

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

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Approved Type 1 Use Regulation

Name of the type of Living Modified Organism	Purple-violet carnation 123.8.8 ( <i>F3'5'H</i> , <i>DFR</i> , <i>Dianthus caryophyllus</i> L.) (OECD UI : FLO-40685-1)
Content of the Type 1 Use of Living Modified Organism	Appreciation of cut flowers, cultivation, storage, transportation, disposal and acts incidental to them.
Method of the Type 1 Use of Living Modified Organism	—

# Outline of the Biological Diversity Risk Assessment

## I. Information concerning preparation of living modified organisms

### 1. Information concerning donor nucleic acid

#### (1) Composition and origins of component elements

Composition of donor nucleic acids and origins of component elements are shown below.

##### i) Expression cassette for selectable marker *surB*

35S: Promoter derived from the cauliflower mosaic virus 35S

*surB*: Acetolactate synthase (ALS) gene isolated from tobacco

*surB* 3': 3' untranslated region of the acetolactate synthase (ALS) gene from tobacco

##### ii) Expression cassette for dihydroflavonol 4-reductase (DFR)

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

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

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

F3'5'H cDNA: Flavonoid 3', 5'-hydroxylase cDNA from Pansy

D8 3': 3' untranslated region of the lipids transfer protein from *Petunia*

##### iv) Others

*lacZ* : $\beta$ -galactosidase gene from *E. coli*

#### (2) Functions of component elements

##### i) Anthocyanin biosynthetic pathway and possible effects of introduced genes in carnation

Outline of anthocyanin biosynthetic pathway is provided in Fig.1 (p4). The anthocyanin biosynthetic pathway is maintained in the plant kingdom and also in the carnation, anthocyanin is synthesized in accordance with the pathway shown in Fig.1 (p4). For the anthocyanin present in the petals of carnation, it is known that 3' and 5' are glycosylated and the glycosides produced contain malyl groups bonded. The pathway shown in Fig.1 (p4) also applies to the synthesis of flavonol which is colorless by itself though can indirectly govern the color of flowers by forming a complex with anthocyanin. It is also known that the pH of vacuole of petal cell can affect the flower color.

The orange-red carnations contain pelargonidin 3,5-(malyl) diglucoside which has a single hydroxyl group (only 4' is hydroxylated) in the B ring of anthocyanin, and the purple-red carnations contain cyanidin 3,5-(malyl) diglucoside which has two hydroxyl

groups (3' and 4' are hydroxylated) in the B ring of anthocyanin. Under natural condition, there never exists such carnation that contains delphinidin 3,5-(maly) diglucoside which has three hydroxyl groups (3', 4', and 5' are hydroxylated) in the B ring of anthocyanin.

