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

Name of Entity: Suntory Holdings Limited
Name of Applicant: Takeshi Niinami, President
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Approved Type 1 Use Regulation

Names of types of living modified organisms	Blue-purple colored carnation tolerant to chlorsulfuron herbicide（*F3'5'H, DFR, sur*B, *Dianthus caryophyllus L.*）（11363, OECD UI: FLO-11363-2）
Content of Type 1 Use of living modified organisms	Use for ornamental purposes, cultivation, storage, transportation, and disposal, and other acts attendant with these.
Method of Type 1 Use of living modified organisms	—

Summary of the Evaluation on Adverse Effect on Biological Diversity

Information Collected for the Evaluation for its Impact on Biodiversity.

Information about Preparation of Living Modified Organisms

1. Information about Preparation of Living Modified Organisms

(1) Information of Donor Nucleic Acid

a. Derivation of construction and elements.

Table 1 below shows the constitution of donor nucleic acid and its components, and Figure 1 (P5) is its configuration map. The nucleotide sequence is shown in Figure 1 of Appendix1 (P1-26) and Figure1 of Appendix 2 (P1-26).

Table 1 Constitution, Origin and Function of Components of Donor Nucelic Acid

Component	Position in Plasmid	Origin and Function
T-DNA Region		
Left Border Region	1-689	Left Border Region derived from Ti plasmid of *Agrobacterium tumefaciens,* to be cut as T-DNA. Tinland 1996; Zupan et al. 2000
Intervening Sequence	690-695	Sequence used for cloning Sambrook et al. 1989
35S Promotor	696-889	35S RNA gene promotor derived from cauliflower mosaic virus (Approx. 0.2kb shorter than normal 35S RNA gene promotor at the 5' end side) Franck et al.1980
Intervening Sequence	890-957	Sequence used for cloning
*sur*B	958-2949	Acetolactate synthase gene derived from tobacco. Imparting chlorsulfuron resistance. Lee et al.1988
*sur*B 3'	2950-4713	3'-Untranslated region of acetolactate acid synthase gene derived from tobacco, Lee et al.1988

Intervening Sequence	4714-4834	Sequence used for cloning
Lac Z promoter	4835-4956	LacZ promotor sequence derived from cloning vector derived from *Escherichia coli*.
DFR genomic DNA	4957-9943	Dihydroflavonol 4-reductase gene derived from petunia (containing promotor, translated region and 3'-untranslated region), Beld et al. 1989
Intervening Sequence	9944-10006	Sequence used for cloning
CHS	10007-11142	Chalcone synthase gene promotor derived from *Antirrhinum*, Sommer H and Saedler H, 1986.
Intervening Sequence	11143-11186	Sequence used for cloning
F3'5'H cDNA	11187-12960	cDNA of flavonoid 3',5'-hydroxylase gene derived from pansy Katsumoto et al. 2007
Intervening Sequence	12961-12981	Sequence used for cloning
D8 3'	12982-13793	3'-Untranslated region of phospholipid transferase gene derived from petunia, Holton 1992
Intervening Sequence	13794-13811	Sequence used for cloning
lacZ	13812-13947	A part of β-galactosidase gene derived from *Escherichia coli*. Sequence used for cloning
Intervening Sequence	13948-13981	Sequence used for cloning
Right Border Region	13982-15825	Right Border Region derived from Ti plasmid of *Agrobacterium tumefaciens,* to be cut as T-DNA. Tinland, B, 1996; Zupan et al. 2000
Exoskeletal Region (Not existing in this recombinant carnation.)		
Intervening Sequence	15826-15835	Sequence used for cloning

pVS1 replicon	15836-23721	Replicon in *A. tumefaciens* derived from *Psudomonas aeruginos*a, Ito et al. 1988; Lazo et al. 1991
Tc resistance	23722-25768	Region containing genes, *Tet*A (24550-25749) and *Tet*A (24550-25749) coding for protein imparting resistance to tetracycline derived from *Escherichia coli*, Backman and Boyer , 1983.
Modified pACYC184 replicon	25769-27296	Replicon of plasmid in *Escherichia coli,* Chang ACY and Cohen SN, 1978 ; Rose 1988
Intervening Sequence	27297-27488	Sequence used for cloning

