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

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Approved Type 1 Use Regulations

Names of Types of Living Modified Organisms	Purple-violet herbicide chlorsulfuron resistant carnation (*F3'5'H*, *DFR*, *dsDFR*, *sur*B , *Dianthus caryophyllus* L.) (25958, OECD UI: IFD-25958-3)
Content of Type 1 Use of Living Modified Organisms	Cultivation in isolated field, storage, transportation, disposal, and acts incidental to them
Method of Type 1 Use of Living Modified Organisms	—

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acids

1) Composition and origins of component elements

The composition and origins of component donor nucleic acids are provided below. Their positional relationship and nucleotide sequence are shown in Figure 1 (p. 11) and Figure 1 of Annex 1 (p. 1-24), respectively.

(a) Selectable marker *sur*B expression cassette

35Ss ：35S RNA gene promoter derived from cauliflower mosaic virus (the 5’-terminal region is about 0.2 kb shorter than that of the common 35S RNA gene promoter) 0.2 kb

*sur*B : Acetolactate synthase gene from tobacco (*Nicotiana tabacum*) 2.0 kb

*sur*B 3’ ：3’ untranslated region of the acetolactate synthase gene from tobacco (*Nicotiana tabacum*) 1.8 kb

(b) Pansy flavonoid 3’,5’- hydroxylase (F3’5’H) expression cassette

CHS ：Chalcone synthase gene promoter derived snapdragon (*Antirrhinum majus*) 1.2 kb

F3’5’H cDNA : cDNA of the flavonoid 3’,5’- hydroxylase gene from pansy (*Viola hortensis*) 1.8 kb

D8 3’ ：3’ untranslated region of the phospholipid transferase gene from petunia (*Petunia hybrida*) 0.8 kb

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

DFR genomic DNA ：Dihydroflavonol 4-reductase gene from petunia (*Petunia hybrida*) (Includes promoter, translated region and 3’ untranslated region) 5.0 kb

(d) Cassette for co-suppression of dihydroflavonol 4-reductase (DFR)

35S : 35S RNA gene promoter from cauliflower mosaic virus 0.4 kb

dsDFR cDNA : cDNA of the dihydroflavonol 4-reductase gene from carnation (*Dianthus caryophyllus*) 0.3 kb (forward) and 0.3 kb (reverse)

5 Intron derived from *DFR* genomic DNA : Intron of the dihydroflavonol 4-reductase gene from petunia (*Petunia hybrida*) 0.2 kb

t35S : 3' untranslated region of the 35S RNA gene from cauliflower mosaic virus 0.2 kb

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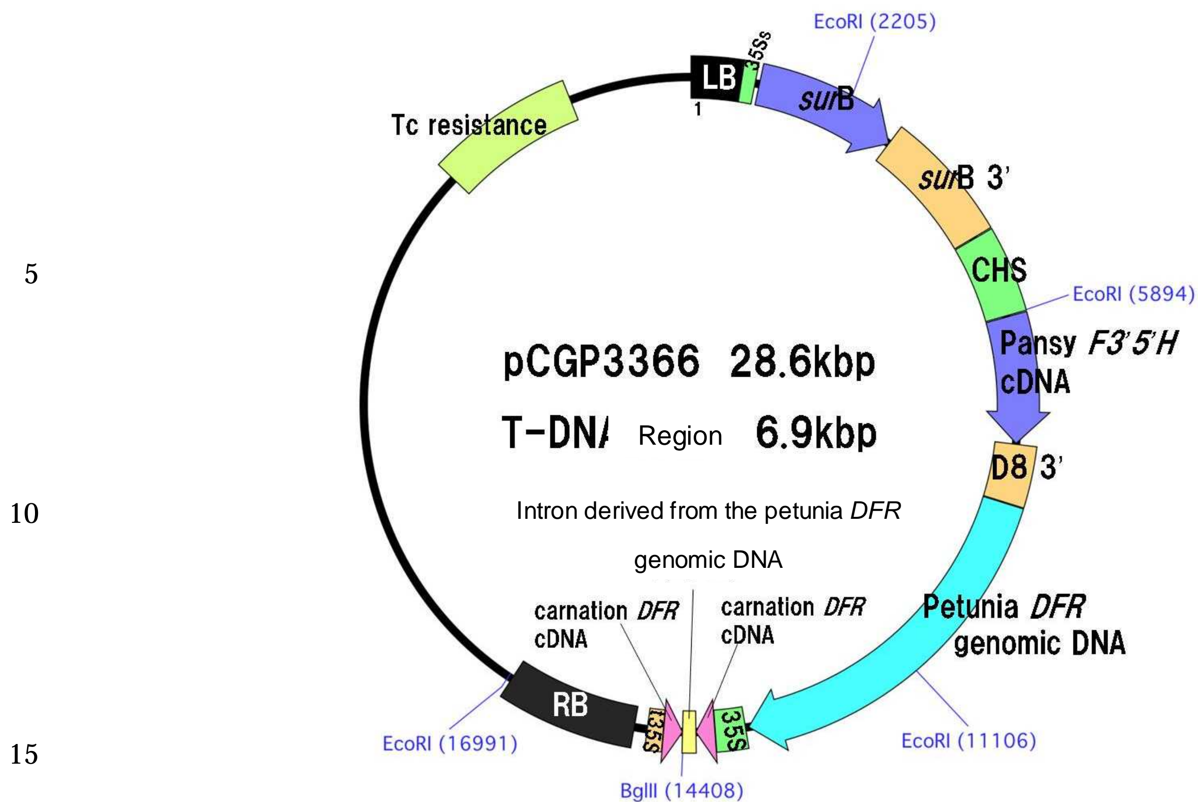


Figure 1 pCGP3366 transformation vector

Three expression cassettes are inserted in the binary vector pWTT2132.

35Ss ：35S RNA gene promoter from cauliflower mosaic virus (Its 5'-terminal region is shorter by about 0.2 kb than that of a common 35S RNA gene promoter)

*sur*B ： Acetolactate synthase gene from tobacco

*sur*B 3' ：3' untranslated region of the acetolactate synthase gene from tobacco

CHS ：Chalcone synthase gene promoter from snapdragon

Pansy *F3'5'H* cDNA ：cDNA of the flavonoid 3',5'- hydroxylase gene from pansy

D8 3' ：3' untranslated region of the phospholipid transferase gene from petunia

Petunia *DFR* genomic DNA：Dihydroflavonol 4-reductase gene from petunia (Promoter, translated region and 3' untranslated region are included)

35S ：35S RNA gene promoter from cauliflower mosaic virus

Carnation *DFR* cDNA ：cDNA of the dihydroflavonol 4-reductase gene from carnation

Intron derived from the petunia *DFR* genomic DNA：
Untranslated region of the dihydroflavonol 4-reductase gene from petunia

t35S ：3' untranslated region of the 35S RNA gene from cauliflower mosaic virus

* The bp position of the cleavage site is indicated in brackets next to each restriction enzyme. Numbering begins at the left border terminal.

