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 123.2.2 （*F3’5’H*, *DFR*, *Dianthus caryophyllus* L.） （OECD UI:FLO-4Ø619-7）
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 Report

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

1. Information concerning a recipient organism or the species to which the recipient organism belongs

(1) Taxonomical position and state of distribution in natural environment

A white carnation cultivar **FE 123**, belonging to *Dianthus caryophyllus* L., *Dianthus*, *Caryophyllaceae*, was used as the recipient organism.

Cultivars of carnation have long been cultivated all over the world, but there has been no report either inside or outside of Japan that cultivars of carnation have become self-seeding.

(2) History and present state of Use

Current carnation cultivars are hybrid cultivars, where the existence of the original species and the origin of cultivation are not clear. It is generally understood that carnations were introduced to Japan in the early 17th century, and commercial production commencing in the late 19th century.

At present carnation cultivars are widely used for indoor ornamental purposes, as one of the three major cut flower species along with roses and chrysanthemums.

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

It is a semi-hardy perennial which favors a cool climate. Within 10-25ºC, it grows well. Usually, it is cultivated in greenhouses, but it is not impossible to overwinter outside in the southwestern regions of Japan.

ii) Mode of propagation or reproduction

(a) Propagation and shedding habits, mode of dispersion, dormancy and longevity of the seed

Occurrence of autogamy and allogamy are both extremely difficult in nature. Seed propagation in nature is almost impossible.

Possibilities of seed shedding and dispersion in nature are extremely low. The seeds do not have dormancy.

A study has been made which confirmed the longevity of the seeds for up to 6 months, stored dry.

(b) Vegetative propagation and its modality, and budding property

Although vegetative propagation through herbaceous cutting is used for commercial production, vegetative propagation does not occur in nature since herbaceous cutting treatment is only valid under adequate environmental conditions. It does not possess budding property under natural conditions.

(c) Degree of autogamy and allogamy, presence of self-incompatibility, possibility of hybridization with wild relatives, and nature to cause apomixis

a. Degree of autogamy and allogamy

Although there are inter-cultivar differences, production of pollen is extremely scarce in general. Also, since there is a difference in the timing of maturation of reproductive organs, i.e., pollens mature earlier than the pistil, autogamy under natural conditions is regarded as being extremely difficult. In addition, it is considered that allogamy rarely occurs in nature due to the properties of the pollen and the difficulty of insect pollination as mentioned later.

b. Presence or absence of self-incompatibility

Although there are inter-cultivar differences, most cultivars can be artificially self-pollinated without representing self-incompatibility.

c. Possibility of hybridization with wild relatives

a) Wild relatives indigenous to Japan

There are four wild relatives indigenous to Japan including *D. superbus* L., *D. kiusianus* Makino, *D. japonicus* Thunb., and *D. shinanensis* (Yatabe) Makino. Other than these, *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams, and *D. superbus* var. *speciosus* Reichb. are distinguished.

b) Possibility of hybridization with wild relatives under natural conditions

There is no report of cases that carnation cultivars and wild relatives indigenous to Japan have hybridized under natural environmental conditions in Japan.

c) Possibility of artificial hybridization with wild relatives

Carnation cultivars can be artificially hybridized between species within the genus of *Dianthus*, and have been bred by artificial hybridization with other species in the genus of *Dianthus*.

Regarding artificial inter-specific hybridization, there is a study which attempted inter-specific hybridization between carnation cultivars and *D.japonicus Thunb*. When carnation cultivars were used as the pollen parent, no seed was obtained, even when embryo culture was utilized. However,

when *D.japonicus Thunb* was used as the pollen parent, the result differed depending on the cultivar of carnations that were used as the mother. A plant that can be considered as the inter-specific hybrid was obtained from only one out of six cultivars tested. It was reported that among the pollinated flowers of this cultivar, 91% formed seeds, 60% of which germinated, but that actual inter-specific hybrid was 50% of the germinated plants. Additionally, there exists a small-flowered spray cultivar (called the gypsy line) that was derived from artificial hybridization between *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams, whose basic species is *D. superbus.* L., and a carnation cultivar.

d) The presence or absence of apomixis causing characteristic

Carnation cultivars do not possess the characteristic to cause apomixis.

d. The mode of pollen transmission and the amount of pollen production

a) The mode of pollen transmission

In nature, there is a possibility that hybridization within the genus of *Dianthus* occurs through insect pollination. But the variety of insects is limited to butterflies and moths. Since the flowers of genus *Dianthus* have their nectaries at their base, only insects with a long proboscis (≥2.5 cm) can reach the nectary. Butterflies with a proboscis as long as 1 cm do not visit *Dianthus* flowers since they cannot reach the nectary. While, since carnation cultivars have a long distance (4-5 cm) between the edge of the petals and the nectary, neither butterflies nor moths can extract the nectar, and few other flower-visiting insects can be observed visiting carnation flowers.