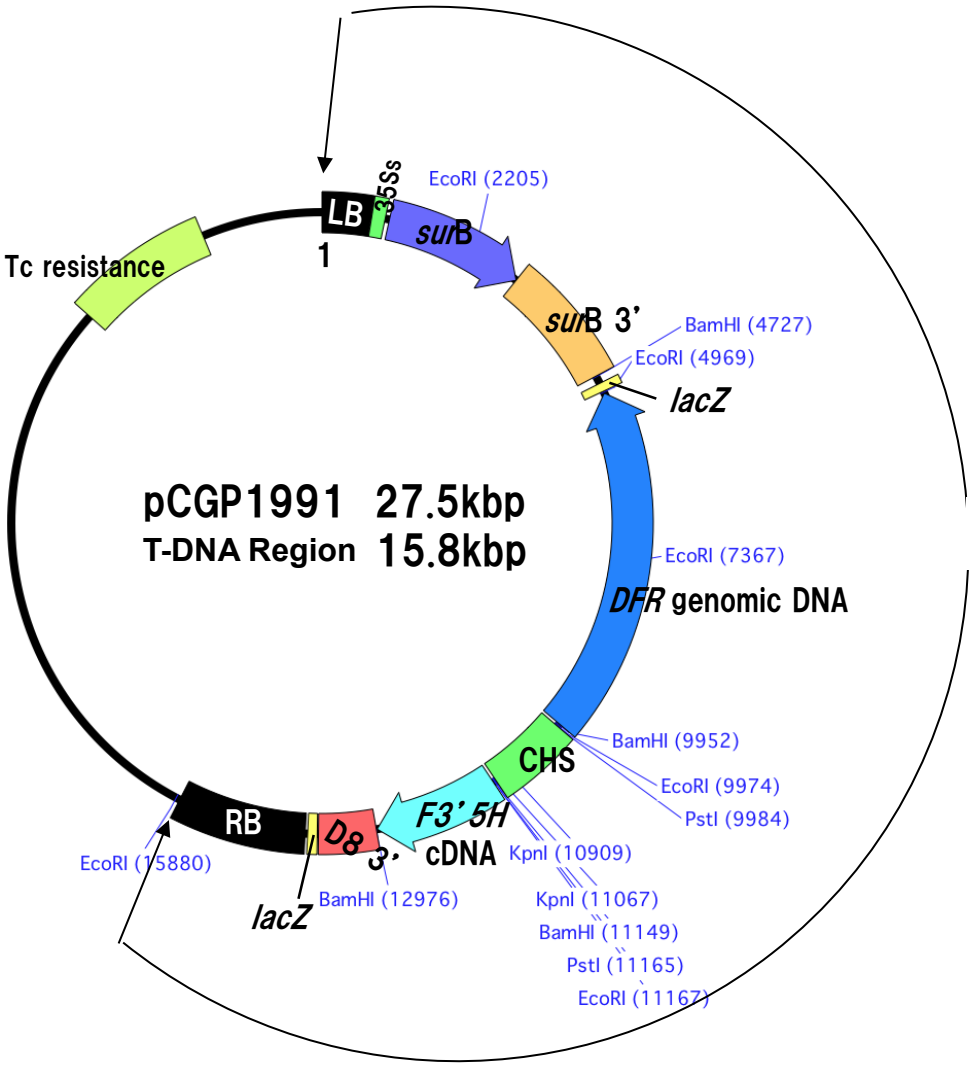


Figure 1. Plasmid map of pCGP1991 used for generating this recombinant carnation

In the process for generating this recombinant carnation, T-DNA region in the above figure is inserted into the genome. The numbers shown with the names of restriction enzymes indicate the cutting positions (bp) when “1” indicates Left Border end.

b. Functions of the components

① Function of each component of donor nucleic acid including objective genes, expression regulatory regions, localization signals, selection markers, and each component in other donor.

Table 1 (P2~4) shows the functions the components of donor nucleic acid used for generating this recombinant carnation.

② Function of proteins produced by the expression of the objective gene and the selection marker, as well as the fact that the protein shares homology with proteins having allergenicity, if applicable.

[*sur*B protein]

This is a variant acetolactate synthase *(ALS)* gene derived from tobacco-cultured cells. Branched amino acids such as valine, leucine and isoleucine are synthesized with the same enzyme because they have similar structures. In microorganisms, L-threonine and pyruvic acid are the precursors for the synthesis of isoleucine and valine, respectively. Both are synthesized by five kinds of common enzymes after L-threonine is converted to 2-oxobutyric acid. The enzyme catalyzing the first reaction is called ALS. When 1-hydroxyethyl-TPP produced by the decarboxylation of the addition compound of pyruvic acid and thiamine-pyrophosphoric acid, is reacted with pyruvic acid of another molecule by ALS, acetobutyric acid is produced, which will become the precursor for synthesizing valine. On the other hand, 2-aceto-2-hydroxybutyric acid that is the precursor of isoleucine is produced when the above 1-hydroxyethyl-TPP reacts with 2-oxobutyric acid. ALS is usually inhibited by sulfonylurea-based chlorsulfuron herbicide, however, it was found that a mutation occurred in *ALS* gene in tobacco-cultured cells growing in the presence of lethal amount of chlorsulfuron, and it consequently exhibited resistance to chlorsulfuron(US patent number 5 141 870). From the above reason, ALS is used as the selection marker for transgenic plants as well. This mutant ALS exhibits the same enzymatic activity as the original ALS. This *ALS* mutant gene is designated as *sur*B gene. It is concluded that the endogenous metabolism was hardly affected by introducing *sur*B gene because the host also has ALS activity. As other kinds of sulfonylurea-based herbicides, there are methylsulfone methyl, tribenuron, thifensulfuron and bensulfuron methyl,

and it is known that this *sur*B gene exhibits resistance to chlorsulfuron and bensulfuron methyl at least (Shimizu et al., 2011). We used chlorsulfuron for selecting this recombinant carnation.

[Flavonoid 3',5'-hydroxylase protein]

Derived from pansy. As shown in Figure 3 (P10), this is an enzyme which hydroxylates dihydroflavonol B-ring, and catalyzes the reaction converting dihydrokaemferol or dihydroquercetin to dihydromyricetin.

[Dihydroflavonol 4-reductase protein]

This enzyme reduces dihydroflavonols (referring to dihydrokaemferol, dihydroquercetin and dihydromyricetin (See Figure 2 and 3)), and produces leucoanthocyanidin (referring to leucopelargonidin, leucocyanidin and leucodelphinidin (See Figure 2 and 3)). Leucoanthocyanidins are direct precursor of anthocyanidins (referring to pelargonidin, cyanidin and delphinidin (See Figure 2 and 3)). DFR has a substrate specificity, and especially the DFR derived from petunia is able to reduce dhydroquercetin and dihydromyricetin as substrates but not dihydrokaemferol (Beld et al., 1989, Huits et al., 1994). Therefore, petunia-derived DFR is considered to be appropriate for producing delphinidin.

To see whether or not those proteins share homology with other proteins which are found to have allergenicity, we conducted a search in November 2014 over Allergen Online version 14 of the University of Nebraska (updated on January 20th 2014) , and didn't observe any sequences that matched the known allergens for more than 6 amino acids.

③ Detail of changes made in the host's metabolic system if applicable

[Flavonoid 3',5'-hydroxylase protein]

By the introduced the flavonoid 3',5'-hydroxylase protein derived from pansy (F3'5'H, Figure 3), dihydrokaemferol or dihydroquercetin is converted to dihydromyricetin. F3'5'H is an enzyme which catalyzes the reaction to hydroxylate flavonoids specifically; therefore it will not modify any other pathways.

