may also depend on the origins of the transferred genes and promoters involved, and these factors govern the expression levels of the transferred genes and the levels of resultantly synthesized anthocyanin (depth of flower color), thereby contributing to provide the lines exhibiting various flower colors (see Annex 8).

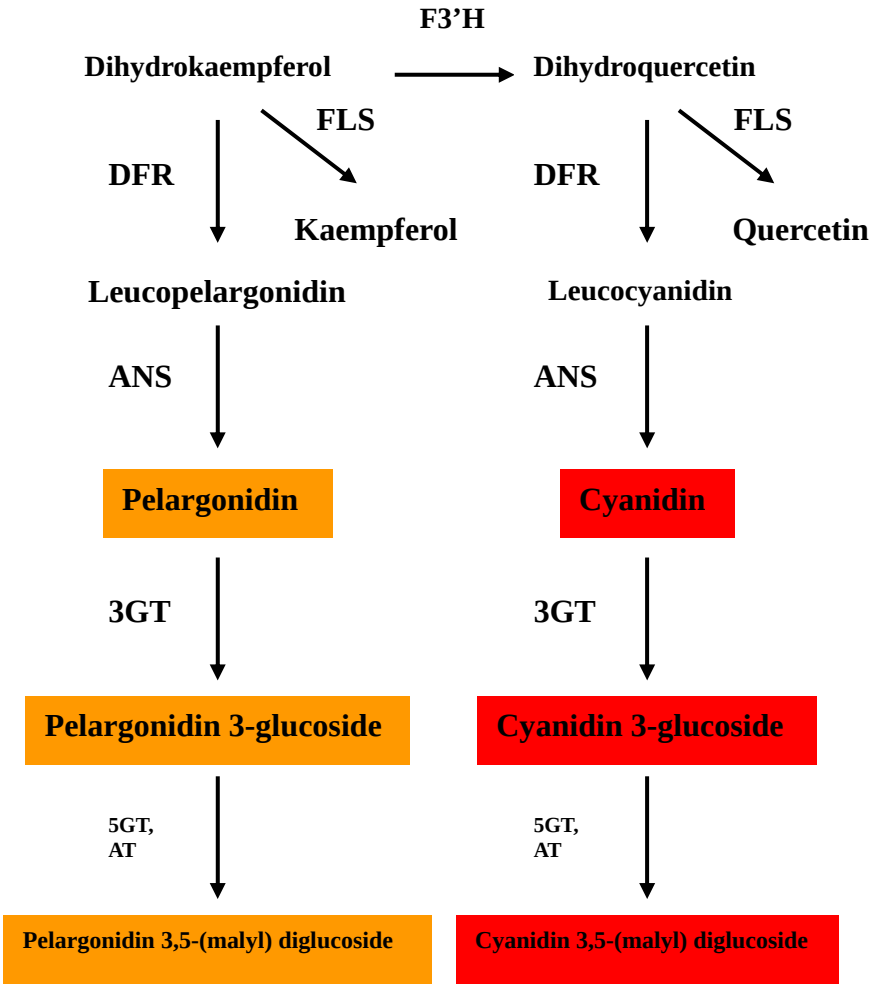


Figure 2 Outline of anthocyanin biosynthesis pathway in the non-recombinant carnation

In the non-recombinant carnation, cyanidin type anthocyanin and/or pelargonidin type anthocyanin are accumulated.

(Note) F3'H: Flavonoid-3'-hydroxylase, FLS: Flavonol synthase, DFR: Dihydroflavonol 4-reductase, ANS: Anthocyanidin synthase, 3GT: Flavonoid 3-glucosyl-transferase, 5GT: Flavonoid 5-glucosyl- transferase, AT: Acyltransferase

