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 chlorsulfuronresistant carnation (*F3'5'H*, *Cyt* b_5, *sur*B, *Dianthus caryophyllus* L.) (26407, OECD UI: IFD-26407-2)
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 acid elements are provided below. Their positional relationship and nucleotide sequence are shown in Figure 1 (p. 10) 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 0.2kb shorter than that of the common 35S RNA gene promoter) 0.2kb

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

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

(b) Petunia cytochrome b_5(*cyt* b_5) expression cassette

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

Cytb$_5$ cDNA : cDNA of the cytochrome b_5 gene from petunia (*Petunia hybrida*)0.5kb

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

(c) Petunia flavonoid3', 5'-hydroxylase (F3'5'H) expression cassette

ANS : Anthocyanidin synthase gene promoter from carnation (*Dianthus caryophyllus*)2.5kb

F3'5'H cDNA :cDNA of the flavonoid 3', 5'-hydroxylase gene from petunia (*Petunia hybrida*)1.5kb

tANS: 3' untranslated region of the anthocyanidin synthase gene from carnation(*Dianthus caryophyllus*)0.8 kb

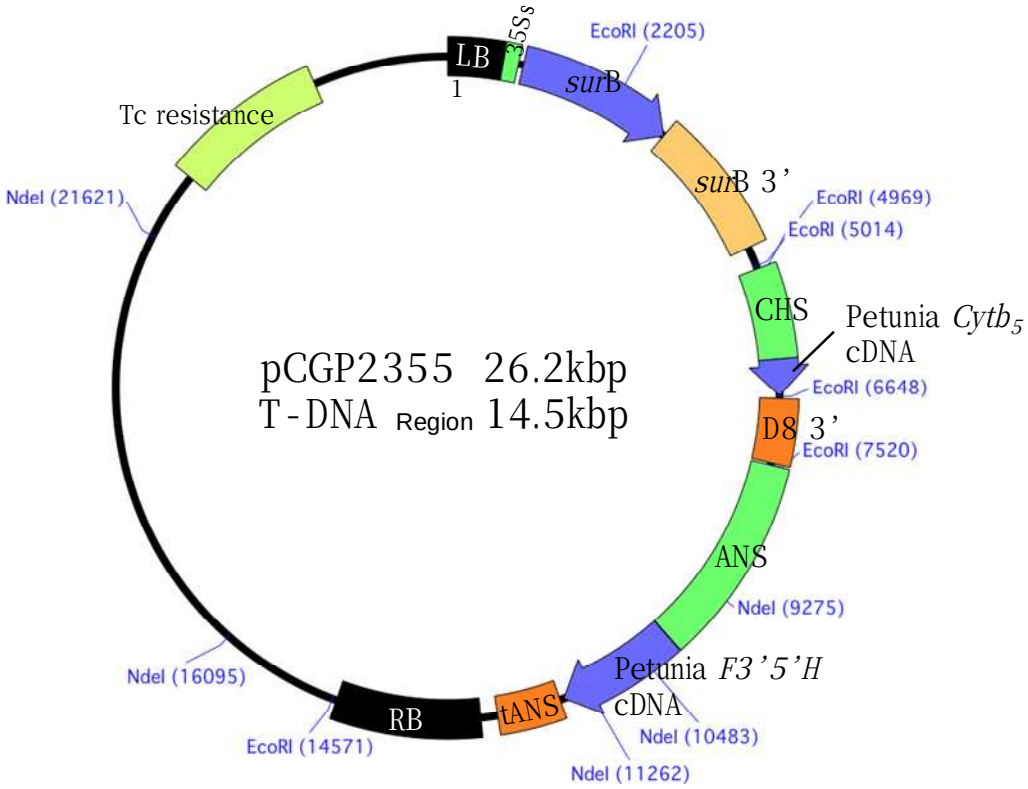

Figure 1. Diagram of transformation vector pCGP2355

Two 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 0.2kb 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

Petunia *Cyt* b_5 cDNA : cDNA of the cytochromeb_5 gene from petunia

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

ANS : Anthocyanidin synthase gene promoter from carnation

Petunia *F3'5'H* cDNA: cDNA of the flavonoid3', 5'-hydroxylase from petunia

tANS : 3' untranslated regionof the anthocyanidin synthase gene from carnation

* 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 26407 (*F3'5'H*, *Cyt* b_5, *sur*B, *Dianthus caryophyllus* L. ; 26407, OECD UI: IFD-26407-2 ; hereinafter referred to as "26407" is shown in Figure 3 (p. 13).