[Dihydroflavonol 4-reductase protein]

By the introduced dihydroflavonol 4-reductase derived from petunia (DFR, Figure

2) Dihydromyricetin is converted to leucodelphinidin. Consequently, delphinidin which does not exist in the host carnation is produced. By flavonol synthase (FLS) of the carnation itself, in addition, dihydromyricetin is converted to myricetin which does not exist in the host carnation. DFR is an enzyme that is specific to dihydroflavonols; therefore, it will not modify any other metabolic pathways.

The following explains the synthetic pathway of anthocyanins and the effect of the transgenes in carnation.

A part of the biosynthetic pathway of anthocyanin is shown in Figure 2 (P9). The biosynthetic pathway of anthocyanins is common in the plant kingdom, and anthocyanins are synthesized in carnation as well by the pathway shown in Figure 2 (P9). It is known that 3-position and 5-position of anthocyanins exiting in the petals of carnations are glycosylated, and mallyl group is bound to these glycosides. Flavonols, which are colorless themselves but influence the flower color indirectly when they form complexes with anthocyanins, are also synthesized in the pathway shown in Figure 2 (P18). In addition, it is known that the pH of vacuoles in a petal cell influences the flower color.

A part of the biosynthetic pathway of anthocyanins in this recombinant carnation is shown in Figure 3 (P10). Carnation containing pelargonidin 3,5-(mallyl) diglucoside in which anthocyanin B-ring has one hydroxyl group (only 4' is hydroxylated) exhibits orange-red color, and carnation containing cyanidin 3,5-(mallyl) diglucoside in which anthocyanin B-ring has two hydroxyl groups (only 3' and 4' are hydroxylated) exhibits slightly purplish red. On the other hand, carnation containing delphinidin 3,5-(mallyl) diglucoside in which anthocyanin B-ring has three hydroxyl groups (3', 4' and 5' are hydroxylated) does not exist in nature.

The hydroxylation pattern of B-ring is determined by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H). These hydroxylation reactions occur at the stage of dihydroflavonols (referring to dihydrokaemferol, dihydroquercetin and dihydromyricetin (See Figure 2 and 3)), and these enzymes hydroxylate dihydrokaemferols.

As dihydroflavonols is the precursor for flavonols as well, pelargonidin 3,5-(mallyl) diglucoside and kaemferol are accumulated in the absence of hydroxylases. Where F3'H exists, cyanidin 3,5- (mallyl) diglucoside and quercetin also exist. Because F3'5'H does not exist in carnation, delphinidin 3,5-(mallyl) diglucoside does not exist, either.

In this context, if petunia-derived *DFR gene* and pansy-derived *F3'5'H gene* are

introduced into the carnations with white flowers because anthocyanidins are not synthesized due to the lack of dihydroflavonol 4-reductase(DFR) activity, delphinidin is produced in the petals, the color of the carnations will turn bluish purple. The produced delphinidin is converted to delphinidin 3,5-(mallyl) diglucoside by enzymes such as endogenous flavonoid 3-glycosidase(3GT). In some carnations with methyltransferase activity, petunidin is produced.

When genes are introduced to a plant, the positions of chromosomally inserted transgenes vary by the transgenic lines; therefore, it is considered that the degree of function of each gene will depend on the inserted positions. Furthermore, there is possibility that it will depend on the origins or promotors of transgenes as well, therefore, they will influence the expression level or the amount of anthocyanins consequently synthesized (flower color intensity), which will result in the lines with various flower colors (See Appendix 8).

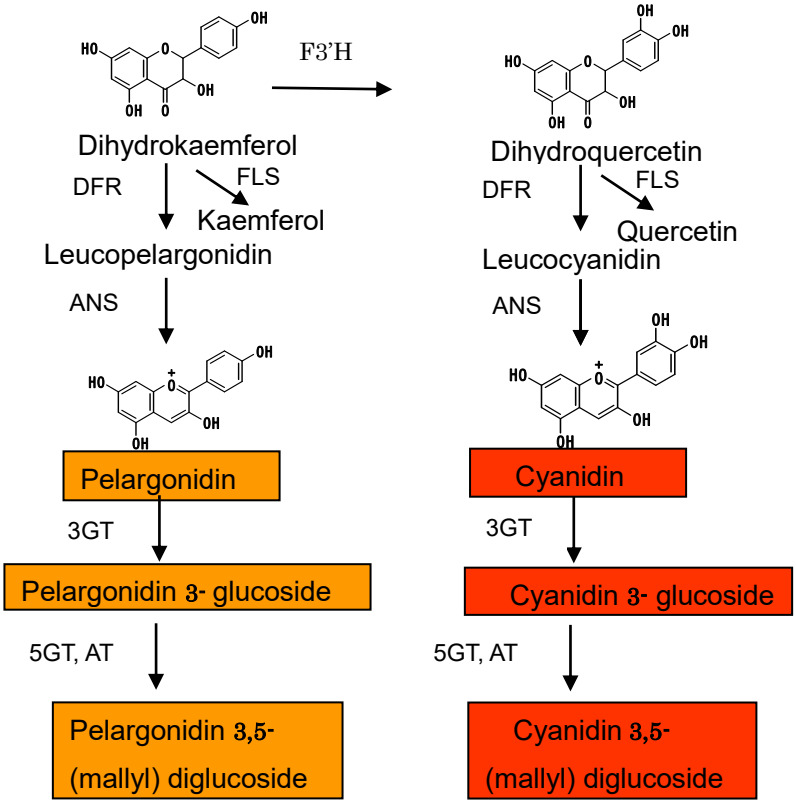
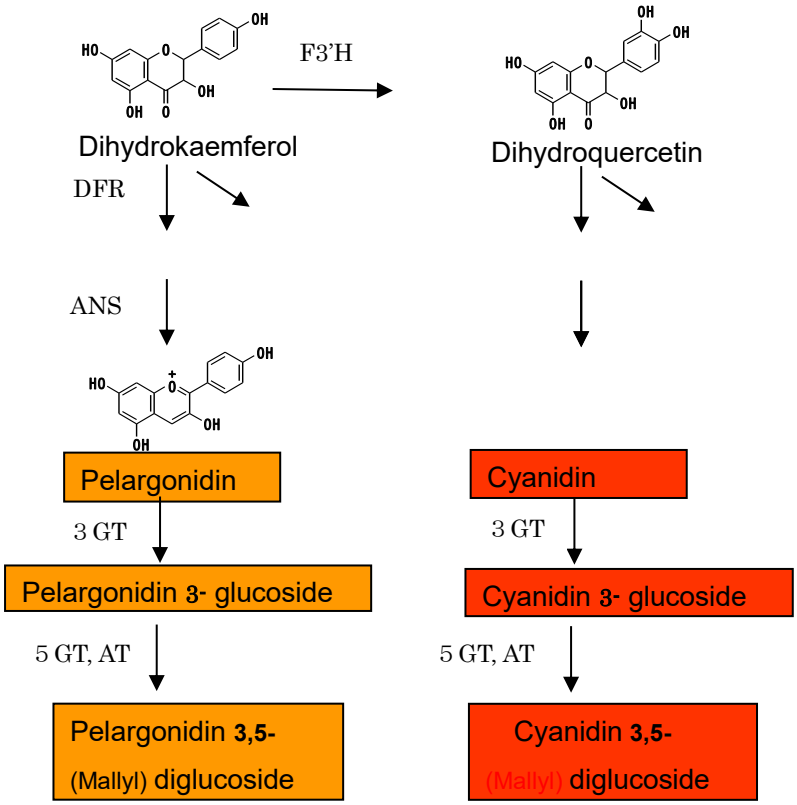