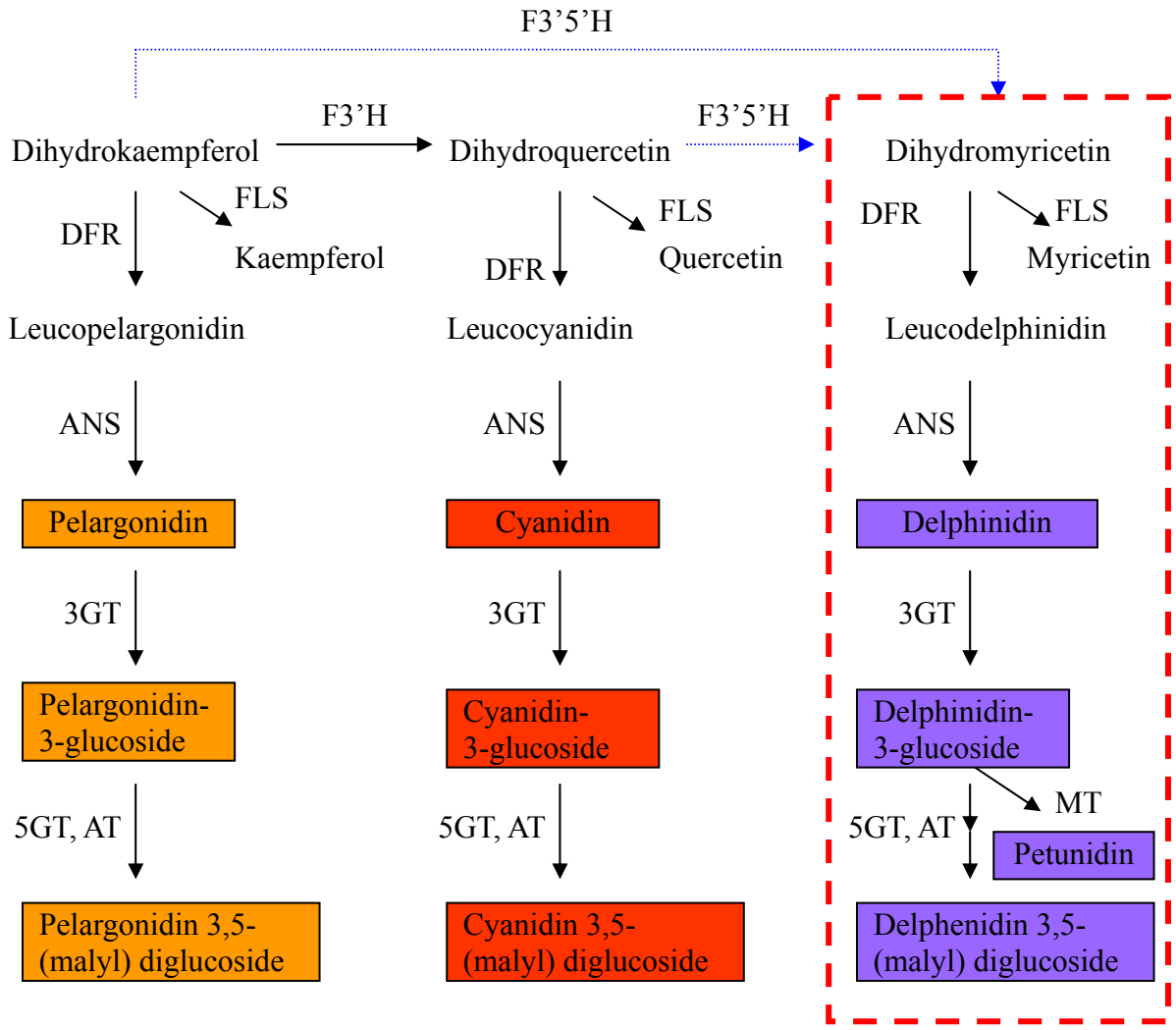


Figure 3 Outline of anthocyanin biosynthesis pathway in this recombinant carnation

In the non-recombinant carnations, the blue dotted pathways do not exist. The other pathways exist in both recombinant and non-recombinant carnation plants. Transferring of the *F3'5'H* gene derived from pansy enables biosynthesis of dihydromyricetin, which results in the accumulation of delphinidin-3-glucoside, a blue anthocyanin, in the petals. In carnation, the delphinidin-3-glucoside is further modified to delphinidin-3,5-(malyl) diglucoside.

(Note) F3'H: Flavonoid 3'-hydroxylase, F3'5'H: Flavonoid 3',5'-hydroxylase, FLS: Flavonol synthase, DFR: Dihydroflavonol 4-reductase, ANS: Anthocyanidin synthase, 3GT: Flavonoid 3-glycosyl-transferase, 5GT: Flavonoid 5-glycosyl-transferase, AT: Acyltransferase, MT: Methyltransferase

* The area enclosed by the red dotted box represents the pathway which is newly synthesized by the function of the transferred gene

(2) Function of component elements

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

a. 35S promoter:

Promoter region from cauliflower mosaic virus *35S RNA* gene. An essential component to allow the downstream neighboring gene to be expressed in the transgenic plant.