2) Function of components of transformation vector

(a) Affects of the transferred genes on anthocyanin biosynthesis in carnation

Part of the anthocyanin biosynthetic pathway is shown in Figure 2 (p. 12). The biosynthetic pathway of anthocyanins is generally conserved among higher plant species and anthocyanins are synthesized via the route shown in Figure 2 (p. 12) in carnation.Non-transgenic carnations produce cyanidin and pelargonidin-related anthocyanins. The 3- and 5-positions of anthocyanins in the petals of carnation are glycosylated and those sugars are bonded to the malyl group. The flavonols quercetin and kaempfero which are themselves colorless and indirectly affect the flower color by forming a complex with anthocyanins, are synthesized from the same biosynthesis pathway shown in Figure 2 (p.12). Petal cell vacuole pH also affects flower color. Flowers of non-transgenic carnation can produce the orange-red pelargonidin 3,5-(malyl) diglucoside, which has one hydroxy group on the B ring of anthocyanins (at the 4-position) is hydroxylated) and the purple-red cyaniding 3,5-(malyl) diglucoside, which has two hydroxy groups on the B ring of the anthocyanin (at the 3- and 4-positions). Non-transgenic carnation can not produce delphinidin3,5-(malyl) diglucoside, which has three hydroxy groups on the B ring of anthocyanin (the 3-, 4-, and 5-positions are hydroxylated).

Hydroxylation of the B ring is determined by expression of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) which hydroxylate dihydroflavonols. Because dihydroflavonols are a precursor of flavonols, pelargonidin 3,5-(malyl) diglucoside and kaempferol accumulate where expression of F3'H and F3'5'H is absent. In the presence of F3'H expression, cyanidin3,5-(malyl) diglucoside and quercetin accumulate. Delphinidin3,5-(malyl) diglucoside is not present in non-transgenic carnation, because F3'5'H is not naturally present in carnation.

Part of the anthocyanin biosynthesis pathway in transgenic carnation line 25958 (*F3'5'H*, *DFR*, *dsDFR*, *sur*B, *Dianthus caryophyllus* L. ; 25958, OECD UI: IFD-25958-3) ; hereinafter referred to as "25958" is shown in Figure 3 (p. 13).

The parental 25958 was derived from has bright red flowers due to pelargonidin and cyaniding accumulation as a result of the activity of F3'H, When the *F3'5'H* and *Cyt* $b_5$genes derived from petunia were transferred, delphinidin was produced in the petals, resulting in purple-violet flowers. The produced delphinidin is converted to delphinidin3,5-(malyl) diglucoside by endogenous flavonoid 3-glycosyltransferase (3GT) and other enzymes.

When a gene is transferred to a plant, the position of the transferred gene on the chromosome is likely to be different in each transgenic line and it is thought that expression of the transferred gene depends on the position it is inserted. The origin and/or promoter associated with the

transferred gene is also likely to affect the level of transferred gene expression. In transgenic carnation the quantity of anthocyanins is affected by these factors and the depth of flower color is therefore different in different lines (see Annex 8).

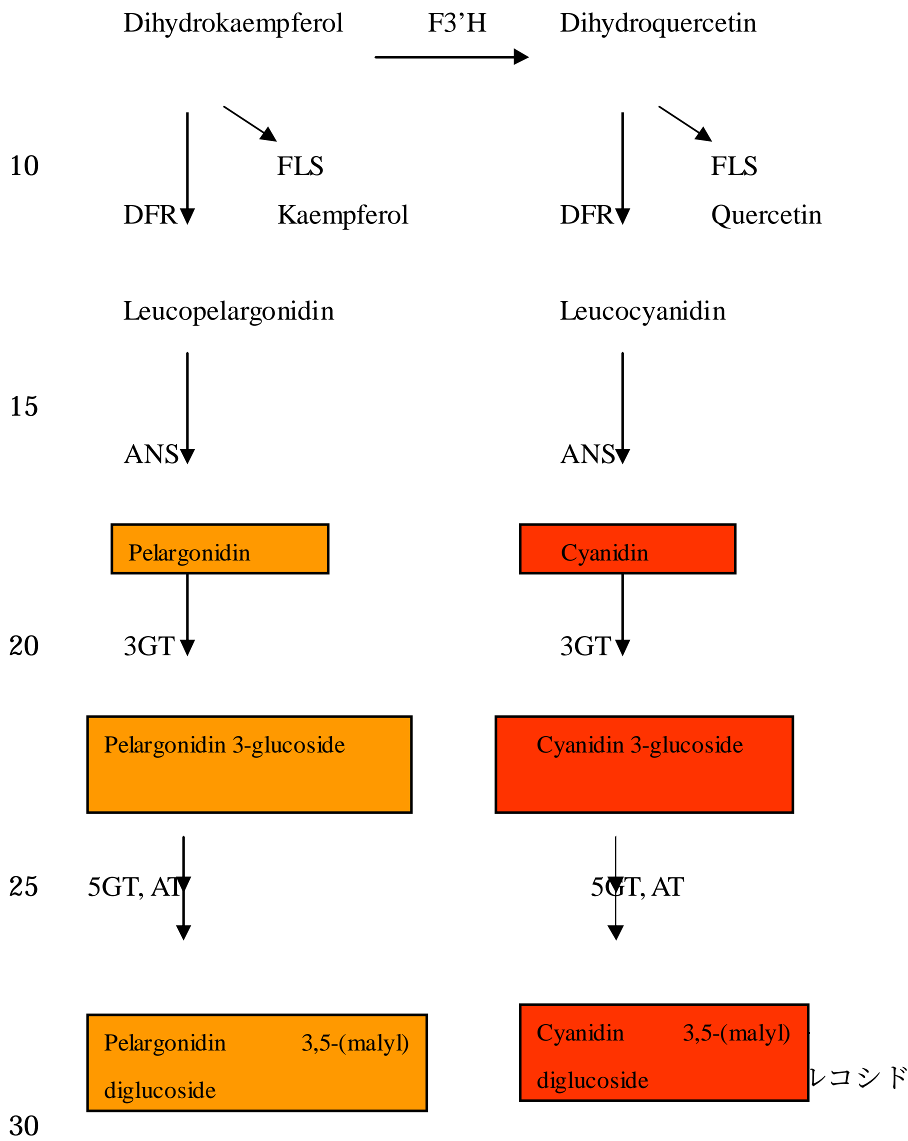


Figure 2 Outline of the biosynthetic pathway of anthocyanins in non-recombinant carnation

Cyanidin-based and pelargonidin-based anthocyanins are accumulated in non-recombinant carnation.