Figure 2. Outline of anthocyanin biosynthetic pathway in non-recombinant carnation

In the non-recombinant carnation, cyanidin type and pelargonidin type Anthocyanin accumulate.

(Note) F3'H: Flavonoid 3'-hydroxylase, FLS: Flavonol synthase, DFR:

Dihydroflavonol 4-reductase, ANS: Anthocyanidin synthase, 3GT: Flavonoid 3-glycosylation enzyme, 5GT: Flavonoid 5- glycosylation enzyme, AT: Acyl transferase



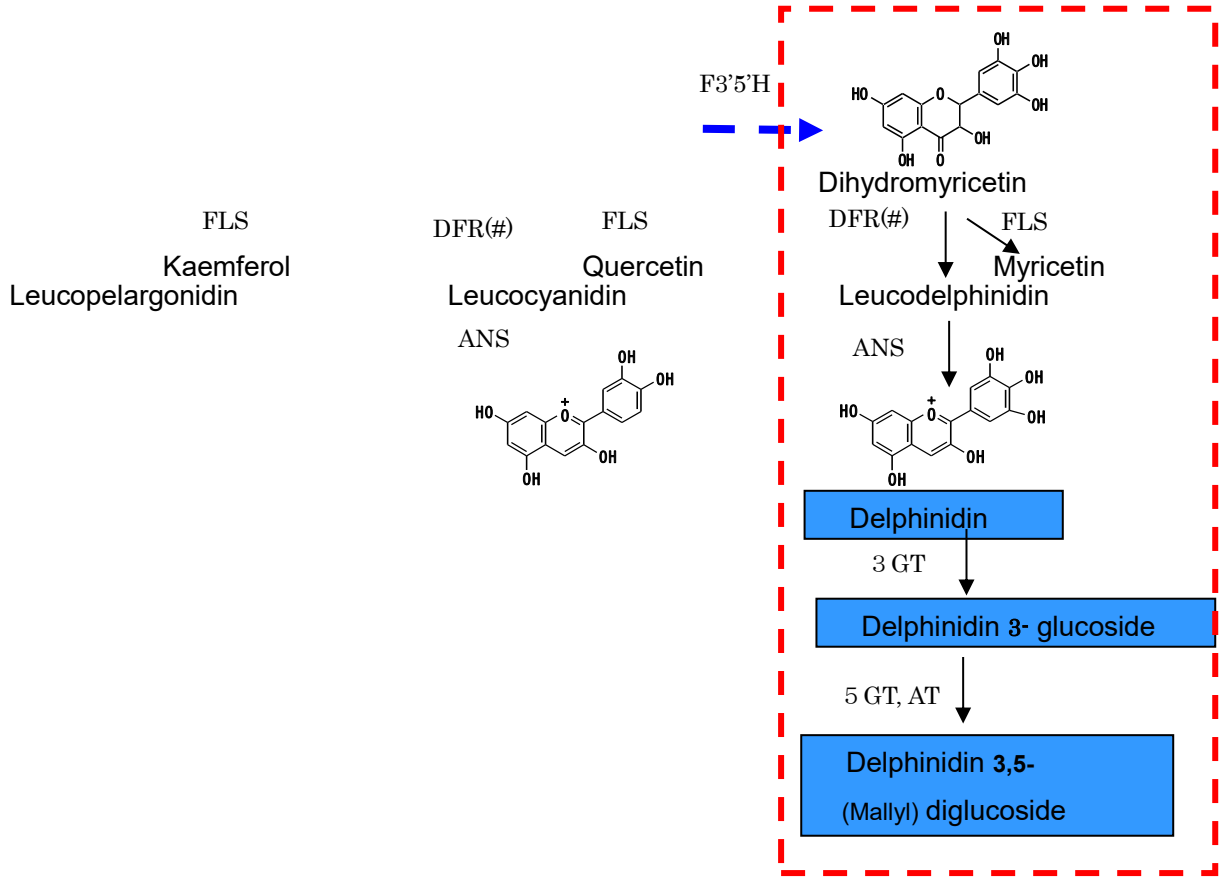


Figure 3. Outline of anthocyanin biosynthesis pathway in this recombinant carnation

The pathway indicated with a blue dashed arrow does not exist in the non-recombinant carnation. But the other pathway exist in both of the recombinant and non-recombinant carnations. Biosynthesizing dihydromyricetin by introducing pansy-derived *F3’5’H gene, a* bluish anthocyanin, delphinidin 3-glucoside is accumulated in the petals. In carnations, it will be further modified to be delphinidin 3,5- (mallyl) 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-glycosylation enzyme, 5GT: Flavonoid 5-glycosylation enzyme, AT: Acyl transferase, MT: Methyl transferase

∗ The pathway newly synthesized by the transgene function is marked with a red dashed line.