The cauliflower mosaic virus possesses circular double-stranded DNA as the genomic DNA and contains the gene expression-regulating site required for autonomous replicating and propagation in the nucleus of host cell by using the gene expression system of the recipient plant. The promoter for the *35S RNA* gene, one of the genes coded on the genome DNA, is known as 35S promoter, and it drives high levels of expression in almost every organ of plant body and at any stage of the growth and therefore, it is frequently applied for expression of foreign genes in plants. (Mitsuhara et al., 1996[21)]).

b. *sur*B gene:

A variant form of the *acetolactate synthase* (*ALS*) gene isolated from tobacco cell culture. The branched-chain-amino-acid valine, leucine, and isoleucine are similar to each other in structure and therefore they are often biosynthesized by the same enzyme. In microorganisms, isoleucine and valine are biosynthesized from their respective precursors, L-threonine and pyruvic acid. After the L-threonine is transformed into 2-oxobutyric acid, both are synthesized by the same five different enzymes. The enzyme catalyzing the first reaction is known as ALS. As a result of reaction of 1-hydroxyethyl-TPP produced by decarboxylation of addition compound of pyruvic acid and thiamin pyrophosphate (TPP) with another one molecule of pyruvic acid with the aid of ALS, acetolactic acid is produced and it acts as the precursor for synthesis of valine. On the other hand, as a result of reaction of the above 1-hydroxyethyl-TPP with 2-oxobutyric acid, 2-aceto-2-hydroxybutyric acid is produced and it acts as the precursor of isoleucine. ALS is normally inhibited by sulfonylurea type herbicide chlorsulfuron, though it is known to offer tolerance to chlorsulfuron as a result of variation of the *ALS* gene in the tobacco cell culture growing in the presence of chlorsulfuron at lethal levels (US Patent Number 5 141 870[22)]). Therefore it is also applied as a selectable marker for the transgenic plant. This variant form of ALS exhibits the same ALS activity as possessed by the original ALS as the enzyme activity. This ALS variant gene is named *sur*B gene. The sulfonylurea type herbicide also includes methylsulfonmethyl, Tribenuron, and Thifenosulfuron. For selection of the recombinant plant, chlorsulfuron was used.

c. *Dihydroflavonol 4-reductase* (*DFR*) gene:

The *dihydroflavonol 4-reductase* (*DFR*) gene from petunia, or a DNA fragment derived from the chromosome DNA including promoter, translated region, and 3' untranslated region.

This enzyme reduces dihydroflavonol and produces leucoanthocyanidin. The leucoanthocyanidin is the direct precursor of anthocyanidin. Among DFRs, DFR derived from petunia can reduce dihydroquercetin and dihydromyricetin as substrates, though it cannot reduce dihydrokaempferol (Beld et al., 1989 [23], Huits et al., 1994 [24]). This suggests that the DFR from petunia is a suitable DFR for production of delphinidin.

d. *Chalcone synthase* (*CHS*) gene promoter:

The *chalcone synthase* (*CHS*) gene promoter from *Antirrhinum majus* (Sommer, 1988 [25]). The *CHS* gene contains a gene fragment of 1.2kb in the 5' side of initiation codon. The *CHS* gene is one of the enzymes which are involved in the synthesis of flavonoids. By using this promoter, the high level of expression of CHS in petal epidermal cells is expected.

e. Flavonoid 3',5'-hydroxylase (F3'5'H) cDNA:

Derived from pansy. As shown in Figure 3 (p.6), it is an enzyme catalyzing the hydroxylation of the B ring of dihydroflavonol, such as converting dihydrokaempferol to dihydromyricetin, or dihydroquercetin to dihydromyricetin.

f. 3' untranslated region of *D8* gene:

The *D8* gene codes the phospholipids transfer protein of Petunia. The sequence used in this recombination is a DNA fragment having the approximate size of 0.8kb, including the region having the approximate size of 150 bp which can be transcribed but not translated (Holton, 1992 [26], International Patent Application PCT/AU/00334: Publication No. WO93/01290 [27]). In cases as in this recombination scheme where three expression cassettes (minimum units for expression of genes from promoter to 3' untranslated region through gene coding region) are linked on a single binary vector for expression of the *sur*B gene, the *DFR* gene, and the *F3'5'H* gene, it is preferred to use different promoters and/or 3' untranslated regions for individual expression cassettes for stable expression of transferred genes. Therefore, 3' untranslated region of the *D8* gene was used as the terminator for *F3'5'H* cDNA expressed in the pansy petals.

g. *lacZ*:

A part of the *lacZ* gene, which codes β-galactosidase of *E. coli*. β-galactosidase is an enzyme which hydrolyses lactose to galactose and glucose. Utilizing this activity, the *lacZ* gene is widely used as a reporter gene. In the vector containing multi-cloning sites in the *lacZ* gene, transferring of any DNA fragments into the multi-cloning sites causes failure of expression of active β-galactosidase. This provides a benefit of increased working efficiency since the presence of

β-galactosidase activity may be used as evidence of freedom from transferring of any DNA fragments. The pWTT2132 vector used in this recombination scheme also contains multi-cloning sites in the *lacZ* gene for the benefit described above. In practice, however, in the binary vector actually transferred in cultivars of carnation, which is shown in Figure 1 (p.3) and Annex 1-Figure 1 (p.1), multi-cloning sites in the pWTT2132 contain other gene expression cassettes transferred and thus, production of proteins offering the β-galactosidase activity is not attained. In addition, the *β-galactosidase* gene promoter does not function in plants and therefore, in transgenic plants, neither active β-galactosidase nor any peptide fragment derived from β-galactosidase could be produced.