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

Flavonoid 5- glycosyltransferase, AT: Acyltransferase

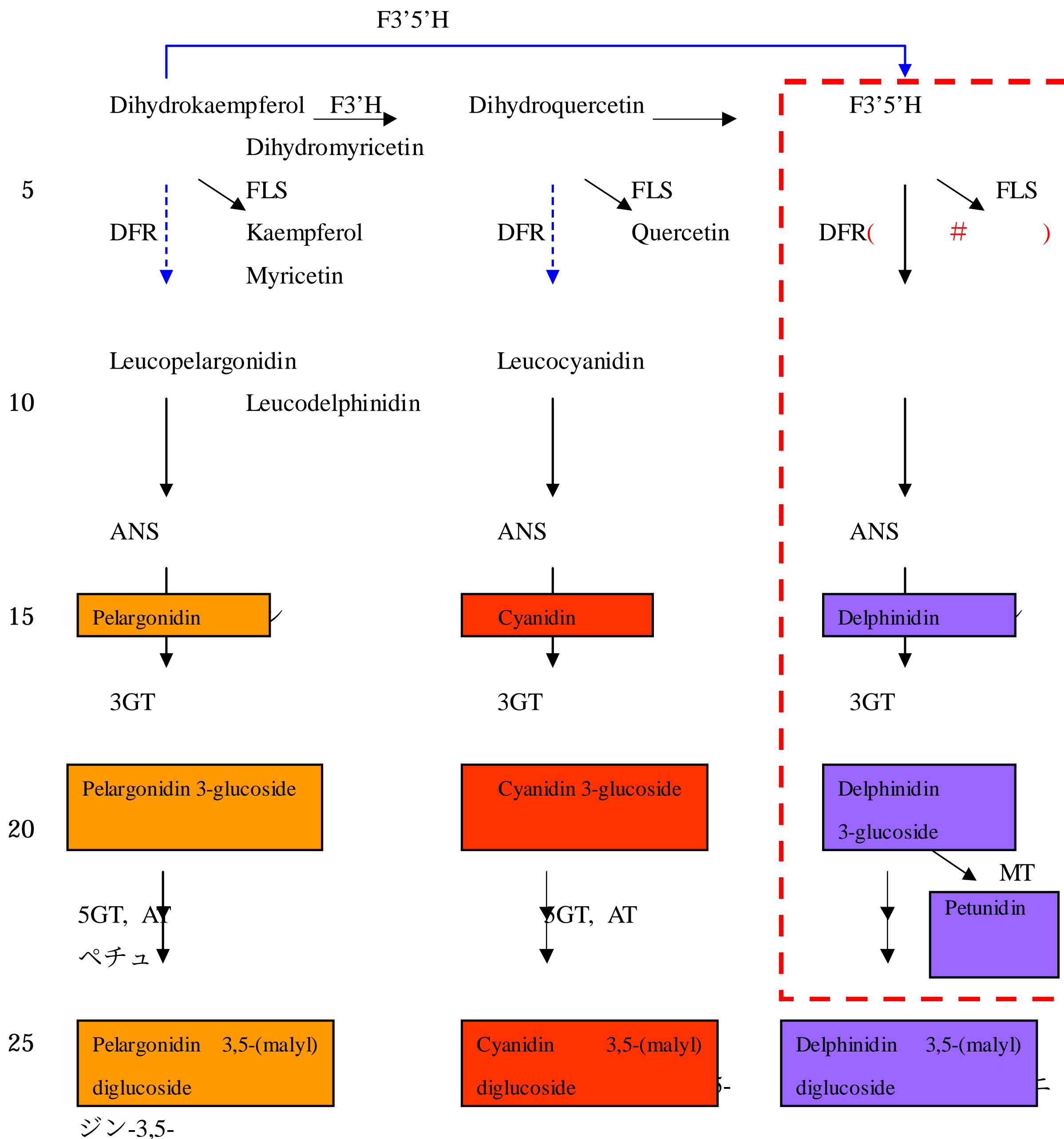


Figure 3 Outline of the biosynthetic pathway of anthocyanins in this recombinant plant

The pathway indicated by a blue arrow does not exist in non-recombinant carnation and the other pathways exist in both recombinant and non-recombinant plants. Transfer of the *F3'5'H* gene derived from pansy, the *DFR* gene derived from petunia, and the *dsDFR* gene derived from carnation can induce biosynthesis of dihydromyricetin to accumulate delphinidin 3-glucoside, which are bluish anthocyanins, in petals. It 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-glycosyltransferase, 5GT: Flavonoid 5-glycosyltransferase, AT: Acyltransferase, MT: Methyltransferase

* The part enclosed in the red dashed line is the pathway synthesized by the function of the transferred gene.

* The pathways indicated by blue dashed lines are inhibited by the *dsDFR* gene of carnation.

* DFR (#) is the enzyme expressed by the transferred gene and appropriate to produce delphinidin.

(b) **Function of components of transformation vector**

(i)Functions of individual component elements of donor nucleic acid, including target gene, regulatory region, localization signal and selectable marker

5 a. 35S promoter:

Promoter region of the *35S RNA* gene derived from cauliflower mosaic virus. It is an essential element for expression in the transgenic plant of the gene downstream of this promoter..

The genomic DNA of cauliflower mosaic virus is cyclic double-stranded DNA and the gene
10 expression regulatory site necessary for self-replication in the nuclei of cells of the recipient organism using the gene expression system in the recipient organism. One of the genes encoded in this genomic DNA, is the *35S RNA* gene. As the promoter of this gene (35S) is expressed at a high level in any stage of growth in almost all organs of a plant, it is often used to express foreign genes in transgenic plants(Mitsuhara et al., 1996[26)]).

15

The 5'-terminal region in this transgenic line is shorter by approximately 0.2 kb than the normal promoter region of the *35S RNA* gene (Franck et al., 1980 [27)]). The 35S promoter was used to express the *sur*B gene.

20 b. *sur*B gene:

Mutation of acetolactate synthase (*ALS*) gene derived from the cultured cells of tobacco.