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

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

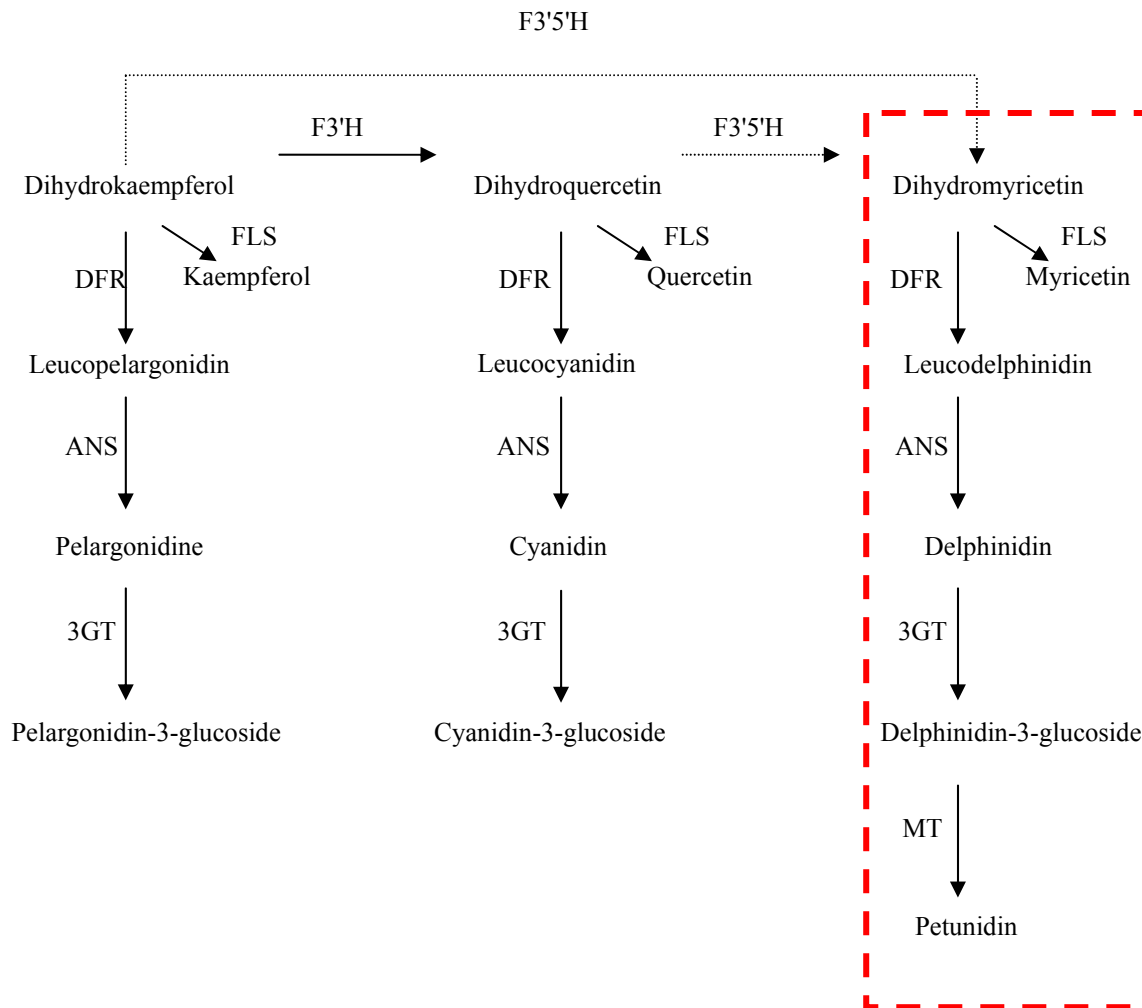


Fig.1 Outline of anthocyanin biosynthetic pathway

The dotted pathways do not exist in the ordinary carnations. Introduction of the F3'5'H gene from *Petunia* enables biosynthesis of dihydromyricetin, which results in the accumulation of delphinidin-3-glucoside, a blue anthocyanin, in the petals. In carnation, the delphinidin-3-glucoside is further modified to delphinidin-3,5-(maly) diglucoside.

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-glucosyl-transferase, MT: Methyltransferase

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

ii) Functions of component elements

(a) Functions of target genes, expression-regulating regions, localization signals, selectable markers and other component elements of donor nucleic acid

a. 35S promoter:

Promoter region from the cauliflower mosaic virus 35S RNA gene. An essential component to allow the downstream neighboring *surB* gene (See the following item b.) to be expressed in the transgenic plant.

The cauliflower mosaic virus possesses circular double-stranded DNA as the genome DNA and contains the gene expression-regulating site required for autonomous replicating and propagation in the nucleus of host cell by using the gene expression system of the recipient plant. The promoter for the 35S RNA gene, one of the genes coded on the genome DNA, is known as 35S promoter, and it drives high levels of expression in almost every organ of plant body and at any stage of the growth and therefore, it is frequently applied for expression of foreign genes in plants. This 35S promoter and other promoters that are expressed in almost all sites in plant body and at any stage of growth are called "constitutive promoter."

b. *surB* gene:

A variant form of the acetolactate synthase (ALS) gene isolated from tobacco cell culture.