The parental 26407 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).

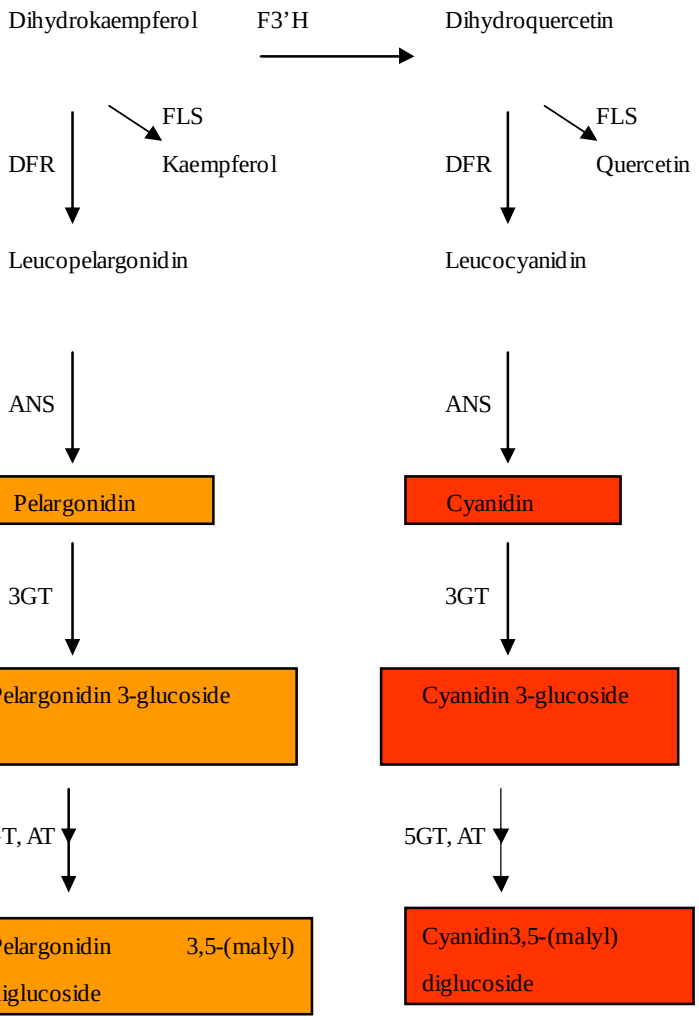

Figure 2. Outline of the biosynthetic pathway of anthocyanins in non-transgenic carnation

Cyanidin-based and pelargonidin-based anthocyanins are accumulated in non-transgenic 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