ALS is essentially for biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine, In microorganisms, isoleucine and valine are biosynthesized from their respective precursors, L-threonine and pyruvic acid by ALS. After L-threonine is transformed into
25 2-oxobutyric acid, they both are synthesized by the five common enzymes. 1-hydroxyethyl-TPP (produced by decarboxylation of pyruvic acid and thiamine pyrophosphate(TPP)) is reacted with pyruvic acid to produce acetobutyric acid, which acts as the precursor for synthesis of valine. 1-hydroxyethyl-TPP may also react with 2-oxobutyric acid to produce 2-aceto-2-hydroxybutyric acid, which acts as the precursor for synthesis of isoleucine. ALS is normally inhibited by
30 chlorsulfuron, a sulfonylurea herbicide. A mutated ALS (SURB), resistant to chlorsulfuron was isolated from cultured cells of tobacco growing in the presence of chlorsulfuron at a lethal level (US patent number 5 141 870 [28)]). SURB, which retains the catalytic properties of normal ALS, is used as a selectable marker for development of transgenic plants.. Other sulfonylurea herbicides include methylsulfone methyl, tribenuron, thifensulfuron and bensulfuron methyl. It is known
35 that this *sur*B gene is resistant to at least chlorsulfuron and bensulfuron methyl (Shimizu et al.,2011 [29)]). Chlorsulfuron was used for selection of 25958 .

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

The chalcone synthase (*CHS*) gene promoter was derived from snapdragon (Sommer, 1988 [30])).and includes a 1.2 kb 5'-terminal from the initiation codon. The *CHS* gene is one of the genes involved in flavonoid synthesis. Use of this promoter is expected to optimize expression level in petal epidermal cells.

d. Cytochrome b_5 (*Cyt* b_5) cDNA:

Cytochrome B5 is found in all animals, plants, and yeasts. The protein provides electrons obtained from NADP or NADPH to fatty acid desaturase and cytochrome P450. The petunia cytochrome b_5 used here functions to transfer electrons specifically to the petunia F3'5'H (De Vetten et al., 1999 [31])). Therefore, it is expected that the combined expression of introduced F3'5'H and *Cyt* b_5 will enhance the function of the petunia F3'5'H, resulting in the efficient production of delphinidin.

e. 3' untranslated region of the *D8* gene:

The *D8* gene codes for a phospholipid transferase from petunia. The sequence used in the transgenic product is a DNA fragment of approximately 0.8 kb which includes the regionof approximately 150 bp which is transcribed but not translated (Holton, 1992 [32]), International patent application PCT/AU/00334: Publication number WO93/01290 [33])). When three expression cassettes (minimum unit for gene expression from the promoter through the gene coding region to 3'untranslated region) are contained in a single binary vector, it is preferable to use different promoters and/or 3'untranslated regions for individual expression cassettes to allow the transferred gene to be stably expressed. Therefore, the 3'untranslated region of the *D8* gene was used as a terminator for *Cyt* b_5 cDNA..

f. Petunia dihydroflavonol 4-reductase (*DFR*) gene:

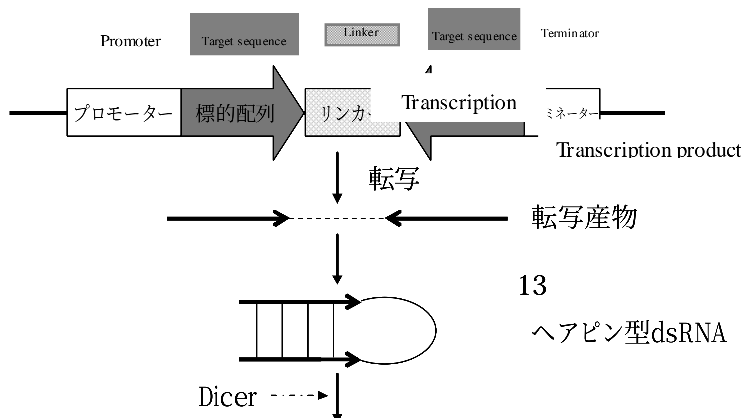
The dihydroflavonol 4-reductase (*DFR*) gene includes promoters, translated region and 3' untranslated regions.

This enzyme reduces dihydroflavonol (dihydrokaempferol, dihydroquercetin, and dihydromyricetin (see Figures 2 and 3)) to produce the leucoanthocyanidin (leucopelargonidin, leucocyanidin, and leucodelphinidin (see Figures 2 and 3)). Leucoanthocyanidin is the direct precursor of anthocyanidin (pelargonidin, cyanidin, and delphinidin (See Figures 2 and 3)). DFR is substrate-specific and the DER derived from petunia is capable of reducing dihydroquercetin and dihydromyricetin as a substrate, but not dihydrokaempferol (Beld et al.,

1989 [33], Huits et al., 1994 [34]) making the DFR derived from petunia suited to delphinidin biosynthesis..

g. Carnation dihydroflavonol 4-reductase (*DFR*) gene (for co-suppresssion):
A carnation *DFR* gene sequence designed to allow transcription of double-stranded RNA to inhibit the function of endogenous DFR, was transferred.

The RNAi method was used to inhibit the expression of the endogenous carnation DFR gene This method, where a 21-23 base pair double-stranded RNA (dsRNA) sequence-specifically efficientl inhibits gene expression, at the level of transcription products. DNA is transcribed into mRNA, but not translated due to cleavage of mRNA. As the diagram in Figure 4 (p. 20) shows, first, dsRNA is processed by the dsRNA specific dicer RNase to short 21-23 base pair siRNA. The siRNA is then unwound into a single-stranded RNA by RNA helicase following which the single-stranded RNA forms a RNA-induced silencing complex (RISC) with proteins. This RISC recognizes the target mRNA and cleaves the target mRNA at the complementary region between siRNA andtarget mRNA, resulting in inhibition of gene expression (Ochiai et al., 2005 [35]; Miki et al., 2005 [36]). In 25958 some of the introns of the petunia *DFR* gene are used as a linker and the transcription cassette, in which the *DFR* cDNAs derived from carnation forms inverted repeats, is transferred. Therefore, the double-strand RNA of the carnation DFR gene is transcribed to degrade mRNA of the gene in the plant cells.