b) The amount of pollen production

Pollen production by current cultivars is extremely scarce or nonexistent. The optimum temperature for pollen production is between 23-26ºC, and the growth of stamens is completely suppressed at temperatures below 17ºC.

e. Fertility, shape, dispersion distance, and longevity of pollen

There is a wide disparity in the fertility of pollen among cultivars, but it has been reported that at least 30% of the pollens are fertile in fertile cultivars. It is reported that the pollens of the Red Sim cultivar, which is the mainstream of carnation cultivars, are less active than those of other cultivars.

Regarding the dispersion distance of the pollen, the pollens of carnation cultivars, which are heavy and sticky and buried deep in the flower, hardly disperse. It was reported that pollens of carnation cultivars have not been detected in the air in Holland despite the fact that carnation cultivars are widely cultivated there.
The longevity of the pollen is 1-2 days, and germination is not observed at all on the 3rd day or after.

iii) Production of harmful substances

As a result of literature review, production of harmful substances from carnation cultivars has not been reported

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

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

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

35S promoter:	Derived from cauliflower mosaic virus
*sur*B coding region:	Derived from tobacco
*sur*B 3' untranslated region:	Derived from tobacco

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

CHS promoter:	Derived from *Antirrhinum majus*
F3'5'H coding region:	Derived from Petunia
D8 3' untranslated region:	Derived from Petunia

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

mac-1 promoter:	Derived from *Agrobacterium* and cauliflower mosaic virus
DFR coding region:	Derived from Petunia
mas 3' untranslated region:	Derived from *Agrobacterium*

(d) Others

lacZ promoter:	Derived from *E.coli*
lacZ coding region:	Derived from *E. coli*

ii) Functions of component elements

(a) 35S promoter

Promoter region from the cauliflower mosaic virus 35S RNA gene. Its downstream neighbouring *sur*B gene is expressed in the transgenic plant.

(b) *sur*B gene

A variant form of the acetolactate synthase (ALS) gene isolated from tobacco cell culture. Used as a selectable marker of transgenic plants since plants introduced with this gene show tolerance to sulfonylurea type herbicides.

(c) Chalcone synthase (CHS) gene promoter

The chalcone synthase gene promoter from *Antirrhinum majus*. CHS 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.

(d) Flavonoid 3’5’-hydroxylase (F3’5’H) cDNA

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

(e) 3’ region of the D8 gene

The D8 gene codes the phospholipids transfer protein of Petunia. The 3’ untranslated region of the D8 gene that is expressed in the petals of Petunia was used as the terminator for the expression of the F3’5’H gene, which is also expressed in the petals of Petunia.

(f) mac-1 promoter

Prepared by inserting an enhancer sequence of cauliflower mosaic virus 35S promoter to the 5’ region of promoter sequence of *Agrobacterium* mannopine synthase. Once inserted into the plant genome, it functions as a constitutive promoter and drives high levels of expression of genes connected downstream, in almost every organ of the plant and at any stage of the growth.

(g) Dihydroflavonol 4-reductase (DFR) cDNA

This enzyme reduces dihydroflavonol and produces leucoanthocyanidin.

(h) mas 3'-terminal sequence

The 3’-terminal sequence of the mannopine synthase gene, as described in (f), and widely used along with the said gene promoter.

(i) lacZ

A part of the lacZ gene, which codes β-galactosidase of *E.coli*. β-galactosidase is an enzyme which hydrolyses lactose to galactose. Utilizing this activity, the lacZ gene is widely used as a reporter gene.