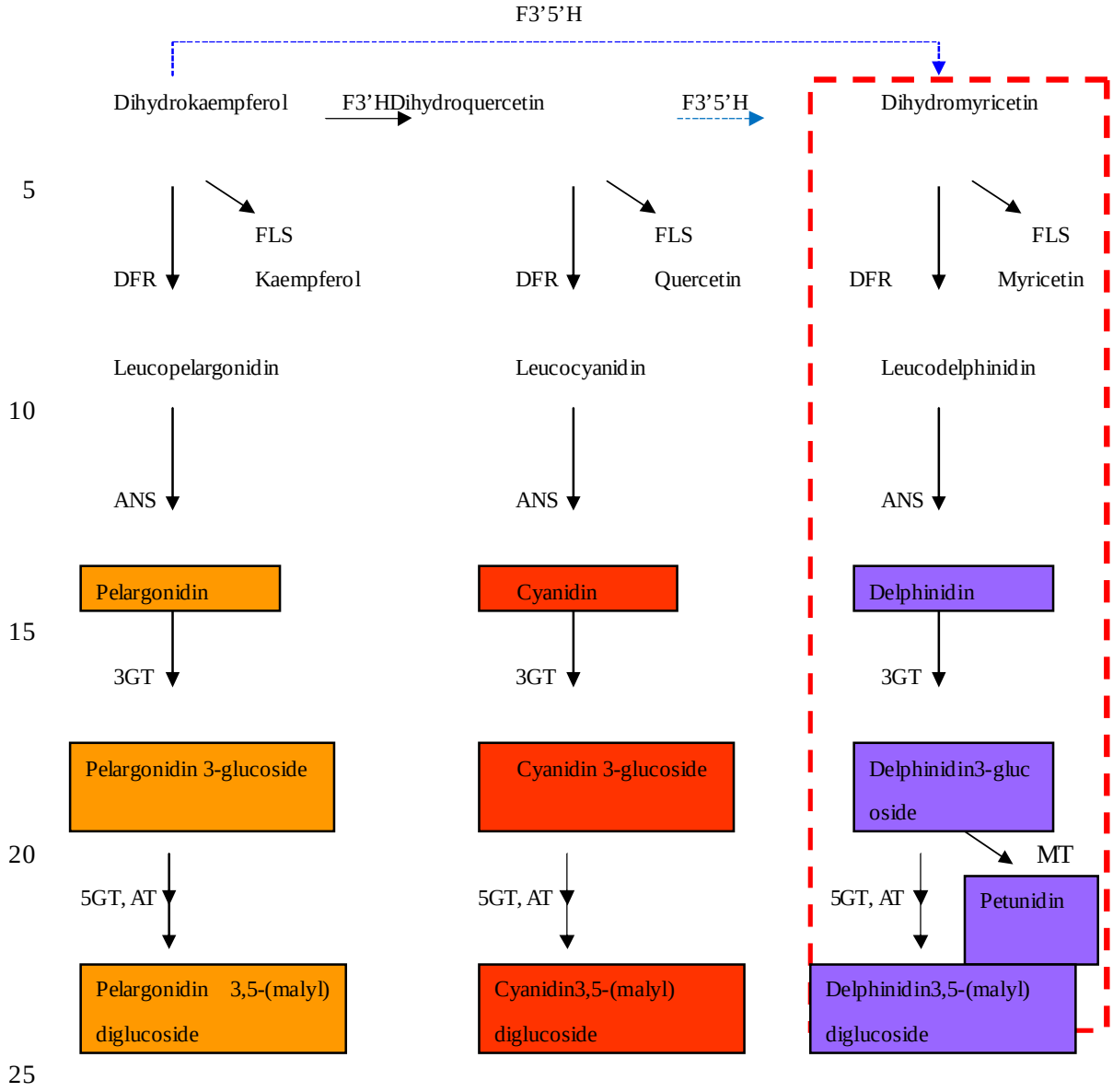

Figure 3 Outline of the biosynthetic pathway of anthocyanins in 26407

The pathways indicated by blue dashed arrows do not exist in non-transgenic carnation. The other pathways exist in both transgenic and non-transgenic plants. Transfer of the *F3'5'H* and gene from petunia results in biosynthesis of dihydromyricetin which is a precursor to biosynthesis of delphinidin 3-glucoside. Delphinidin 3-glucoside. is further modified to delphinidin 3,5-(malyl) diglucoside in 26407..

(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 of the pathway enclosed within the red dashed line is that part synthesized as a result of expression of the transferred genes.

(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

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 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]).

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 *surB* gene.

b. *surB* 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 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 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 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 26407. .

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. ANS promoter:

The anthocyanidin synthase (*ANS*) gene promoter derived from carnation contains a 2.5 kb 5'-terminal fragment from the initiation codon. The *ANS* gene is one of the genes associated with flavonoid synthesis and catalyzes the reaction in which colored anthocyanidin (pelargonidin, cyanidin, and delphinidin (see Figures 2 and 3)) are produced from colorless leucoanthocyanidin (leucopelargonidin, leucocyanidin, and leucodelphinidin (see Figures 2 and 3)). The use of this promoter is expected to increase the level of expression in the petal epithelial cells.

g. cDNAof the flavonoid 3', 5'-hydroxylase (F3'5'H):

As shown in Figure 3 (p.13), the F3'5'H enzyme hydroxylates the B ring of the dihydroflavonol

(dihydrokaempferol, dihydroquercetin, and dihydromyricetin(see Figures 2 and 3)). Dihydrokaempferol or dihydroquercetin are converted to dihydromyricetin.

h. ANS terminator:

The terminator of the anthocyanidin synthase (*ANS*) gene from carnation. It contains a 0.8 kb gene fragment.