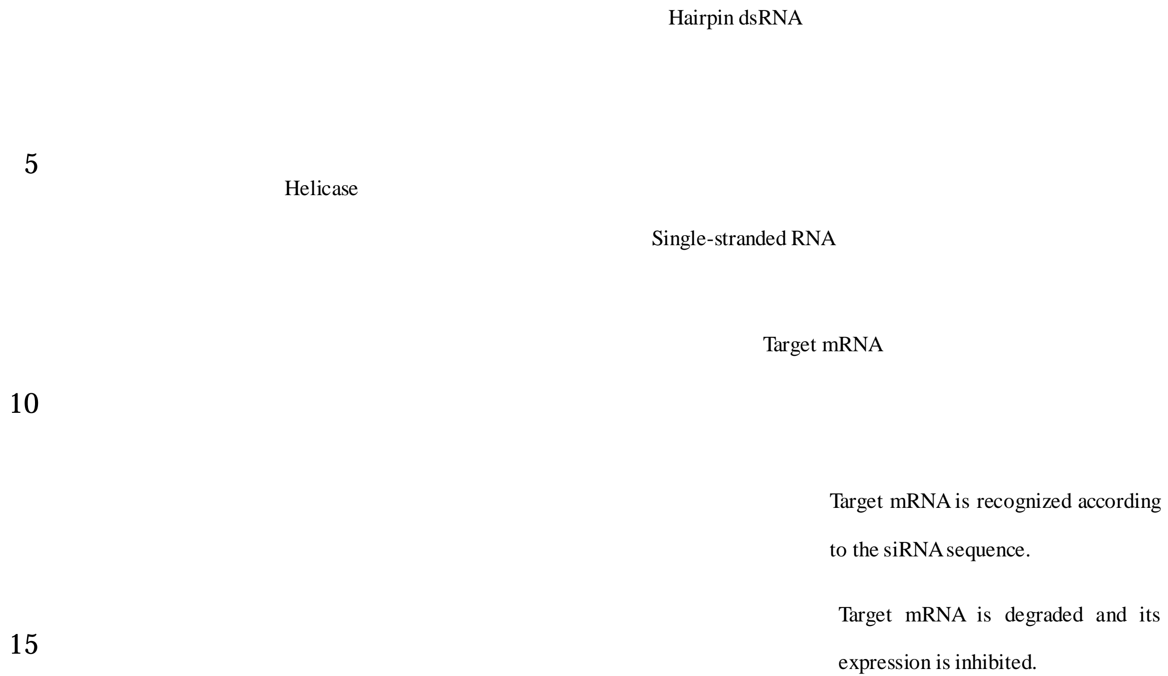


Figure 4 Outline of the RNAi method

h. 35S terminator:

The terminator region of *35S RNA* gene from cauliflower mosaic virus. containing a 0.2 kb gene fragment.

5 (ii) Functions of proteins produced by the expression of target gene and selectable markers, and whether the produced protein is homologous with any protein that is known to possess any allergenicity

The F3'5'H from pansy converts dihydrokaempferol or dihydroquercetin to dihydromyricetin, and 10 the DFR derived petunia converts dihydroquercetin and dihydromyricetin to leucocyanidin and leucodelphinidin, respectively. The ALS proteins encoded by the *sur*B gene confers resistance to the herbicide. chlorsulfuron

The results of the search in May 2012 using Allergen Online version 12 (updated on February 7, 15 2012), the allergen database of the University of Nebraska, showed that these proteins were not homologous to any proteins identified to be allergic.

(iii) Any change caused to the metabolic system of the recipient organism

20 The transferred F3'5'H converts dihydrokaempferol or dihydroquercetin to dihydromyricetin. The transferred *dsDFR* gene inhibits the translation of endogenous DFR, resulting in inhibition of the conversion of dihydrokaempferol and dihydroquercetin to leucopelargonidin and leucocyanidin, respectively. The transferred petunia DFR converts dihydromyricetin to leucodelphinidin. In consequence, delphinidin, which is absent in the recipient carnation, is produced. Dihydromyricetin 25 is converted by flavonol synthase (FLS) in 25958) to myricetin, which is absent in the recipient carnation variety.

(2) Information concerning transformation vector

1) Name and origin

The vector pCGP3366 used for the development of 25958 was constructed from synthetic plasmid pWTT2132 derived from *E. coli* and *Agrobacterium* (US DNAP Corp.). It contains the tetracycline resistance gene derived from plasmid pSC101 in *E. coli*, the multicloning site derived from *E. coli*, and T-DNA left and right border sequences derived from *Agrobacterium* t*umefaciens*

2) Properties

(a) Number of base pairs and nucleotide sequence

The number of base pairs in pCGP3366 is 28,599 bp. The nucleotide sequence of the T-DNA is shown in Figure 1 of Annex 2 (p. 1-27).

(b) Presence or absence of nucleotide sequence having specific functions, if present, and their functions

As a selectable marker of *E. coli*, the tetracycline resistance gene is present outside the T-DNA region. As a selectable marker of the transgenic plant the *sur*B gene, which confers resistance to chlorsulfuron herbicide, is present.

(c) Presence or absence of infectious characteristics and, if present, information concerning the range of the recipient organism

This vector is not infectious.

(3) Method of preparing living modified organism

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

The outline of the structure of the binary vector pCGP3366 and its nucleotide sequence are shown in Figure 1 (p. 11) and Figure 1 in Annex 1 (p. 1-24), respectively. The size is approximately 28.6 kbp and the size of the T-DNA region between left and right borders is approximately. 16.9 kbp. In the T-DNA region transferred to the recipient organism, the *sur*B gene is intended to serve as a selectable marker and the pansy *F3'5'H*, the petunia *DFR*, and the carnation *dsDFR* genes for flower color modification.

2) Method of transferring nucleic acid to the recipient organism

The *Agrobacterium* co-cultivation method (US Patent number 5 589 613 [37)]) was used for transformation. In October 2004, the line of *Agrobacterium tumefaciens* line Ag10 was inoculated with the surface-sterilized stem explants of CON4, 25958 was isolated in November 2005.25958 is maintained by vegetative propagation.

3) Maintenance of living modified organisms

25958 is maintained by vegetative propagation. A unit for the application of this transgenic plant is the current generation

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

A selectable medium containing chlorsulfuron (1-5µg/l) was used.for selection of the transgenic plant.