∗ DFR marked with (#) mark is an enzyme newly expressed by the transgenes, and it is suitable to produce delphinidin because it efficiently reduces dihydromyricetin. This DFR can reduce dihydroquercetin as well.

(2) Information of Vector

A Name and Origin

The vector pCGP1991 used for generating this recombinant carnation was constructed based on *Escherichia coli* and *Agrobacterium*-derived synthetic plasmid pWTT2132 (DNAP USA). It contains tetracycline-resistant genes derived from plasmid pSC101 retained by *Escherichia coli and* a multi-cloning site derived from *Escherichia coli,* as well as *Agrobacterium*-derived T-DNA Left Border and Right Border sequences.

B Characteristics

① Nucleotide number and sequence of vector

The nucleotide number of pCGP1991 is 27,488 bp, and the nucleotide sequence of T-DNA is shown in Figure 1 (p.1-21) of Appendix 1.

② Specific function of base sequence if any

Tetracycline-resistant gene imparting tetracycline-resistance is included in the selection marker for construct vector in *Escherichia coli* but this gene is not introduced to this recombinant carnation. As the selection marker for this recombinant, surB gene imparting resistance to chlorsulfuron herbicide is introduced to this recombinant.

③ Whether or not vector has infectivity, and the host range information if it has infectivity

This vector does not contain any sequence with known infectivity.

(3) Preparation method for living modified organisms

A. Constitution of whole nucleic acid transferred into the host

Figure1 (P5) shows the position and direction of the components in vector pCGP1991 used for generating this recombinant carnation, and the cutting positions by restriction enzymes.

B. Method to transfer nucleic acid into the host

Agrobacterium mediated method was used for introducing nucleic acid into the host.

We inoculated 10 seedlings of *Agrobacterium tumefaciens* into the surface-sterilized UNESCO stalk pieces from October to November 1995, then obtained the blue-purple colored recombinant DNA organisms from July to November 1996. They are currently maintained by vegetative propagation.

C. Growing process of living modified organisms

This recombinant carnation is maintaining by propagating the present generation with the introduced genes by vegetative propagation.

① How to select cells to which nucleic acid is transferred

We used the selection medium containing chlorsulfuron (1-5μg/l) for selecting this recombinant carnation.

② Presence or absence of remaining *Agrobacterium* cells if nucleic acid is transferred by *Agrobacterium* method

We removed the *Agrobacterium* used for generating this recombinant carnation by adding Ticarcillin to the medium at tissue culture. We also spread the extract from the leaves of this recombinant DNA organism on the selection medium capable of growing *Agrobacterium* having transgenes, and checked whether any *Agrobacterium*-containing the transgenes was remaining or not. However, no colony like *Agrobacterium* was observed.

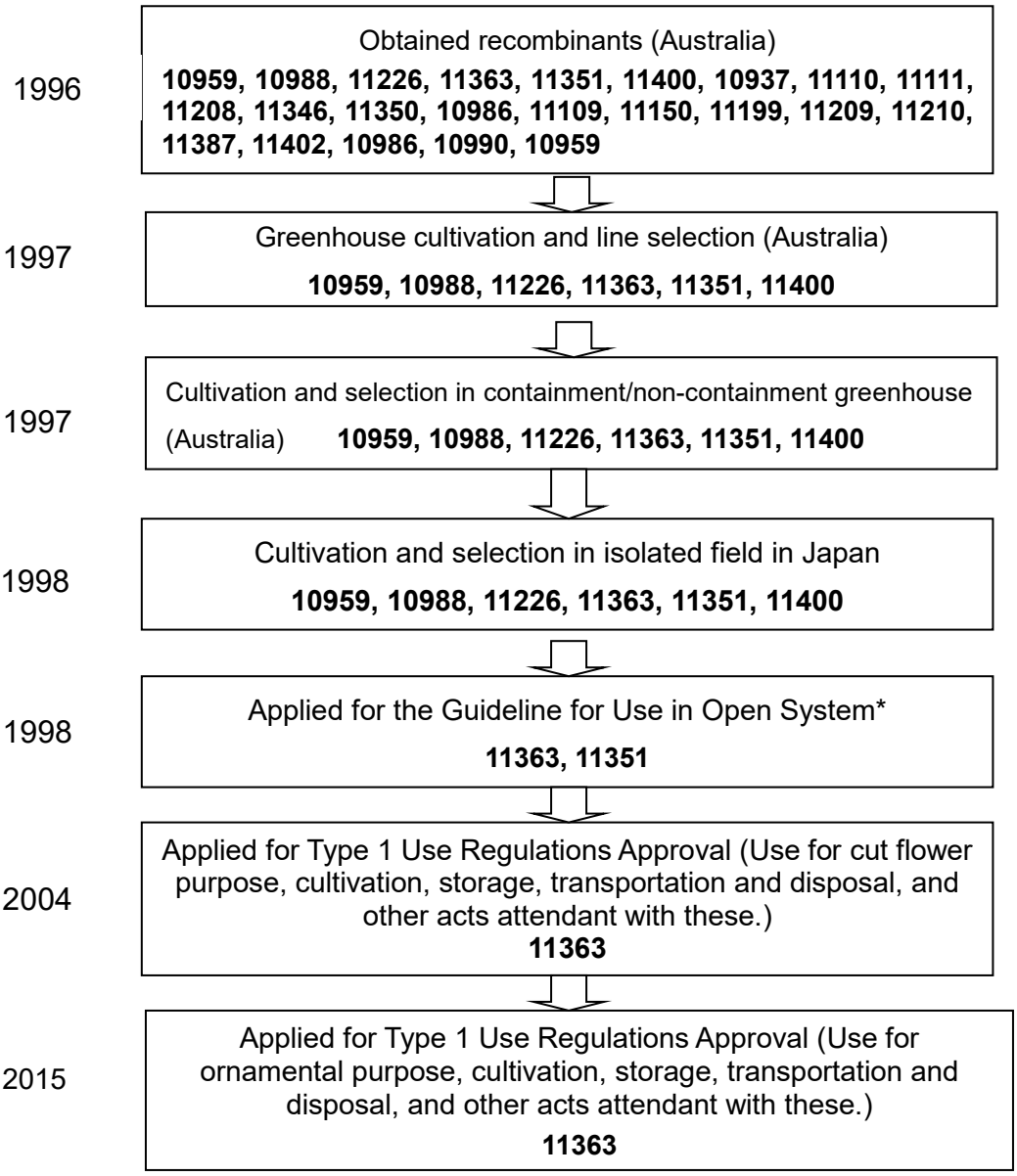
Also, none of the tetracycline gene being a part of the outer skeletal region of plasmid was detected from this recombinant carnation either by PCR method or Southern blotting method (Appendix 10). *Vir*G gene that *Agrobacterium* has on Ti plasmid was not detected by PCR method using genome DNA obtained from various portions of the scion (Appendix 9, P1-3). In addition, although we have maintained