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

The F3'5'H derived from pansy converts dihydrokaempferol to dihydromyricetin, or dihydroquercetin to dihydromyricetin, and the DFR derived from petunia converts dihydroquercetin and dihydromyricetin to leucocyanidin and leucodelphinidin respectively. In addition, the ALS, a protein encoded by the *sur*B gene isolated from tobacco, exhibits tolerance to chlorsulfuron herbicide.

As to whether or not these proteins offer homology with any protein which is known to possess any allergenicity, "Non-Food Allergen sequence" in the "Allergen sequence db" was searched using the database SWISS-PROT with the result that there was no homology identified with these proteins.

In addition, the *β-galactosidase* gene promoter does not function in plants and therefore, in transgenic plants, neither active β-galactosidase nor any peptide fragment derived from β-galactosidase could be produced and thus this would not become an allergen.

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

By the F3'5'H derived from pansy, dihydrokaempferol is converted to dihydromyricetin, or dihydroquercetin to dihydromyricetin. In addition, by the DFR derived from petunia, dihydroquercetin and dihydromyricetin are converted to leucocyanidin and leucodelphinidin respectively. Consequently, delphinidin is produced.

(2) Information concerning vectors

1) Name and origin

A synthetic plasmid derived from *E. coli* and *Agrobacterium*, pWTT2132 (DNA Plant Technology (DNAP) in the US), was used as the vector. It contains the tetracycline-resistant gene derived from the plasmid pSC101 possessed by *E. coli*, multi-cloning sites derived from *E. coli*, and T-DNA left border and right border sequences derived from *Agrobacterium*.

2) Properties

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

pWTT2132 is a 18,648 bp binary vector, and the nucleotide sequence is provided in Annex 2-Figure 1 (p.1).

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

pWTT2132 represents tetracycline resistance. It contains the *sur*B gene (derived from tobacco), which gives tolerance to chlorsulfuron herbicide and used as a selectable marker, and T-DNA left border and right border sequences. Replication origin (ori) contains pVS1 ori from *Pseudomonus aeruginosa* and pACYC184 ori derived from *E. coli*. To the plants, only the sections within the left border and right border are transferred.

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

Infectivity with any other bacteria is not identified.

(3) Method of preparing living modified organisms

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

Structure of the binary vector pCGP1991 is outlined in Figure 1 (p.3), and the nucleotide sequence is provided in Annex 1-Figure 1 (p.1). The binary vector pCGP1991 has the approximate size of 27.5 kbp, including approximately 14.0 kbp of the T-DNA region between the left border and right border. The T-DNA region which is transferred to the recipient plant contains the *sur*B gene to be used as the selectable marker for the recombinant plant and the petunia *DFR* gene and the pansy *F3'5'H* gene for the modification of flower color.

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

The *Agrobacterium* method (US Patent number 5 589 613 [28)]) was used for the plant transformation.

From May 1996 to October 1997, *Agrobacterium tumefaciens* Agl0 strain was inoculated to the stem pieces of surface sterilized seedlings of carnation cultivar FE123. Light purple recombinants were obtained from July 1997 to August 1998. At present, they are maintained by vegetative propagation.

3) Processes of rearing of living modified organisms

This recombinant carnation is raised based on the assumption that the gene transferred generation proliferates by vegetative propagation. The application for approval of this recombinant carnation is intended only for the current generation of recombination.