(b) Presence or absence of remaining *Agrobacterium* where the *Agrobacterium* method is used for transferring nucleic acid

Agrobacterium used for the production of 25958 was removed by the addition of ticarcillin to the tissue culture medium. To determine whether any residual *Agrobacterium* was present, extracts from leaves of 25958 were smeared on a selectable medium enabling growth of *Agrobacterium* carrying the transferred gene only., As no colonies considered to be *Agrobacterium* were observed it was concluded that there was no residual *Agrobacterium* vector in 25958

(c) Cultural history of the following lines: cells to which the nucleic acid was transferred; the selected lines in which transferred nucleic acid was initially confirmed; the line subjected to isolated field tests; and the line used for collection of other information for assessment of adverse effects on biological diversity

2004-2005

Transgenics were obtained. (Australia)

50 lines; 25928 to 25977

2005-2006

First selection (Australia)
(Selected by the characterization of parameters such as flower color, number of petals, and plant length)
Lines: 25938, 25939, 25943, 25944, 25947, 25953, 25957, 25958, 25964, 25965, 25973, 25975, 25976

2007

Closed greenhouse and special screened greenhouse tests (Australia);
Second selection in a normal greenhouse (Colombia)
(Selected by comprehensive assessment of growth characteristics, productivity, characteristics of cut flowers (flower color, flower types, length of cut flowers, etc.))

Line: 25958

（Note）A unit for the application of this transgenic plant is the current generation

(4) Location of nucleic acid transferred to cells and stability of expression of introduced traits

(a) Location of transferred nucleic acid

5 The *F3'5'H* gene derived from pansy, one of the nucleic acid transferred into 25958, is located in the chromosome. Its translation product, F3'5'H, is translated in the cytoplasm, and then the N-terminal signal peptide of the F3'5'H is recognized and translocated to the endoplasmic reticulum (ER), resulting in fulfillment of its proper enzymatic function. The transferred exogenous nucleic acid is normally inserted in the chromosome. Southern blot analysis showed that a single copy of the
10 transferred nucleic acid exists in 25958 (see Annex 3, p. 1-14), and delphinidin is actually produced by the function of F3'5'H, the translation product of the *F3'5'H* gene. Therefore, it is thought that the genes on the T-DNA, including the *F3'5'H* gene, are present on chromosomes. In addition, the probability of the gene transfer into the genome of the organelle is very low by the *Agrobacterium* method, which also supports the idea the transferred nucleic acids exists on chromosomes.
15

(b) The number of copies of introduced transferred nucleic acid and stability across multiple generations

From Southern blot and sequence analysis it was shown a single copy of the transferred sequence
20 exists in the genome of 25958. (see Annex 3, p. 1-14).

As 25958 is grow by vegetative propagation stability of intergenerational transmission by recombination has not been analyzed. 25958 has been propagated continuously since 2005 and vegetative propagation has been repeated in several countries., There have been no cases showing
25 different flower color, suggesting the transferred nucleic acids are stable in 25958

(c) Positional relationships where multiple copies exist

Not applicable.
30

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

The expression of the transferred pansy *F3'5'H*, petunia *DFR*, and *sur*B genes was analyzed by
35 Northern blotting. For the pansy *F3'5'H* and petunia *DFR* genes, signals specific to the transferred genes and with the expected molecular weight, were detected only in the petals of 25958.. These

results indicate that the expression of the genes inserted into the genome was stable. *sur*B gene signals were detected in petals, leaves and roots, with the expected molecular weight (see Annex 3, p. 15-16). The flower color of the individual grown by vegetative propagation is homogeneous and there have been no cases showing any flower color other than bright purple.

Therefore, the expression of the genes inserted into the genome is stable.

The medium added with chlorsulfuron is used only in the tissue culture of 25958, which is stably resistant to chlorsulfuron, based on the expression of the *sur*B gene (see Annex 6, p. 31).

(e) 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

25958 does not contain any transmissible sequences. Therefore the genes transferred to 25958 are never transmitted..

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

Southern blot analysis allows specific detection and identification of 25958. Approximately 10 μg of chromosomal DNA is sufficient for detection. A PCR_based unique identification protocol, specific for 25958 also been developed. It was confirmed that by this method 25958 can be detected using as little as 1 ng of genomic DNA ..

Methods of detection and identification of living modified organisms -- See Annex 4, p. 1-2.
Their sensitivity and reliability -- See Annex 4, p. 3.

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

(a) Specific physiological or ecological characteristics due to expression of products encoded by transferred nucleic acid

After the pansy *F3'5'H* and petunia *DFR* genes were overexpressed in the recipient organism and the expression of the endogenous *DFR* gene was suppressed, delphinidin was produced and the color of the flower changed to bright purple (see Annex 5, p. 2-6 and Annex 6, p. 10-14).

The pansy *F3'5'H* is expressed in petals, since it is regulated by a petal specific promoter. The petunia *DFR* is intrinsically expressed in petals. The carnation *dsDFR* is driven by a constitutive promoter and so the dsRNA is transcribed in all organs. However, the endogenous *DFR* is specifically expressed in petals, suggested that the *DFR* transcript is degraded only in petals and its expression is inhibited.

By spraying chlorsulfuron on young plants in an isolated field it was determined that the expression of the *sur*B gene transferred as a selectable marker conferred resistance to the herbicide chlorsulfuron (see Annex 6, p. 31).

(b) 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

The following is based on the data from the tests performed in the closed greenhouse and special screened greenhouse of Florigene Pty. Ltd. in Australia in 2008 and 2009, the closed greenhouse in the Suntory Research Center from 2008 to 2010, and the Tsuyama-Misaki Field of NISSHOKU Corporation in 2011 and 2012. As with the recipient organism CON4, 25958 was not cultivated by seed propagation.

a. Morphological and growth characteristics

The recipient organism and 25958 were cultivated in a special screened greenhouse to evaluate the characteristics of stem length at flowering time , number of petals, flower diameter and anther viability, length and width. The results showed that statistically significant differences were observed between the recipient organism and 25958 for flower diameter and the form, length and width of anther (χ^2 test or t test, level of significance of 5%) (see Annex 5, p 7 and 12). The diameter of the flower of the recipient organism was 5.9 ± 0.5 cm, compared to 5.5 ± 0.4 cm for 25958. 38 healthy and 50 non-healthy anthers and 27 healthy and 117 non-healthy anthers were observed in the recipient organism and 25958, respectively. The length and width of anther of the recipient organism and 25958 were 1.7 ± 0.4 mm and 0.7 ± 0.2 mm, and 1.4 ± 0.3 mm and 0.5 ± 0.2 mm, respectively.