the original plant of the recombinant DNA organism in tissue culture for more than 15 years because they are vegetatively propagated, no propagation of *Agrobacterium* was observed in the culture medium.

Therefore, we have reached the conclusion that there is no transgene-containing *Agrobacterium* remaining in this transgenic carnation.

③ The breeding process regarding the cell with nucleic acid transferred the line in which the existing condition of a copy of the transferred nucleic acid was confirmed, the line subjected to an Isolated Field Experiment, Other lines used for collecting the information necessary to their the Biological Diversity Risk Assessment Tests.

As carnations are propagated vegitatively, this recombinant carnation is the T0 generation of the transformation. We selected superior lines from many independent recombinant lines.



* “Guidelines for the Use of Recombinant DNA Organisms in Agriculture, Forestry and Fisheries” (From 1989 to 2004)

(4) Existing condition of nucleic acid transferred into a cell and stability of phenotypic expression by this nucleic acid

① The location where the copy of transferred nucleic acid exists

From the result of Southern blot analysis, it was concluded that the nucleic acid transferred into this recombinant carnation existed in three locations. As all the sequences adjacent to the introduced genes matched the sequence for carnation genome, the transferred nucleic acids are thought to exist chromosomally (See Appendix 3-1, P3-6 and Appendix 3-2, P9).

② Copy number of transferred nucleic acid and transmitting stability of nucleic acid copies through generations

By performing Southern blot analysis, it was concluded that the transferred sequences existed at three positions in this recombinant carnation. The transferred sequence is thought to be the total or partial length from LB to RB of T-DNA (See Appendix 3, P2). By the way, we have not analyzed the transmitting stability through generations because all these recombinant DNA organisms are produced by vegetative propagation and only the T0 transformant exists.

Although the vegetative propagation of this carnation since its generation in 1996, it has never exhibited any different colors; therefore, it is considered that the transferred nucleic acid exists stably in this recombinant DNA organism.

③ If there are more than two copies existing on chromosome, whether they are neighboring or separately placed to each other

They were considered to be located separately because the transferred nucleic acid exists in scaffold having different carnation draft genome sequences (Table1, Appendix 3-2, P9).

④ Stability of expression between individuals or generations under the natural conditions, regarding the characteristics specified in ① in (6).

The Northern blot analysis was performed on the expression in petals of the transferred pansy F3’5’H gene and petunia DFR gene. In this analysis, the signals specific to the transgenes were detected only in this recombinant carnation, and it was revealed that the inserted genes in the genome were expressing (See Appendix 3-1, P7). In addition, the flower color generated as a result of the transgene expression is blue-purple in this recombinant carnation and the expression is stable. Other individual carnations reproduced by vegetative

propagation also have maintained the same blue-purple color without any exception.

From the above, it is concluded that the expression of the genes inserted to the genome is stable.

Moreover, chlorsulfuron-added medium is used for this recombinant carnation only in the case of tissue culture, but it has stable resistance to chlorsulfuron due to the expression of *sur*B gene.

⑤ If there is possibility that the transferred nucleic acid will be transmitted to wild plants and animals by a virus infection or via any other routes, whether it is transmissible or not and the level of transmission if it is.

—

(5) Method to detect and identify the living modified organisms as well as its detection sensitivity and reliability

It is possible to specifically detect and identify this recombinant carnation by Southern blot analysis (Appendix 3-1, P3-6). Regarding its detection sensitivity, it is detectable by using about 20μg chromosomal DNA (Appendix 3-1, P3-6).

Furthermore, the conditions enabling specific detection and identification of this recombinant carnation was clarified by creating PCR primers based on the information of genome sequences around the region of T-DNA inserted into this recombinant carnation genome (Appendix 4, P4-6). The detection sensitivity was 10 ng (Appendix 4, P6).

(6) Difference from the host or taxonomic species the host belongs to

① Detail of physiological and ecological properties imparted by expression of the copy of transferred nucleic acid

As a result of making overexpression of pansy *F3'5'H* gene and petunia *DFR* gene in the host, delphinidin was produced and the flowers turned to blue-purple (See Appendix 5 P3-5, Appendix6 P9-10).

Pansy *F3'5'H* expresses on the petals because it is under the control of petal-specific promoter, and petunia *DFR* expresses in the petal which is its original organ because it has introduced genomic DNA fragment containing the promotor region.

In addition, it was confirmed by using chlorsulfuron-added medium that chlorsulfuron tolerance was imparted by the expression of *sur*B gene which was

introduced as a selection marker.