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

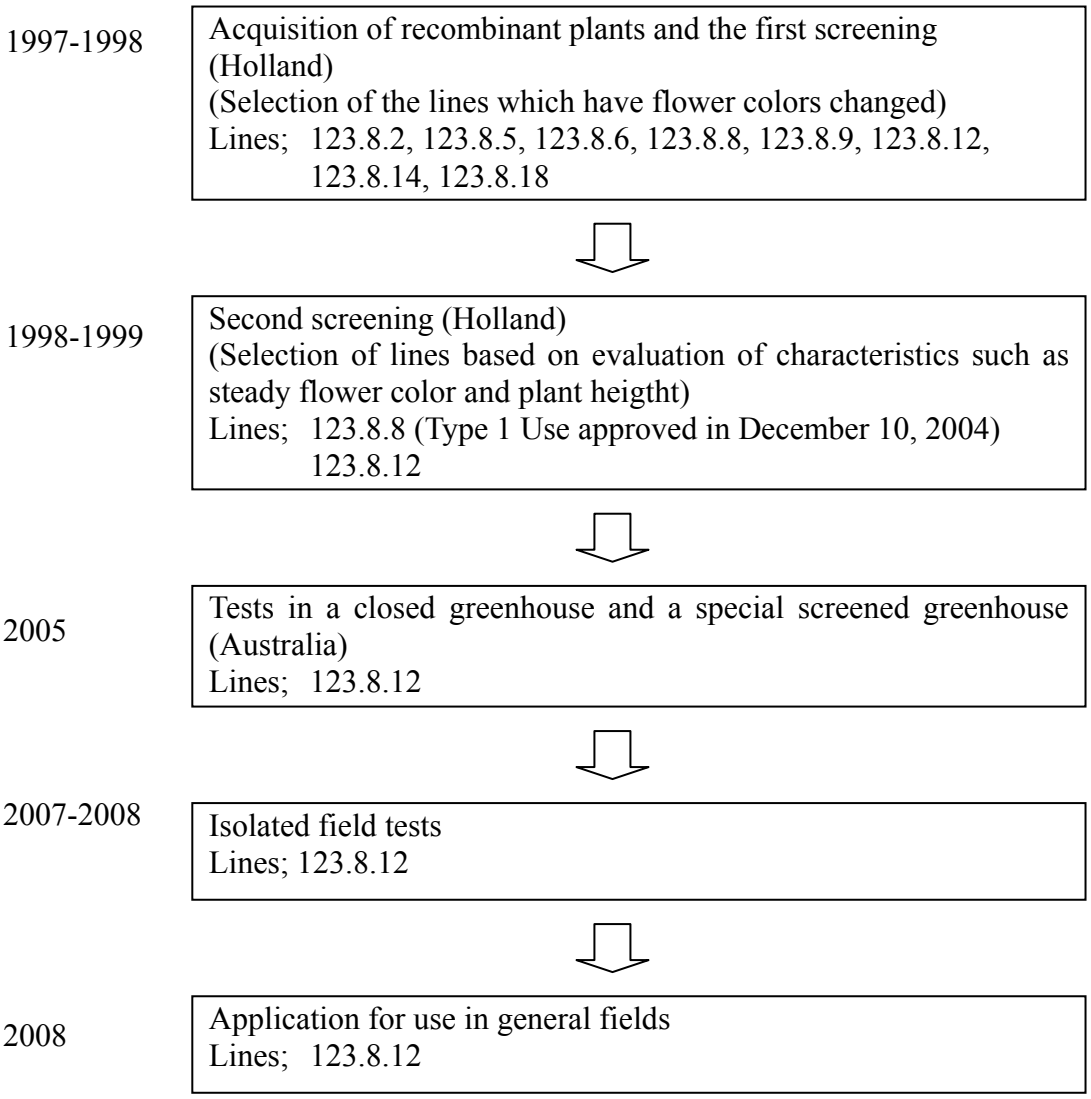
For selection of recombinants, a selective medium containing chlorsulfuron (1-5μg/L) was used.

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

By applying extracts from the leaves of this recombinant carnation plant onto the selective medium allowing *Agrobacterium* which contains the transferred gene to grow, observation was made for any growing colony to identify any residual *Agrobacterium* which contains the transferred gene. However, there was no growing colony observed which was considered *Agrobacterium*.

Consequently, it was judged that there is no residual *Agrobacterium* which contains the transferred gene in this recombinant carnation.

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



(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

The transferred nucleic acid is considered to exist on the chromosome of this recombinant carnation. Eternally transferred nucleic acids are normally transferred onto the chromosome. In practice, however, though with extremely low probability, transferred nucleic acids may be transferred into the organellar genome of chloroplast, etc. The pansy *F3'5'H* gene, one of the nucleic acids transferred to the recombinant plant, intrinsically exists on the nuclear genome, and the translation product F3'5'H is translated in the cytoplasm then transferred to the endoplasmic reticulum (ER), thereby offering the intrinsic enzyme function. Assuming the *F3'5'H* gene is transferred to the organellar genome, the translation product cannot move from the organelle to ER and then, it is considered to fail to offer the intrinsic function. However, in this recombinant carnation, delphinidin is actually produced by the function of F3'5'H which is the translation product of the *F3'5'H* gene. This suggests that the *F3'5'H* gene and the other genes on the T-DNA are considered to exist on the chromosome. In addition, as a result of Southern blotting analysis, it is considered that there are several copies of transferred nucleic acid in this recombinant carnation, though, based on the above understanding, it is considered that at least one of the copies exists on the nuclear genome (see Annex 3, p.1-5). In addition, in consideration of the fact that the *Agrobacterium* method has a very low probability of transferring the genes into organellar genome, it is considered likely that the transferred nucleic acids are mostly or all present in the nuclear genome.