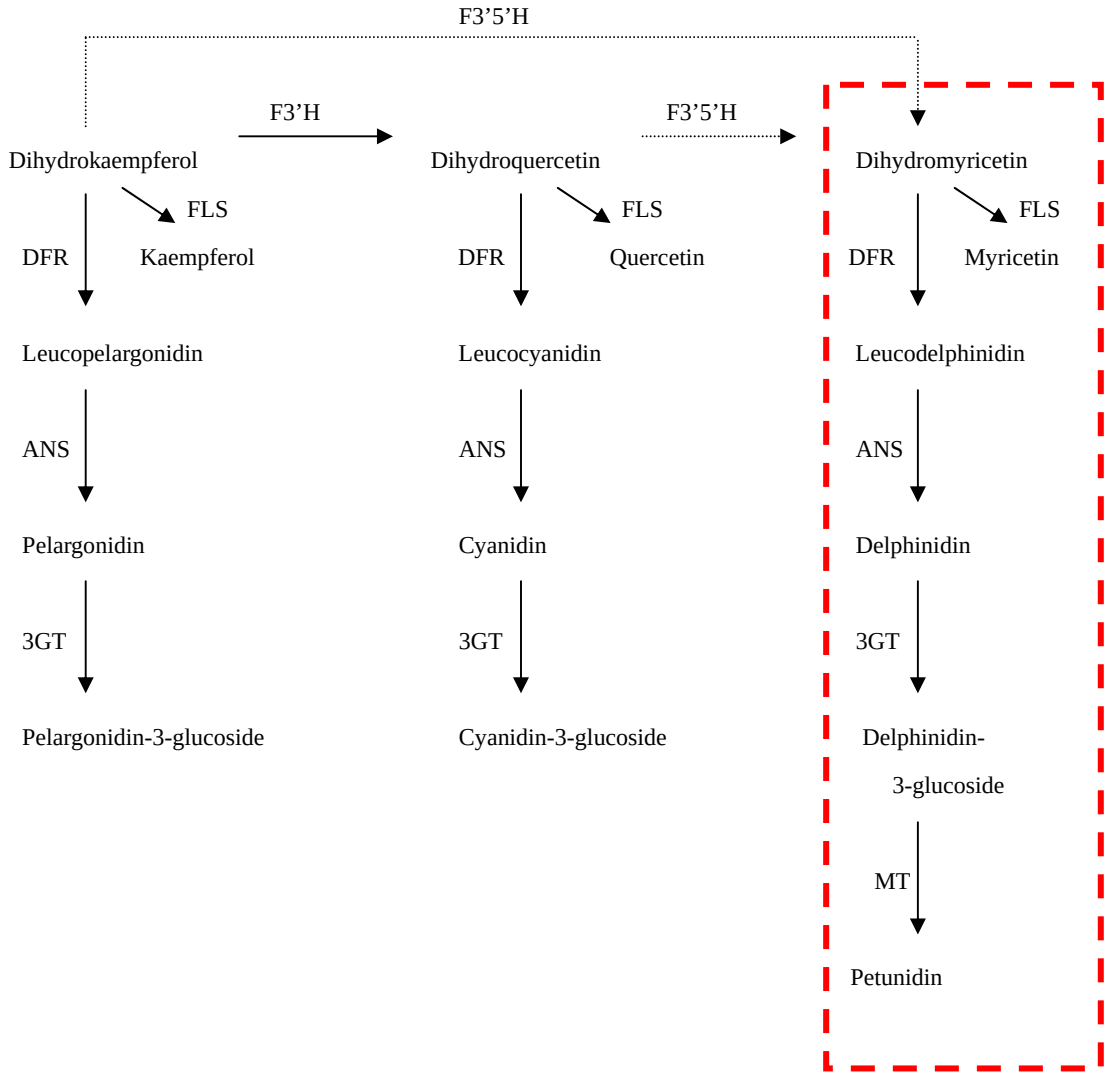

Fig.1 Outline of anthocyanin biosynthetic pathway

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. 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 dotted box represents the pathway which is newly synthesized by the function of the introduced gene.

(2) Information concerning vector

i) Name and origin

A synthetic plasmid derived from *E.coli* and *Agrobacterium*, pWTT2132 (DNA

Plant Technology, USA), was used as the vector.

ii) Properties

pWTT2132 is an approximately 19 kb binary vector, and its translocation to other bacteria has not been observed. It represents tetracycline resistance. It contains the *sur*B gene (derived from tobacco), which gives resistance to chlorsulfuron herbicide and used as a selectable marker, as well as T-DNA left border and right border sequences.

(3) Method of preparing living modified organisms

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

The binary vector pCGP1470 has the approximate size of 26.5 kbp, including approximately 13.25 kbp 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 *sur*B gene to be used as the selectable marker for the transgenic plant and Petunia DFR and F3'5'H genes for the modification of flower color.

ii) Method of transferring nucleic acid transferred in the recipient organism

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

iii) Processes of rearing of living modified organisms

The background to the development of this "purple-violet carnation 123.2.2 （F3'5'H、DFR、*Dianthus caryophyllus* L.）" (hereinafter referred to as the recombinant) is as follows.