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

c. Dihydroflavonol 4-reductase (DFR) gene:

This is the dihydroflavonol 4-reductase (DFR) gene of petunia, and DNA fragment derived from chromatic DNA including promoter, translated region and 3' untranslated region.

This enzyme reduces dihydroflavonol and produces leucoanthocyanidin. The leucoanthocyanidin is the direct precursor of anthocyanidin. Among DFRs, the DFR derived from petunia can reduce dihydroquercetin and dihydromyricetin as substrates, though it cannot reduce dihydrokaempferol. This suggests that the DFR from petunia is a suitable DFR for production of delphinidin.

d. Chalcone synthase (CHS) gene promoter:

The chalcone synthase (CHS) gene promoter from *Antirrhinum majus*. The CHS gene is one of the enzymes which are involved in the synthesis of flavonoids. By using this promoter, the high level of expression of CHS in petal epidermal cells is expected.

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

Derived from Pansy. As shown in Fig.1 (p4), it is an enzyme catalyzing the hydroxylation of the B ring of dihydroflavonols, such as converting dihydrokaempferol to dihydromyricetin, or dihydroquercetin to dihydromyricetin.

f. 3' untranslated region of the D8 gene:

The D8 gene codes the phospholipids transfer protein of Petunia. In cases as in this recombination scheme where three expression cassettes (minimum units for expression of genes from promoter to 3' untranslated region through gene coding region) are linked on a single vector for expression of *surB* gene, DFR gene, and F3'5'H gene, it is preferred to use different promoters and/or 3' untranslated regions for individual expression cassettes for stable expression of introduced genes. Therefore, 3' untranslated region of the D8 gene was used as the terminator for F3'5'H cDNA expressed in the petunia petals.

g. *lacZ*:

A part of the *lacZ* gene, which codes  $\beta$ -galactosidase of *E. coli*.  $\beta$ -galactosidase is an enzyme which hydrolyses lactose to galactose and glucose. Utilizing this activity, the *lacZ* gene is widely used as a reporter gene. In the vectors containing multi-cloning sites in the *lacZ* gene, insertion of any DNA fragments into the multi-cloning sites causes failure of expression of active  $\beta$ -galactosidase. This provides a benefit of increased working efficiency since the presence of  $\beta$ -galactosidase activity may be used as evidence of freedom from insertion of any DNA fragments. The pWTT2132 vector used in this recombination scheme also contains multi-cloning sites in the *lacZ* gene for the benefit described above. In practice, however, in the binary vector actually introduced in cultivars of carnation, multi-cloning sites in the pWTT2132 contain other gene expression cassettes inserted and thus, production of proteins offering the  $\beta$ -galactosidase activity is not attained. In addition,  $\beta$ -galactosidase gene promoter does not function in plants and therefore, in transgenic plants, neither  $\beta$ -galactosidase nor any peptide fragment

derived from  $\beta$ -galactosidase could be produced and thus this would not become an allergen.

- (b) Functions of proteins produced by the expression of target genes and selectable markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

The F3'5'H derived from pansy converts dihydrokaempferol to dihydromyricetin, or dihydroquercetin to dihydromyricetin, and the DFR derived from petunia converts dihydroquercetin and dihydromyricetin to leucocyanidin and leucodelphinidin respectively. In addition, the *surB* isolated from tobacco provides tolerance to chlorsulfuron herbicide.

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

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

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

## 2. Information concerning vector

- (1) Name and origin

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

- (2) Properties

- i) Number of bases and base sequence of vector

pWTT2132 is a 18,648bp binary vector.

- ii) Functions specific to any base sequence

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

- iii) Capability of infection of vector and the information relating to its host-range if the vector is found infectious

Infectivity with any other bacteria is not identified.