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, three signals were detected when the pansy *F3'5'H* gene was used as a probe, and one signal was detected when the *sur*B gene was used as a probe. This suggests that T-DNA has been transferred in the genome of carnation (see Annex 3, p.1-5).

The recombinants have been all proliferated by the vegetative propagation and then, the stability of inheritance through multiple generations has not been identified. To examine the stability of the transferred genes when vegetative propagation is repeated, Southern blotting analysis was conducted using the plant bodies vegetative-propagated in different periods (June 2005 and November 2007). As a result, in all the plant bodies examined, the identical signal pattern was obtained. This suggests that the transferred genes in this recombinant carnation stably exist even if vegetative propagation is repeated (see Annex 3, p.6-8).

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

As a result of Southern blotting analysis, signals were detected in several fragments of the pansy *F3'5'H* gene and then it is considered that there are several copies of transferred nucleic acid. There may be a possibility that several copies of the transferred genes would exist on a single fragment appearing as one signal, though there is no result obtained from the analyses on the flanking sequence of nucleic acids transferred onto

chromosome, showing several copies are present adjacent to each other. Consequently, it is considered that the transferred genes are located separately.

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

Regarding the expression of the transferred pansy *F3'5'H* gene and the petunia *DFR* gene in the petals, Northern blotting analyses were carried out. As a result, for the *F3'5'H* gene, signals, which are specific to the transferred genes, were detected only in the recombinants, which indicate expression of the genes transferred into the genome (see Annex 3, p.25-26). For the petunia *DFR* gene, the level of expression was too low to detect and then additional RT-PCR analyses were carried out. As a result, amplification products, which are specific to the gene, were detected (see Annex 3, p.27-28). Consequently, it was found that the transferred gene in the genome is expressed.

Additionally, to examine the stability of expression of transferred genes when vegetative propagation is repeated, RT-PCR analyses were carried out using the plant bodies vegetative-propagated in different periods (June 2005 and November 2007). As a result, in all the plant bodies examined, amplification products, which are specific to the transferred genes, were detected (see Annex 3, p.29-30). Consequently, it was found that the transferred gene in the genome is stably expressed even if vegetative propagation is repeated.

The color of flowers obtained from the expression of the transferred genes is a stable light purple in this recombinant carnation (see Annex 5, p.3-7 and Annex 6, p.7-11), which indicates the expression of the transferred genes in the genome. Also for the individuals proliferated by vegetative propagation, the uniformity of flower color is maintained, and there is no case reported referring to occurrence of any flower color other than light purple.

Consequently, stable expression of the genes transferred into the genome is expected.

In addition, only in the case of tissue culture of this recombinant carnation, chlorsulfuron is added to the medium, though stable chlorsulfuron tolerance is available by expression of the *sur*B gene.

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

There are some viruses identified which can infect carnation, though occurrence of viruses is decreasing due to the recent widespread use of virus-free seedlings. In fact, there is no case reported referring to the fact that transferred genes are transmitted to wild animals and wild plants through horizontal transmission due to viral infection. Therefore, it is considered extremely low that the transferred nucleic acid in this recombinant carnation plant would be transmitted to wild animals and wild plants due to viral infection.

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

The genomic sequence in the neighboring regions of T-DNA transferred in the genome of this recombinant plant was identified. Based on the sequence information, PCR primers were prepared and the conditions allowing detection and identification specifically for this recombinant plant were determined. This method was confirmed to allow detection of the recombinant plant by using the genome DNA of 500 ng at a minimum for reaction.

More information regarding the methods of detection and identification of living modified organisms can be found in Annex 4, p.1-2.

Their sensitivity and reliability can be found in Annex 4, p.3.

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

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

By transferring the pansy *F3'5'H* gene and the petunia *DFR* gene to the recipient organism and succeeding over-expression of the genes in petals, delphinidin was produced and the flower color changed to light purple (see Annex 5, p.3-7 and Annex 6, p.7-11). The pansy F3'5'H is considered expressed in petals since it uses a petal-specific promoter, and the petunia DFR is considered expressed in petals, the organ where the gene is intrinsically expressed, since it contains the DNA fragments including the promoter derived from chromosome DNA transferred.

In addition, it was also identified by use of the chlorsulfuron-added medium that expression of the *sur*B gene transferred as a selectable marker contributes to offer tolerance to herbicide chlorsulfuron.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

These were evaluated based on the data from the tests carried out in the closed greenhouse and special screened greenhouse which are located inside Florigene Pty. Ltd. in Australia in 2005, the tests carried out in the isolated field which is located in the Tsuyama-Misaki field of Nihon-Shokusei Co., in 2007 to 2008. Like the recipient organism, cultivation by harvested seeds is not carried out for the recombinant plant.