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

The F3'5'H derived from petunia converts dihydrokaempferol or dihydroquercetin to dihydromyricetin, and the *Cyt* b_5 derived from petunia increases the activity of F3'5'H. The ALS proteins encoded by the *sur*B gene derived from tobacco are resistant to the herbicide chlorsulfuron .

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

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

The F3'5'H derived from petunia converts dihydrokaempferol or dihydroquercetin to dihydromyricetin. As a result, delphinidin is produced. Dihydromyricetin is converted by the flavonol synthase (FLS) in carnation to myricetin, which is absent in the recipient carnation variety.

(2) Information concerning transformation vector

1) Name and origin

The vector pCGP2355 used for the development of 26407 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 tumefaciens*.

2) Properties

(a) Number of base pairs and nucleotide sequence

The number of base pairs in the pCGP2355 is 26,179bp. The nucleotide sequence of 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 pCGP2355 and its nucleotide sequence are shown in Figure 1 (p. 10) and Figure 1 in Annex 1 (p. 1-24), respectively. The size is approximately 26.2 kbp and the size of the T-DNA region between left and right borders is approximately 14.5kbp. In the T-DNA region transferred to the recipient organism, the *sur*B gene is intended to serve as a selectable marker and the petunia *F3'5'H* and petunia *Cyt* b_5 genes for flower color modification .

2) Method of transferring nucleic to the recipient organism

The *Agrobacteriumco-cultivation* method (US Patent number 5 589 613 [34)]) was used for transformation. In October 2004, *Agrobacterium tumefaciens line* Ag10 was inoculated with surface-sterilized stem explants of CON4, 26407 was isolated in July 2005. 26407 is maintained by vegetative propagation.

3) Maintenance of living modified organisms

26407 is maintained by vegetative propagation.

(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 26407 was removed by the addition of ticarcillin to the tissue culture medium. To determine whether any residual *Agrobacterium* was present, extracts from leaves of 26407 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 26407.

(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; 26406 to 26455
	⇩
2005-2006	First selection (Australia) (Selected by the characterization of parameters such as flower color, the number of petals, and plant length) Lines: 26406, 26407, 26409, 26411, 26436
	⇩
2007	Closed greenhouse and special screened greenhouse tests (Australia); Second selection in a normal greenhouse (Colombia) (Selected by comprehensive assessment, including growth characteristics, productivity, characteristics of cut flowers (flower color, flower types, length of cut flowers, etc.)) Line: 26407

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

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

(a) Location of transferred nucleic acid

The *F3'5'H* gene derived from petunia, one of the nucleic acid transferred into 26407, 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 transferred nucleic acid exists in 26407(see Annex 3, p. 1-12), 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.

(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 exists in the genome of 26407 (see Annex 3, p. 1-12).

As 26407 is grow by vegetative propagation stability of intergenerational transmission by recombinantion has not been analyzed. These transgenic plants have been propagated continuously since 2005 and vegetative propagation has been repeated in several countries., There have been no cases showing different flower color, suggesting the transferred nucleic acids are stable in 26407.

(c) Positional relationships where multiple copies exist

Not applicable.

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

The expression of the transferred petunia *F3'5'H*, petunia *Cyt* b_5, and *surB* genes in petals, leaves, and roots was analyzed by Northern blot analysis. For the petunia *F3'5'H* and petunia *Cyt* b_5 genes,

signals specific to the transferred genes and with the expected molecular weight, were detected only in the petals of 26407. These results revealed that the expression of the genes inserted into the genome was stable. *sur*B gene signals were detected in petals, leaves and roots of 26407, , and with the expected molecular weight (see Annex 3, p. 13-14). The flower color of the individual grown by vegetative propagation is homogeneous and there have been no cases showing any flower color other than dark red 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 26407, which is stably resistant to chlorsulfuron, based on the expression of the *surB* gene (see Annex 6, p. 30).