### 3. Method of preparing living modified organisms

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

The binary vector pCGP1991 has the approximate size of 27.5kbp, including approximately 14.0kbp of the T-DNA region between the left border and right border. The T-DNA region which is introduced to the recipient plant contains the *surB* gene to be used as the selectable marker for the recombinant plant and petunia DFR and pansy F3'5'H genes for the modification of flower color.

#### (2) Method of transferring nucleic acid transferred in the recipient organism

The *Agrobacterium* method was used for the plant transformation.

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

#### (3) Processes of rearing of living modified organisms

##### i) Method of selecting the cell into which nucleic acid is transferred

For selection of recombinants, a selective medium containing chlorsulfuron (1-5 $\mu$ g/l) was used.

##### ii) Presence of any residual body cell of *Agrobacterium* in the case where the *Agrobacterium* method is used for transferring the nucleic acid

By applying extracts from the leaves of this recombinant plant onto the medium, observation was made for any growing colony to identify any residual *Agrobacterium*. There was no colony observed which was considered *Agrobacterium*.

Consequently, it was judged that there is no residual *Agrobacterium* in this recombinant.

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

#### (1) Location of the copy of transferred nucleic acid

The transferred nucleic acid is located on the chromosome of the recombinant plant.

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

Southern blotting analyses revealed that five copies of transferred nucleic acid exist in the genome of the recombinant. The transferred sequence is considered to represent the entire length from LB to RB of T-DNA, or a part of the sequence from LB to RB. The recombinants are all produced by vegetative propagation and thus, available species are limited to the transformants in the current generation at the time of preparation. Consequently, stability of the inheritance through multiple generations is not analyzed.



- (3) Nearby or separate location of multiple copies, if present, on the chromosome

It is considered that the transferred nucleic acids are located separately.

- (4) The stability of the expression among individuals and generations under natural conditions with respect to the characteristics shown specifically in 6 (1)

Regarding the expression of introduced pansy F3'5'H and petunia DFR genes in the petals, Northern blotting analyses were carried out. As a result, signals which are specific to the introduced genes were detected only in the recombinants, which indicate expression of the genes inserted into the genome. In addition, the color of flowers obtained from the expression of the introduced genes is a stable purple-violet in this recombinant. Also for the individual living modified organisms bred by vegetative propagation, the color of flowers is consistently maintained, and there is no case reported referring to occurrence of any flower color other than purple-violet.

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

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

#### **5. Methods of detection and identification of living modified organisms and their sensitivity and reliability**

The genome sequence in the neighboring regions of T-DNA inserted in the genome of this recombinant was identified. Based on the sequence information, PCR primer was prepared and then with the PCR method using the primer, the conditions allowing detection and identification specifically for this recombinant were determined. In addition, this method was confirmed to allow detection of this recombinant by using the genome DNA of 10ng at a minimum for reaction.

#### **6. Difference from the recipient organism or the species to which the recipient organism belongs**

- (1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acids

By introducing pansy F3'5'H and DFR genes to the recipient organism and succeeding overexpression of the genes in petals, delphinidin was produced and the flower color changed to purple-violet. The genes are considered at steady-state levels of expression in petals for the pansy F3'5'H that uses a petal-specific promoter and in petals which is the proper organ of expression for the petunia DFR that introduces DNA fragment derived from chromatic DNA including promoter.

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

- (2) The degree of difference between the recombinant plant and the species to which the recipient organism belongs, if any, with respect to the physiological or ecological characteristics listed below

These were evaluated based on the data from the tests carried out in the closed system/non-closed system greenhouse which is located inside Suntory Co., Ltd in 1999, and the tests carried out in the isolated field which is located inside Suntory Co., Ltd in 2000. Like the recipient organism, cultivation by harvested seeds is not carried out for the recombinant plant.