The recipient organism and 25958 were cultivated in an isolated field to evaluate the growth characters plant height, time-dependent change in the number of nodes, and flowering time and anther number, length and width, number of petals, and flower diameter as morphological characters. The results showed that statistically significant differences were observed between the recipient

organism and 25958 for petal number and anther number length and width(t test, level of significance of 5%) (see Annex 6, p 18). The number of petals of the recipient organism and 25958 were 42.9 ± 5.8 and 37.9 ± 5.7, respectively. The number of anther of the recipient organism and 25958 were 4.5 ± 2.9 and 1.5 ± 1.4, respectively. The length and width of anther of the recipient organism were 3.2 ± 0.5 mm and 2.4 ± 0.5 mm, compared to 2.7 ± 0.5 mm and 1.8 ± 0.5 mm for 25958..

b. Cold resistance and heat resistance at the early stage of growth

Cultivars of carnation are not fertilized under natural conditions, and thus do not set seeds. Since seed propagation is only possible by artificial means, the cold and heat resistance of seeds could not be determined.

c. Wintering ability and summer survival of the mature plant

Cultivars of carnation prefer temperatures around 20°C, and therefore they are cultivated in temperature-controlled greenhouses in the hot summer season in Japan. However, the recipient organism and 25958 have survived the summer in Japan and showed no visible differences in the growth characteristic of plant height. Both lines have been cultivated for six years in Melbourne, Australia, where the greenhouse temperature rises up to 43-45°C in summer. Considering the maximum temperature in the summer is around 35°C in an average year in Japan and considering the results from Melbourne, both lines are considered to survive summer. Since there have been no findings on mature plants under winter conditions in Japan, in which the minimum temperature drops below zero, wintering ability of the mature plants was evaluated in the isolated field tests. In those tests all individuals overwintered and no differences were observed between the recipient organisms and 25958 (see Annex 6, p 23-24).

d. Fertility and pollen size

Anthers from plants of the recipient organisms and 25958, which were both grown in the special screened greenhouse, were visually examined for the presence of anthers and pollen. The, viability, germination and pollen size were statistically significantly different (χ^2 test or t test, level of significance of 5%) (see Annex 5, p. 8-10). The number of viable and non-viable pollen in the recipient organisms was 1085 pollen/10 flowers and 148/10, respectively, compared to 751/10 and 222/10, respectively for 25958. Number of germinated and ungerminated pollen in the recipient organisms were 32 pollen/10 flowers and 968/10, respectively, compared with 10/10 and 990/10 For

25958. Pollen diameter for the recipient organisms and 25958 was 56.4±7.1 µm and 60.1±9.4 µm, respectively. Pollen diameter was also measured from flowers cultivated in the isolated field test., A statistically significant difference was observed in the size between the recipient organisms and 25958 (t test, level of significance of 5%) (see Annex 6, p. 19-21). The average diameter of pollen from the recipient organism and 25958 was 49.0±5.3 and 45.1±4.1 µm, respectively.

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

Cultivars of carnation are not fertilized under natural conditions, and thus they do not set seeds. Since seed propagation is possible only by artificial means, the production, shedding habit, dormancy, and germination rate of the seed were not evaluated.

f. Crossability

Since the presence of pollen was observed in both the recipient organisms and 25958 plants grown in the special screened greenhouse, (see Annex 5, p. 7), they were artificially crossed with wild species in the closed greenhouse in the Suntory Research Center. The wild species used was *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams. This is the most widely distributed wild *Dianthus* species in Japan and is known to be compatible with some cultivars of carnation. The results showed that seeds were formed in both the recipient organism and 25958 but the respective crossing rates were only 0.012% and 0.018% for the recipient organism and 25958 respectively. All the seeds obtained from both the recipient organism and 25958 were planted. Only one seed, obtained from crossing with 25958, germinated (see Annex 5, p. 11). The low frequency of successful hybridization indicates a low possibility of out crossing without artificial intervention.

The crossing rate with the wild species *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams, was evaluated in an isolated field test, under natural conditions. Seeds collected from *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams were germinated and using PCR method it was determined whether the transferred genes present in 25958 were present. No T_DNA genes were detected (see Annex 6, p. 22).

g. Production of harmful substances

Cultivars of carnation have been cultivated and used for a long time., No production of harmful substances from carnation has been reported in any country, including Japan.

In order to determine whether the transferred genes could cause production of any harmful

substances in 25958, the recipient organism and 25958 were cultivated in a special screened greenhouse, after which the germination of lettuce seeds were examined in plowing-in and succeeding crop tests. The results showed no statistically significant difference in seedling fresh weight between the recipient organism and 25958 (t test, significance level is 5%) (see Annex 5, p.
5 14-15). There was no statistically significant differences in the number of fungi, bacteria, and actinomycetes cultured from the recipient organism and 25958 (t test, significance level is 5%) (see Annex 5, p. 16).

The same experiments were carried out where the recipient organism and 25958 were cultivated in
10 an isolated field The results showed no statistically significant difference in seeedling fresh weight between the recipient organism and 25958 (t test, significance level is 5%) (see Annex 6, p. 25-26). There was no statistically significant differences in the number of fungi, bacteria, and actinomycetes cultured from the recipient organism and 25958 (t test, significance level is 5%) (see Annex 6, p. 27).

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It has never been reported that ALS, Cyt b_5, F3'5'H, delphinidin, petunidin, or myricetin are harmful These are the newly produced compounds and proteins in 25958..

II. Review by persons with specialized knowledge and experience concerning adverse effects on biological diversity

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, a review was made by experts with specialized knowledge and experience concerning adverse effects on biological diversity Results of the review are listed below.

(1) Item-by-item assessment of adverse effects on biological diversity

25958 was developed using the transformation vector pCGP3366. This construct is based on plasmid pWTT2132 derived from *E. coli* and *Agrobacterium*. It has been shown by Southern blot analysis that a single copy of the *sur*B gene (acetolactate synthase gene), the *F3'5'H* gene (flavonoid 3'5'-hydroxylase gene), the *DFR* gene (dihydroflavonol 4-reductase gene), and the *dsDFR* gene (dihydroflavonol 4-reductase gene) are present in 25958.

Northern blot analysis confirmed that the expression of the transferred *F3'5'H*, *DFR*, and *dsDFR* genes was confined to the petals, whle the *sur*B gene was expressed in petals, leaves and roots. The endogenous *DFR* gene activity was suppressed in petals of 25958.. The expression of the transferred *sur*B gene was confirmed in the tissue culture of 25958 by growth in medium containing the herbicide chlorsulfuron and by applying herbicide chlorsulfuron to plants 25958 plants are propagated by cuttings and only the transgenic organism, that is, the current generation is present. Therefore the safety of transmission between multiple generations has not been analyzed.

1) Competitiveness

Cultivars of carnation have a long history of use in Japan. There have been no reports of escape from cultivation.

Characteristics relating to competitiveness (growth characteristics such as the stem length and reproduction and breeding characteristics) were examined in 25958 and the recipient control plant.