(a) Morphological and growth characteristics

The recipient organism and the recombinant plant were cultivated in a closed greenhouse and special screened greenhouse to identify the growth characteristics, i.e., stem length at flowering time, and the morphological characteristics, i.e., survival rate of anthers, length of anther, width of anther, number of petals, and diameter of flower. In the survival rate of anthers among the items examined, a

statistically significant difference was observed between the recipient organism and the recombinant plant (Student *t* test, level of significance of 5%). Specifically, the survival rate of anthers was 73.3% for the recipient organism compared to 20.0% for the recombinant plant. Regarding the stem length at flowering time, length of anther, width of anther, number of petals, and diameter of flower, no significant difference was observed between the recipient organism and the recombinant plant (see Annex 5, p.8, p.10).

In addition, the recipient organism and the recombinant plant were cultivated in an isolated field to identify the growth characteristics, i.e., plant height, changes in the number of nodes over time, and flowering time, and the morphological characteristics, i.e., number of anthers, length of anther, width of anther, number of petals, and diameter of flower. In the diameter of flower among the items examined, a statistically significant difference was observed between the recipient organism and the recombinant plant (Student *t* test, level of significance of 5%). Specifically, the diameter of flower was 7.5±0.2 cm on average for the recipient organism compared to 7.3±0.2 cm on average for the recombinant plant.

Regarding the plant height, number of nodes, number of anthers, length of anther, width of anther, and the number of petals, no significant difference was observed between the recipient organism and the recombinant plant. For flowering time, a large difference was not observed between the recipient organism and the recombinant plant (see Annex 6, p.12-15).

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

Cultivars of carnation are not fertilizable under natural conditions and thus they do not set any seed. Propagation by seed is available only by cultural practices and therefore cold-resistance and heat-tolerance at the early stage of growth of seed were not evaluated.

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

Carnation cultivars favor a cool climate at around 20°C and therefore they are cultivated in temperature-controlled greenhouses in hot summer season in Japan. However, in the eight-years experience in cultivation of the recipient organism and the recombinant plant in Melbourne in Australia where the temperature in vinyl greenhouses can rise to up to 43-45°C in summer, both species have survived the summers and no visible difference has been observed in plant height and other growth characteristics. In consideration of a possible maximum temperature of around 35°C during the summer in Japan in an average year and also the results in Melbourne, it is considered that both plants could successfully survive summer. However, since there is no information available about mature plant in the winter condition in Japan in which the minimum temperature becomes below zero, wintering ability of mature plant was examined in the isolated field tests. As a result, all the individuals examined successfully survived the winter, and no difference was observed between the recipient organism and the recombinant plant (see Annex 6, p.18-19).

(d) Fertility and size of the pollen

The anthers and the pollens contained therein of the recipient organism and the recombinant plant grown in the non-closed greenhouse and special screened greenhouse were examined visually, and the existence of pollen was not confirmed (see Annex 5, p.8). Also for the recipient organism and the recombinant plant cultivated in the isolated field, the existence of pollen was not confirmed (see Annex 6, p.16-17).

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

Cultivars of carnation are not fertilizable under natural conditions and thus they do not set any seed. Propagation by seed is available only by cultural practices and therefore production, shedding habit, dormancy and germination rate of seed were not evaluated.

(f) Crossability

As a result of examination for the existence of pollen of the recipient organism and the recombinant plant cultivated in the closed greenhouse, special screened greenhouse and isolated field, no pollen was confirmed in both recipient organism and the recombinant plant (see Annex 5, p.8 and Annex 6, p.16-17).

(g) Productivity of harmful substances

Cultivars of carnation have long been cultivated and used, though production of harmful substances by carnation cultivars has not been reported in any country, including Japan.

In order to identify the possibility that the transferred genes could affect the metabolism of the recombinant plant and cause productivity of any harmful substance, a plow-in test and succeeding crop test were carried out for any effect on germination of lettuce seeds. As a result, no statistically significant difference was observed between the recipient organism and the recombinant plant cultivated in the closed greenhouse, special screened greenhouse and isolated field (see Annex 5, p.12-13 and Annex 6, p.20-21). In addition, as a result of soil microflora test, no statistically significant difference was observed in the number of fungi, bacteria, and actinomycetes between the recipient organism and the recombinant plant (see Annex 5, p.14 and Annex 6, p.22). Moreover, there is no report that delphinidin and myricetin produced newly in this recombinant carnation by the transferred genes would be harmful.