(a) Morphological and growth characteristics

The recipient organism and the recombinant plant were cultivated in a vinyl greenhouse in the isolated field to identify the morphological and growth characteristics, i.e., plant height, number of nodes, flowering time, flower diameter, number of petals, anther length and anther width. In the number of petals and anther width, there were statistically significant differences (Student's *t* test at 5% significance level) between the recipient organisms and the recombinant plant. Concretely, the average number of petals of the recipient organisms and the recombinant plants were  $50.0 \pm 4.0$  and  $61.0 \pm 5.0$ . In addition, the average anther width of the recipient organisms and the recombinant plants were  $1.3 \pm 0.3\text{mm}$  and  $1.0 \pm 0.3\text{mm}$ . No significant difference was observed between the recipient organism and the recombinant in plant height, number of nodes, flowering time, flower diameter and anther length. Consequently, it was considered that there is no difference in the morphological and growth characteristics between the recipient organism and the recombinant plant.

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

Cultivars of carnation are not fertilizable under natural conditions and thus they do not set any seed. Propagation by seed is available only by cultural practices and therefore chilling-tolerance and heat-tolerance at the early stage of growth of the plant derived from seed was not evaluated. For the chilling-tolerance at the early stage of growth of the plant derived from herbaceous cutting, no difference was observed in growth between the recipient organism and the recombinant from the wintering ability test in which seedlings of 10-15cm in plant height and about one in number of nodes were cultivated outdoors in the isolated field and thus, it is considered that there is no difference between the recipient organism and the recombinant. In addition, based on the facts that in the normal cultivation of carnation, herbaceous cutting is performed in spring and matured plant body is attained in summer and that any herbaceous cutting in summer or winter is performed under artificial conditions in greenhouses, heat-tolerance at the early stage of growth was not evaluated.

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

As a result of outdoor cultivation of the recipient organism and the recombinant plant in isolated fields, all the plants could overwinter and no difference was observed in the growth. Thus, it was considered that there is no difference in the wintering ability between the recipient organism and the recombinant plant.

Summer survival of matured plants was not evaluated since carnation cultivars favor a cool climate at around 20°C and therefore they are cultivated in temperature-controlled greenhouses in hot summer season in Japan. However, in the five-years experience in cultivation of the recipient organism and the recombinant in Melbourne in Australia where the temperature in vinyl greenhouses can rise up to 43-45°C in summer, both species have survived the summers and no visible difference has been observed in plant height and other growth characteristics. In consideration of a possible maximum

temperature of around 35°C during the summer in Japan in an average year and also the results in Melbourne, it was considered that both plants could successfully survive summer.

(d) Fertility and size of the pollen

The pollens of the recipient organism and the recombinant plant cultivated in the non-closed system greenhouse were examined visually, and the existence of pollen was confirmed. Then, fertility of pollens was examined by acetocarmine staining. As a result, fertility of pollens was found in the recipient organism and the recombinant plant. In addition, the germination of the pollen tube was examined using germinating medium, and germination was observed for pollen from both plants. However, the germination rate was low and no significant difference was found between the recipient organism and the recombinant plant. Consequently, it was considered that there is no difference in the fertility of pollen between the recipient organism and the recombinant plants.

In addition, the size of pollens was examined under a microscope, and no difference was observed between the recipient organism and recombinant plants.

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

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

(f) Crossability

Pollens existed in both of the recipient organism and the recombinant plants which were cultivated in the non-closed system greenhouses, and germinability was confirmed, though no statistically significant difference was observed. In the growth characteristics including flowering time of the recipient organism and the recombinant plants cultivated in isolated fields, the significant difference was observed in the number of petals and anther width, but the difference was not the one to cause effects to the insect pollination. In practice, no significant difference was found in the observation of flower-visiting insects, and insects to get into flowers were not found. Consequently, it was considered that there is no difference in the crossability between the recipient organism and the recombinant plants.

(g) Productivity of harmful substances

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

In addition, as a result of a plow-in test and succeeding crop test for any effect on germination of lettuce seeds in order to identify the possibility that the introduced gene could affect the metabolism of the recombinant and cause productivity of any harmful substance, no difference was observed between the recipient organism and the recombinant plants cultivated in the isolated field. Moreover, the results of a soil microflora test showed no difference in the number of eumycetes, bacteria, and actinomycetes between the recipient organism and recombinant plants.