The results from the study performed in the isolated field between 2011 and 2012 showed that the

numbers of the petals, number of anthers, anther length and anther width were lower in 25958 than the recipient control organism. Although these differences were possibly caused by long term cultivation in the medium containing higher concentration of plant hormone, none of the differences observed were big enough to be outside the range seen in non-transgenic cultivars of carnation

The results from the study on the reproduction and breeding characteristics in the isolated field showed that pollen diameter was lower in 25958 than that the recipient control organism, This difference is unlikely to affect competitiveness..

As a result of expression of the transferred traits, delphinidin, petunidin and myricetin were produced in the petals of 25958 and the flower color was changed. Therefore, it was expected that the change of the flower color could change flower visiting insect fauna. Insects were scarcely observed visiting carnation flowers and the changes of flower color did not affect the number and species of the insects visiting flowers when 25958 was cultivated in an isolated field. It is therefore unlikely that the change in flower color caused by the production of delphinidin and other flavonoids in 25958 affected on the surrounding biological diversity.

25958 is resistant to chlorsulfuron but the chlorsulfuron resistance trait is unlikely to be competitiveness under natural conditions, where herbicides are unlikely to be used..

Based on the above facts, it was judged that the conclusions made by the applicant are reasonable; a)that wild animals and wild plants likely to be affected from this transgenic plant cannot be specified b)25958 poses no significant risk of adverse effects on biological diversity attributable to competitiveness.

2) Production of harmful substances

Cultivars of carnation have long been used in Japan and there has been no report that carnation produce any harmful substances affecting the growth and habitat of wild animals and plants.

The results of soil microflora tests and plowing-in and succeeding crop tests performed to examine the production of harmful substances (those which secreted from the roots to affect other plants and soil microorganisms and those which the plant harbors and affects other plants after the death of the plant) showed no differences between 25958 and the recipient control organism.

Although 25958 produces delphinidin, petunidin, myricetin and other flavonoids those substances

are also contained in the petals of bluish pansy and petunia and have not been reported to be harmful to other wild animals and plants..

In addition, it has been confirmed in homology searches,that the *sur*B, DFR, and F3'5'H proteins produced by 25958 do not have similar amino acid sequences to any known allergens..

Based on the above, it was judged that the conclusion made by the applicant are reasonable; a) that the wild animals and wild plants likely to be affected from 25958 cannot be specified b) that 25958 poses no significant risk of adverse effects on biological diversity attributable to production of harmful substances..

3) Crossability

Some cultivars of carnation can be crossed with wild species of the genus *Dianthus* and there are four species growing wild in Japan,; *Dianthus superbus L. var. superbus*, *Dianthus kiusianus*, *Dianthus japonicas*, and *Dianthus shinanensis*,. Two sub-species of *Dianthus superbus* have also been identified; , *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams and *Dianthus superbus var. speciosus. T*he possibilities of crossing of these related wild species with 25958 were evaluated with respect to the following three points..

(i) Characteristics of pollen

Cultivars of carnation produce little or no pollen and if present, its fertility is low. The life span of pollen is just 1-2 days, and the germinability is completely lost on day 3. Statistically significant differences in number of healthy anthers, pollen viability, and pollen size were observed between 25958 and the control.

However, 25958 was unlikely to cross under natural conditions based on the following points: 1) Both 25958 and the recipient control organism produce little pollen, 2) the number of healthy anthers is lower in 25958, 3) Pollen viability was lower in 25958 4) differences in pollen size were observed between 25958 and the recipient control organism, but there was no difference in appearance of pollen, and the germination rate of 25958 is lower than that of the recipient control organism, 5) the results of the examination of natural crossing ability in the isolated field showed that the T-DNA genes did not transmit to wild *Dianthus,* 6) there have been no reports that cultivars of carnation have crossed with related wild species present in Japan, and 7) there have been no reports from overseas that cultivars of carnation have crossed with related wild species under natural

conditions or that hybrid individuals have established in the wild, either by insect or wind pollination.

If hybrids were produced, they would be unlikely to affect wild plants because newly produced pigments, the *DFR*, *F3'5'H*, and *sur*B genes, or changes in flower color do not affect flower visiting insects and the newly developed chlorsulfuron resistance does not increase competitiveness under natural conditions, where herbicides are unlikely to be used.

(ii) Possibility of crossing by insect pollination

Since cultivars of carnation have a long distance between the edge of the petal and the nectary (4-5 cm), only butterflies or moths can potentially reach the nectar, and few other flower-visiting insects are observed visiting the flowers. It is confirmed that since the nectary of the related wild species of the genus *Dianthus* is at the base of the flower only insects with long proboscis (2.5 cm or more) can reach the nectary,. Although insects visit the related wild species, the characteristics, such as flower shape, of 25958 and cultivars are similar and therefore insect pollination is unlikely to occur between this transgenic carnation and the related wild species.

(iii) Possibility of crossing by wind pollination

Since the anthers of cultivars of carnation are covered in petals, the amount of pollen is very low, and the pollen is sticky, the possibility of pollen scattering by wind is extremely low. As the anthers of 25958 are also covered in petals,pollen is unlikely to be scattered by wind. It has been reported in the Netherlands that pollen of cultivars is not detected in the air, although cultivars are widely cultivated in that country.

Based on the above, it was judged that the conclusion made by the applicant is reasonable.; a) that 25958 is unlikely to be crossed with related wild species b) that 25958 poses no significant risk of adverse effects on biological diversity attributable to 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 25958, in accordance with the Type 1 Use Regulation, causes adverse effects on biological diversity in Japan. It was judged that the conclusions made by the applicant, outlined above, are reasonable.

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List of Annexes for Purple-violet herbicide chlorsulfuron resistant carnation (*F3'5'H*, *DFR*, *dsDFR*, *sur*B, *Dianthus caryophyllus* L.) (25958, OECD UI: IFD-25958-3)

Annex 1 Structure of the entire nucleic acid transferred to the recipient organism (Non-disclosure confidential information)

Annex 2 Information concerning transformation vector (Non-disclosure confidential information)

Annex 3 Location of nucleic acid transferred to cells and stability of expression of introduced traits (Non-disclosure confidential information)

Annex 4 Methods of detection and identification of living modified organisms and their sensitivity and reliability (Non-disclosure confidential information)

Annex 5 Results from tests in the closed greenhouse and special screened greenhouse (Non-disclosure confidential information)

Annex 6 Results from tests in the isolated field (Non-disclosure confidential information)

Annex 7 Information obtained in other countries (Non-disclosure confidential information)

Annex 8 Relationship between flower color and the composition of anthocyanidin in purple-violet carnation lines (Non-disclosure confidential information)