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

Carnation cultivars (*Dianthus caryophyllus* L.) have long been cultivated and used also in Japan, though there has been no case reported referring to wild growing in natural environment in any country, including Japan.

As a result of examination on the characteristics relating to competitiveness, a statistically significant difference was observed between this recombinant carnation and the recipient organism regarding the survival rate of anthers in the special screened greenhouse test and the diameter of flower in the isolated field test. However, based on the findings that the existence of pollen in the anthers of recipient organism and recombinant carnation was not observed and that no significant difference was observed in all the characteristics examined but the survival rate of anthers and diameter of flower, it is considered unlikely that the differences in survival rate of anthers and diameter of flower could enhance the competitiveness of this recombinant carnation plant.

In this recombinant carnation plant, due to delphinidin and myricetin produced in petals with expression of the transferred *DFR* gene and the *F3'5'H* gene, the flower color changed to purple-violet. However, based on the fact confirmed by the investigation of flower visiting insects in the isolated filed using this recombinant carnation plant that there is almost no insect observed visiting the flowers of carnation, it is considered extremely low that cultivation of this recombinant carnation plant could change the flower visiting insect fauna.

In addition, this recombinant cultivar of carnation is conferred with the tolerance to chlorsulfuron due to the *sur*B gene, though chlorsulfuron is considered not to become a selective pressure under natural environment and thus it is considered unlikely that this trait could enhance the competitiveness of this recombinant cultivar of carnation.

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

(2) Productivity of harmful substances

Productivity of any harmful substances by carnation cultivars to wild animals and wild

plants has not been reported.

This recombinant carnation induces biosynthesis of bluish anthocyanins due to the expression of the transferred genes. However, they are also contained in the petals of bluish pansy and petunia, and they are not reported to possess any harmful effect on the other wild animals and wild plants.

In addition, as a result of investigation of the productivity of this recombinant carnation of any harmful substances (the substances secreted from the roots which can affect other plants and microorganisms in soil, the substances existing in the plant body which affect other plants after dying), no significant difference from the non-recombinant carnation was observed.

As a result of amino acid sequence homology search, it was confirmed that the ALS protein, the DFR protein, and the F3'5'H protein do not share structurally related homologous sequences with any known allergens.

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 carnation poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

Some limited cultivars of carnation can cross with wild relatives of the genus *Dianthus* and in Japan, 4 species and 2 variants are growing wild. The possibility of crossing of this recombinant carnation with those wild relatives was evaluated in terms of the characteristics of pollen, insect pollination, and wind pollination.

(i) Characteristics of pollen: Pollen production by cultivars of carnation is scarce or nonexistent, and the fertility of pollen, even if existent, is low. In addition, the longevity of pollen is 1-2 days, and germination is not observed at all on the 3rd day or after. Based on the characteristics of carnation cultivars described above, crossing of this recombinant carnation under natural condition is considered very difficult. Moreover, as a result of examination of existence and germination rate of pollen of the recipient organism and the recombinant carnation, existence of anthers was observed, though existence of pollen was not observed. Therefore, the possibility of crossing of this recombinant carnation is considered least. However, should this recombinant carnation produce pollens, there is a possibility of crossing with wild relatives through insect pollination and/or wind pollination and then these factors were also evaluated.

(ii) Possibility of crossing by insect pollination: Carnation cultivars have a long distance between the edge of the petals and the nectary and then neither butterflies nor moths can extract the nectar, and few other flower-visiting insects can be observed visiting carnation flowers. Since the flowers of wild relatives of the genus *Dianthus* have their nectaries at their base, only insects with a long proboscis can reach the nectary, and butterflies and other insects with a shorter proboscis never visit *Dianthus* flowers. Ants may visit the flowers, though the sphere of activity of ants is limited to several meters and it is generally known that the secretion of ants

inactivates the pollens; therefore, transmission of pollens by ants is scarce. Due to the similarity of this recombinant carnation to cultivars in flower shape and other characteristics, the possibility of crossing by insect pollination is considered least.

(iii) Possibility of crossing by wind pollination: For carnation cultivars, the anthers are covered in petals, pollen production is scarce, and pollens are very sticky, then it is considered extremely low that the pollens would disperse due to the wind transmission. Similarly to cultivars, anthers of this recombinant carnation are also buried in petals and thus the possibility of pollen dispersion is low. It was reported that pollens of carnation cultivars have not been detected in air in Holland despite the fact that carnation cultivars are widely cultivated there.

Based on the above understanding, it is considered very seldom that this recombinant carnation could cross with wild relatives. Therefore, the following conclusion made by the applicant was judged reasonable: There is no risk of Adverse Effect on Biological Diversity caused by crossability.

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 carnation 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 reasonable.

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