Consequently, it was considered that there is no difference in the productivity of harmful substances between the recipient organism and the recombinant plants.

## **II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity**

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism applied based on the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." Results of the review are listed below.

### **1. Item-by-item assessment of Adverse Effect on Biological Diversity**

#### **(1) Competitiveness**

Carnation cultivars (*Dianthus caryophyllus* L.) have been cultivated in various countries including Japan, though they are known not to become self-seeding. In addition, they are also known to have an extremely low possibility of crossing other than in cultural practices.

This recombinant cultivar of carnation features the color of flowers changed to purple-violet due to the introduction of F3'5'H and DFR genes, and it may affect the types and/or behaviors of flower-visiting insects. However, the number of petals of the recombinant cultivars of carnation is much increasing compared to the non-recombinant carnation cultivars. Thus, this recombinant plant has less possibility to be crossed by wind pollination and insect pollination than the carnation cultivars, and this recombinant plant is considered not to improve pollination ability.

In addition, this recombinant cultivar of carnation features the tolerance to sulfonylurea type herbicide due to the introduced *surB* gene, though the herbicide is not considered to become a selection pressure under natural environmental conditions.

Furthermore, as a result of an investigation in the isolated field in Japan, it is considered that no difference between the recombinant carnation and the non-recombinant carnation cultivars is found regarding the characteristics which are attributable to competitiveness under natural conditions in Japan.

Based on the above, the following conclusion by the applicant was judged reasonable: There are no wild animals or plants identified which may be subjected to the effects attributable to competitiveness and there is no risk of Adverse Effect on Biological Diversity caused by competitiveness.

#### **(2) Productivity of harmful substances**

Production of any harmful substances by carnation cultivars to wild animals and plants has not been reported.

This recombinant cultivar of carnation produces flavonoid 3' 5'-hydroxylase by the pansy F3'5'H, and dihydroflavonol 4-reductase by petunia DFR. These enzymes cause the biosynthesis of bluish anthocyanins which are not possessed by non-recombinant cultivars

of carnation but contained in the petals of bluish pansies or petunias, and they have been reported not to cause any harm to other wild animals and plants.

In addition, this recombinant cultivar of carnation also produces a variant form of the acetolactate synthase (ALS) due to the *surB* derived from tobacco cell culture, though this enzyme is known to offer the same activity as possessed by ALS which is inherent in every plant body in addition to the feature of sulfonylurea type herbicide tolerance.

Furthermore, in isolated fields in Japan, this recombinant cultivar of carnation has been investigated for production of any harmful substances to other plants and microorganisms in a series of tests of plow-in, succeeding crop and soil microflora with the result that there is no significant difference from non-recombinant carnation cultivars.

Based on the above, the following conclusion by the applicant was judged reasonable: There are no specific wild animals or plants identified which may be subjected to the effect attributable to the productivity of harmful substances and there is no risk of Adverse Effect on Biological Diversity caused by the productivity of harmful substances.

### (3) Crossability

It is known that there is virtually no possibility that carnation cultivars can cross in any manner other than cultural practices. This recombinant plant features a significantly smaller number of petals compared to carnation cultivars though it has no distinct difference from carnation cultivars in those fundamental structures of the floral organ in that the petals are dense and the stamens and pistils are covered by the petals, and it is also confirmed that the pollens have no fertility.

Based on the above, there may be no possibility that nucleic acid transferred to the recombinant carnation cultivar could be introgressed to any of the four types of plants belonging to *Dianthus* spp. indigenous to Japan. Therefore, the following conclusion by the applicant was judged reasonable: There is no species that may be subjected to the effect attributable to crossability and thus there is no risk of Adverse Effect on Biological Diversity caused by crossability.

## 2. Conclusion

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant cultivar of carnation in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is valid.