(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

26407 does not contain any transmissible sequences. Therefore the genes transferred to 26407 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 26407. Approximately 10 µg of chromosomal DNA is sufficient for detection. A PCR_based unique identification protocol, specific for 26407 also been developed. It was confirmed that by this method 26407 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.
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 petunia *F3'5'H* and petunia *Cyt* b_5 genes were expressed in the recipient organism, delphinidin was produced and the color of the flower changed to dark red purple(see Annex 5, p. 2-6

and Annex 6, p. 9-13). The petunia *F3'5'H* and petunia *Cyt* b_5 are expressed in petals

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. 30).

(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 test data in the closed greenhouse in the Suntory Research Center in 2008 to 2010, and the test data in the Tsuyama-Misaki Field of NISSHOKU Corporation in 2011 and 2012. As with the recipient organism CON4, the transgenic plants were not cultivated by seed propagation.

a. Morphological and growth characteristics

The recipient organism and 26407 were cultivated in a special screened greenhouse to evaluate the stem length at flowering time, viability, length and width of anther, the number of petals and the diameter of flower as morphological characteristics. The results showed that statistically significant differences were observed between the recipient organism and 26407 for the stem length, anther viability and the number of petals (χ^2test or t test, level of significance of 5%) (see Annex 5, p 7 and 12). The stem length of the recipient organism was 99.1 ± 5.2cm, compared with that of 26407 which was 94.7 ± 3.8cm. 38 viable and 50 non-viable anthers and 17 viable and 93 non-viable anthers were observed in the recipient organism and 26407, respectively. The numbers of petals of the recipient organism and 26407 were 43.8±4.4 and 37.1±6.0, respectively.

The recipient organisms and 26407 were cultivated in an isolated field to evaluate plant height, time-dependent change in the number of nodes, and flowering time as growth characteristics, and the number, length and width of anther, the number of petals and flower diameter as morphological characteristics. The results showed that statistically significant differences were observed between the recipient organism and 26407 for plant height on July 1 and the numbers of petals and anthers (t test, level of significance of 5%) (see Annex 6, p 14-15, 17). The plant height on July 1 of the recipient organism and 26407 were 54.1 ± 8.3 cm and 63.6 ± 5.9 cm, respectively. The number of petals of the recipient organism and 26407 were 42.9±5.8 and 30.4±5.2, respectively. The number of

anthers of the recipient organism and 26407 were 4.5±2.9 and 1.5±1.8, respectively.

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

Cultivars of carnation are not fertilized under natural conditions, and thus they 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 26407 have survived the summers and showed no visible differences in growth characteristics including 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 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 isolated field tests. All individuals overwintered and no differences were observed between the recipient organism and 26407 (see Annex 6, p 22-23).

d. Fertility and pollen size

Anthers and pollen from the recipient organisms and 26407, which were both grown in the special screened greenhouse, were visually examined for the presence of pollen Pollen size and viability were statistically significantly different between the recipient organism and 26407 (χ^2test or t test, level of significance of 5%) (see Annex 5, p. 8-10). Viable and non-viable pollen counts were 1085 pollen/10 flowers and 148/10, respectively for the recipient plant, and 1050/10 and 230/10, respectively for 26407. The diameter of pollen from the recipient organism and 26407were 56.4±7.1µm and 59.9 ±9.7µm, respectively.

Germination and size of the pollen were observed and statistically significant differences were observed in the size of pollen between the recipient organisms and 26407 (t test, level of significance of 5%) (see Annex 6, p. 20). The diameter of a pollen grain of the recipient organism and 26407 were 49.0±5.3 and 43.6 ± 5.9 µm, respectively. There were no statistically significant differences

between the recipient organism and 26407 for percentage germination (see Annex 6, p. 18).

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 26407 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 species is the most widely distributed *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 26407 but the respective crossing rates were low at 0.012% and 0.004% for the recipient organism and 26407, respectively. Moreover, all the seeds of both the recipient organism and 26407 did not germinate (see Annex 5, p. 11).