② Whether there is physiological or ecological difference between genetically modified crops and the taxonomic species that their hosts belong to, and the degree of difference if any

From 1977 to 1998, Isolated Field Experiment was performed in the special net (containment) greenhouse and the isolated field in Suntory Research Center. We used this recombinant carnation as the test sample, and used the culivated variety UNESCO as the non-recombinant control carnation. Just like its host, it is not cultivated by seed propagation.

a Morphological and growth characteristics

While cultivating the host and this recombinant carnation in the special net greenhouse, we researched the growth characteristics such as stem length at flowering, and the morphological characters such as the number of petals, the flower diameter, as well as the length and width of anthers. As a result, there was statistically significant difference (significance level 5%, t test) observed between the host and this recombinant organism in terms of the petal number and flower diameter as well as the anther length and width (See Appendix5 P8). As for the number of petals, the host had 46.0±4.2 pieces, and on the other hand, the recombinant had 39.0±8.5 pieces. The flower diameter was 3.2±4.4 cm for the host and 3.97±5.5 cm for the recombinant. The anther length and width were 2.2±0.9mm and 0.7±0.2mm respectively for the host, on the other hand, 3.00±1.1mm and 1.00±0.4mm for the recombinant. Since there are many garden species of carnations with a variety of flower sizes and forms, and this recombinant flower is included in the range of garden species and its pollen is not capable of germination, it was concluded that it has no adverse effect on biological diversity even though there is significant difference.

While cultivating the host and this recombinant carnation in the greenhouse in the isolated field, we examined the morphological and growth characteristics such as plant height, number of nodes, flowering period, as well as anther length and width, but there was no statistically significant difference observed between the host and the recombinant in terms those characteristics (See Appendix 6 P11). Therefore, it was concluded that there was no difference between the host and this recombinant organism in the morphological and growth characteristics.

b Low/high temperature tolerance in initial growth stage

The cultivated variety of carnations is neither fertilized nor forming seeds in the natural condition. As seed propagation can only be performed by artificial means, we did not study the high/low temperature tolerance of seed-derived plants in its

initial growth stage. As for the low-temperature tolerance of young plants, which is derived from cutting propagation, we conducted a wintering character test by planting seedlings 10-15cm in height and having about 1 node in the isolated field. But no difference was observed between the host and the recombinant plants in their growth, therefore, it is concluded that there is no difference in the low-temperature as well (See Appendix 6 P19). In addition, the study on high-temperature tolerance in the initial growth stage was not conducted because cutting propagation for carnations is usually performed in spring time and the plant becomes mature in summer, or in the case that cutting is performed in summer or winter in a greenhouse under the artificial condition.

c Wintering ability of full-grown plant

We cultivated the host and the recombinant carnations in the isolated field, and found that all of them survived in winter and no difference was observed in their growth (See Appendix 5, P19). For this reason, it was concluded that there is no difference between the host and this recombinant in terms of wintering ability. The summering ability of adult plants was not studied because the cultivar carnations like cool temperature, about 20 degree C; therefore, in a hot summer environment in Japan they are only cultivated in the greenhouse at the artificially controlled temperature. However, there is an example that the hosts and this recombinant have been cultivated for 7 years in Melbourne, Australia where the highest temperature in the greenhouse reaches 43 – 45 degrees C in summer, but both of them have survived summers and no difference has been observed in the growth such as plant height. Taking account of this result in Melbourne and the average highest temperature in summer in Japan, which is approximately 35 degrees C, it is concluded that both of them are able to survive Japanese summer as well.

d Fertility and size of pollen

As a result of visual monitoring of the anthers and pollens contained in the host and this recombinant carnation, which were grown in the special net greenhouse, pollens were observed in both of them (See Appendix5 P6). We conducted a germination test with these pollens but no germination was observed (See Appendix 5, P7).

In addition, when we cultivated the host and this recombinant carnation in the isolated field, pollens were visually observed in their anthers. However, no difference was observed in the size of these pollens by examining under microscope (See Appendix 6, P14). After that, we conducted the pollen germination test, however, no

germination was observed, either (See Appendix 6, P15-16).

e Production amount, shattering habit, dormancy and germination rate of seeds

No cultivated varieties of carnations are fertilized nor form seeds in the natural condition. As seed propagation is only possible by artificial means, the study was not conducted regarding the production amount, shattering habit, dormancy, or germination rate of the seeds.

f Crossing rate

There were pollens existing in both of the host and this recombinant carnation, however, no germination was observed in their pollen tubes (See Appendix 6, P15-16). In crossbreeding the pollens of the host and this recombinant to cultivated variety carnation and pink (Dianthus), seed formation was not observed, either.

On the other hand, as a result of artificial crossbreeding, the pistils were observed to be fertile and some seeds were formed, however, there was no difference observed between the host and this recombinant carnation, and the number of seeds was less than other kinds of cultivated variety carnations (See Appendix6, P18). Regarding their growth characteristics including the blooming period, there was no statistically significant difference observed between the host and this recombinant carnation (See Appendix 6, P11-12). From the above, it was concluded that there was no difference in the crossing rate between the host and this recombinant carnation.

g Productivity of harmful substances

We have experienced a long-term usage of the cultivated variety carnations, but the harmful substances produced by cultivated variety carnations has not been reported in any countries including Japan.

In order to clarify the possibility of transgenes to influence the metabolism of this recombinant carnation and produce harmful substances, we performed a plowing test and a post cultivation soli test using the seeds of Chinese cabbage in the special net greenhouse, and the seeds of lettuce in the isolated field. In both cases, no statistically significant difference (significance level at 5 % in t-test) was observed between the host and this recombinant carnation in terms of germination rate and

fresh weight of a seedling (See Appendix 5 P10-11 and Appendix 6, P20-21).

In addition, as a result of soil microflora test conducted in the special net greenhouse and the isolated field, no statistically significant difference (significance level at 5% in t-test) was observed between the host and this recombinant carnation in terms of the number of bacteria, actinomyces and filamentous fungi (See Appendix 5, P12 and Appendix 6, P22).