In an isolated field test, the crossing rate with the wild species, *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams, under natural conditions was evaluated. Seeds were collected from *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams growing adjacent to 26407 to evaluate, using a PCR method, whether the T-DNA genes of 26407 were transmitted to *D. superbus* var. *longicalicinus*. No T-DNA genes were detected (see Annex 6, p. 21).

g. Production of harmful substances

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

In order to determine the possibility that the transferred genes could affect the metabolism of 26407, causing production of harmful substances, alleopathic experiments were carried out. In these experiments, the recipient organism and 26407, were cultivated in a special screened greenhouse, and the germination of lettuce seeds were examined in plowing-in and succeeding crop tests. The results showed statistically significant differences in fresh weights of seedlings between the recipient organism and 26407 treatments (t test, significance level 5%) (see Annex 5, p. 14-15).In plowing-in tests, the fresh weights of seedlings of the recipient organism and 26407 were

1053.5±304.6 and 1237.4 ± 296.5mg, respectively. In the succeeding crop tests, the fresh weights of seedlings of the recipient organism and 26407 were 1056.6±417.4 and 1220.4 ± 476.5mg, respectively. The same experiments were carried out where the recipient organism and 26407 were cultivated in an isolated field. The results showed no statistically significant differences in fresh weights of seedlings between the recipient organism and 26407 (t test, significance level is 5%) (see Annex 6, p. 24, 25).

There was no statistically significant differences in the number of fungi, bacteria, and actinomycetes cultured from the recipient organism and 26407 (t test, significance level is 5%) (see Annex 5, p. 16 and Annex 6, p. 26).

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 26407.

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 persons (experts)with specialized knowledge and experience concerning adverse effect on biological diversity. Results of the review are listed below.

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

26407 was developed using the pCGP2355 construct. pCGP2335 is based on the 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 derived from tobacco(acetolactate synthase gene), the *Cyt* $b_5$gene derived from petunia (cytochrome$b_5$gene) and the *F3'5'H*gene derived from petunia (flavonoid3'5'-hydroxylase gene) are present on the chromosome of 26407.

Northern blot analysis confirmed that expression of the transferred *Cyt* $b_5$and *F3'5'H* genes was confined to the petals while the *sur*B gene was expressed in the petals, leaves and roots. The expression of the transferred *sur*B gene was confirmed in the tissue culture of 26407 by growth in medium containing the herbicide chlorsulfuron and by applying herbicide chlorsulfuron to plants. 26407 plants are propagated by cuttings and only the transgenic organisms, that is, the current generation is present, and 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 26407 and the recipient control plant.

The results from the study on the growth characteristics performed in the isolated field in 2011 and 2012 showed that the numbers of the petals and anthers of 26407 were lower than the recipient control. 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 the diameter of pollen grains from 26407 was smaller than that of the recipient control.,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 26407 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 26407 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 26407 affected on the surrounding biological diversity.

26407 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)26407 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 26407and the recipient control organism.

Although 26407 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, Cyt b_5, and F3'5'H proteins produced by 26407 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 26407 cannot be specified b) that 26407 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 26407 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 frequency of healthy anthers, degree of fertility of pollen, and pollen size were observed between 26407 and the control

However, 26407 was unlikely to cross under natural conditions based on the following points: 1) Both 26407 and the recipient control organism produce little pollen, 2) the number of healthy anthers of 26407 is fewer than that of the recipient control organism, 3) differences in pollen size were observed between 26407 and the recipient control organism, but there was no difference in appearance of pollen, and germination rate of 26407 was lower than that of the recipient control organism, 4) the results of the examination of natural crossing ability in the isolated field showed that the T-DNA of 26407 was not transmitted to seeds, 5) there have been no reports that cultivars of carnation have crossed with related wild species present in Japan, and 6) 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 *sur*B、*Cyt* $b_{5\,z}$, and *F3'5'H* 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 26407 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. The anthers of 26407 are also covered in petals, as with cultivars of carnation, pollen so it 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 the country.

Based on the above, it was judged that the conclusion made by the applicant is reasonable.; a) that 26407 is unlikely to be crossed with related wild species b) that 26407 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 26407, 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 chlorsulfuron resistant carnation (*F3'5'H*, *Cyt* b_5, *sur*B, *Dianthus caryophyllus* L.) (26407, OECD UI: IFD-26407-2)

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

Annex 2 Information concerning transformation vectors (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)