No.2 Results of review meeting for Adverse Effect Assessment 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) Results of Assessment on Adverse Effect on Biological Diversity

This recombinant carnation is generated by introducing *Escherichia coli* and T-DNA region of synthetic plasmid pCGP1991 originating from *Agrobacterium* by the *Agrobacterium* mediated method.

In this recombinant carnation, plural copies of *F3'5'H gene* encoding pansy-derived flavonoid 3',5'-hydroxylase, *DFR* gene encoding petunia-derived dihydroflavonol 4-reductase, and *sur*B gene encoding tobacco-derived acetplactate acid synthase are integrated at each of the three chromosomal positions. The expression of the target gene was confirmed by observing that (1) RNA is expressed by Northern blotting method and (2) the flower color of the individual plants selected after repeating vegetative propagation turned from white to blue-purple and it is expressed stably in the later generations.

(A) Competitiveness

Carnations have been cultivated in Japan for years, however, there have been no reports in any countries that these cultivated species escaped out of the greenhouse and grew in the natural environment.

In 1998, we cultivated this recombinant carnation as well as the non-recombinant carnation in the isolated field (vinyl greenhouse), and investigated the plant height, the number of nodes, flowering period, and the length and width of anthers, but no statistically significant difference was observed.

It was presumed to be possible that the color of this recombinant carnation was changed by the delphinidin produced in the petals due to the transgene expression and this color change influences the flower-visiting insect fauna. However, as a result of the comparative study on the flower-visiting insects between the other type of recombinant carnations producing the same substances such as delphinidin and the non-recombinant carnation, it is confirmed that no statistically significant difference was observed in terms of the number of those insects.

In addition, although this recombinant carnation is resistant to chlorsulfuron

herbicide, it is hard to expect that this resistance will improve its competitiveness in the natural environment where herbicide is unlikely to be sprayed.

From the above facts, we have reached the judgement that there is no risk of this recombinant carnation affecting on the biological diversity because of the competitiveness.

(B) Productivity of harmful substances

Carnations have been cultivated in Japan for years; however, there have been no reports in any countries that these cultivated species affected the growth and/or population of flora and fauna in the surrounding area.

Dihydroflavonol 4-reductase and flavonoid 3',5'-hydroxylase, which are produced by this recombinant carnation are the enzymes expressing in petunia and pansy, and it has not been reported that these enzymes had any harmful effects on wild animals and plants.

In fact, in the plowing test and post cultivation soil test, no statistically significant difference was observed between this recombinant carnation and the non-recombinant carnation in terms of the germination rate and fresh weight of seedlings of Chinese cabbage and lettuce. In the soil microflora test, there was no statistically significant difference observed between this recombinant carnation and the non-recombinant carnation in terms of the number of bacteria, actinomyces and filamentous fungi.

From the above, we have reached the judgement that there is no risk of this recombinant carnation affecting on the biological diversity due to the productivity of harmful substances.

(C) Crossability

Some of the cultivated varietyies of carnations are capable of being crossed artificially with wild relatives growing in Japan, such as *Dianthus superbus* L. var. *superbus, Dianthus kiusianus*, *Dianthus japonicus, Dianthus shinanensis, Dianthus superbus* L. var. *and Dianthus superbus* var. *speciosus*, however, there was no report that any of them hybridized with the wild relatives under natural conditions in Japan.

Additionally, it is also considered that the possibility of this recombinant carnation to be crossed with the wild relatives is very low because the pollens of its host are not capable of germination, insect pollination is very unlikely to happen because there are few insects visiting the flowers due to a long distance to the honey glands,

as well as that pollens are unlikely to be dispersed by wind due to its high viscosity. In fact, it was confirmed that no seeds were formed in the experiment of crossbreeding pollens of this recombinant carnation and the host carnation with *Dianthus superbus* L. var. *longicalycinus* and other cultivated carnations.

From the above, we have judged that there is no risk of this recombinant carnation affecting on the biological diversity due to its crossability.

(2) Conclusion based on the Biological Diversity Risk Assessment Report

Based on all of the abovementioned facts, we have reached a judgment that the conclusion of the Assessment on Adverse Effect on Biological Diversity, stating that there shall be no risk of affecting on the biological diversity in Japan if this recombinant carnation is used according to Type 1 Use Regulations, is reasonable.

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Appendix List of Blue-Purple Colored Carnation Tolerant to Chlorsulfuron Herbicide（*F3'5'H*, *DFR*, *sur*B, *Dianthus caryophyllus* L.）（11363, OECD UI: FLO-363-2）

Appendix 1 Total Construction of Nucleic Acid Introduced into the Host (Confidential / Not Open to the Public)

Appendix 2 Information of Vector (Confidential / Not Open to the Public)

Appendix 3-1 Existing Condition and Phenotypic Expression Stability of Introduced Nucleic Acid (Confidential / Not Open to the Public)

Appendix 3-2 Structural Analysis of Chromosomally Inserted Genes (Confidential / Not Open to the Public)

Appendix 4 Method to Detect and Identify the Living Modified Organisms As Well As Its Detection Sensitivity and Reliability (Confidential / Not Open to the Public)

Appendix 5 Result of Experiments in Containment / Non-containment Greenhouse (Confidential / Not Open to the Public)

Appendix 6 Result of Experiments in Isolated Field (Confidential / Not Open to the Public)

Appendix 7 Information Obtained From Overseas Usage (Confidential / Not Open to the Public)

Appendix 8 Relationship Between the Difference of Flower Colors among Blue-Purple Carnation Lines and the Composition of Anthocyanidin (Confidential / Not Open to the Public)

Appendix 9 Verification of the Persistence of Agrobacterium (Confidential / Not Open to the Public)

Appendix10 Verification of the Presence of Tetracycline-Resistant Genes

(Confidential / Not Open to the Public)

Appendix 11 Research on Flower Visiting Insects for Blue-purple Carnations
(Confidential / Not Open to the Public)