From May to September 1996, *Agrobacterium tumefaciens* Agl0 strain was inoculated to the stem pieces of surface sterilized seedlings of greenhouse-grown carnation cultivars. Purple-violet recombinants were obtained from July 1997 to August 1998. At present, they are maintained by vegetative propagation. The obtained recombinants have acquired chlorsulfuron resistance due to expression of the *sur*B gene. The residual *Agrobacterium* was not observed in these recombinants.

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

i) Location of the copy of transferred nucleic acid

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

ii) The number of copies of transferred nucleic acid and stability of its inheritance through multiple generations.

Southern blot analyses revealed that six copies of transferred nucleic acid exist in

the genome of the recombinant.

iii) Nearby or separate location of multiple copies, if present, on the chromosome

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

iv) The stability of the expression among individuals and generations under natural conditions with respect to the characteristics shown specifically in (5) i).

Regarding the stability of the phenotypic expression of introduced petunia F3’5’H and DFR genes, Northern blot analyses were carried out. As a result, signals which are specific to the introduced genes were detected only in the recombinants, which indicates stable expression of the genes inserted into the genome. After development, this recombinant plant has been successively cultivated, and the color of the flowers is a stable purple-violet. Also, due to the expression of the *sur*B gene, the plant stably exhibits chlorsulfuron resistance.

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

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

By introducing petunia F3’5’H and DFR genes to the recipient organism, delphinidin was produced and the flower color changed to purple-violet (Table 1 and Fig. 2).

In addition, due to the expression of the *sur*B gene which was introduced as the selectable marker, herbicide chlorsulfuron resistance is accompanied.

ii) 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 isolated field which is located inside Green Gold Biosystem Co., Ltd in 2003. Like the recipient organism, cultivation by harvested seeds is not carried out for the recombinant plant.

(a) Morphological and growth characteristics

The recombinant plant and the recipient organism were cultivated in the greenhouse, and the growing characteristics, in other words, plant height, number of nodes, flowering time, diameter of flower, length of stem, number of petals, length of anther, and width of anther were investigated. Among these parameters, statistically significant difference (Student t test: significance level 5%) was found in the number of petals. Specifically, the average number of petals of the recipient organism was 56.9 ± 4.6, while that of the recombinant plant was 48.9 ± 7.4 in tests carried out in the isolated field. As for plant height, number of nodes, flowering time, diameter of flower, length of stem, length of anther, and width of

anther, no significant difference was found. Consequently, it was considered that there is no difference in the growing characteristics between the recipient organism and the recombinant plant.

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

Since carnation cultivars do not propagate by seed or by vegetative parts other than cultural practices, and since this recombinant plant does not set seed under natural conditions, these parameters were not evaluated.

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

As a result of outdoor cultivation of the recombinant plant and the recipient organism, 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.

(d) Fertility and size of the pollen

The pollens of the recombinant plant and the recipient organism were examined visually, and the existence of pollen was confirmed in both plants. The pollen fertility was checked by acetocarmine staining, and the fertility was confirmed for the pollen of both plants. The germination of the pollen was examined using germinating medium, and germination was observed for pollen from both plants. There was no significant difference in the presence of pollen and the rate of germination of pollen between the recombinant and recipient plants. Consequently, it was considered that there is no difference in the fertility of pollen between the recipient organism and the recombinant plants.

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

Since carnation cultivars do not propagate by seed or by vegetative parts other than cultural practices, and since this recombinant plant does not set seed under natural conditions, these parameters were not evaluated.

(f) Hybridization

Pollens existed in both of the recipient organism and recombinant plants, and their germinating ability was confirmed. Also, no significant difference was observed in the traits related to hybridization, such as germination rate of the pollen, between the recipient organism and the recombinant plants. Although there was a significant difference in the number of petals among the parameters for growing characteristics, the difference did not affect insect pollination. Since the anthers of carnation cultivars exist deep under the petals, it is regarded that there is little possibility of pollen dispersion by wind due to the structure of the flower. Consequently, it was considered that there is no difference in the hybridization between the recipient organism and the recombinant plants

. Table 1 Analysis of anthocyanidin in petals

	Recipient organism(FE123)	Recombinant（123.2.2）
Delphinidin (μg/g)	0	478.9
Petunidin (μg/g)	0	12.6
Cyanidin (μg/g)	0	17.8

Fig. 2-1　Flower of the recipient organism（FE123）

Fig. 2-2　Flower of the recombinant plant（123.2.2）

(g) Production of harmful substances

As a result of literature review, production of harmful substances by carnation cultivars has not been reported. Also, there was no difference in the adverse effect on germination of lettuce seeds in the plow-in test and succeeding crop test between the recipient organism and the recombinant plants. In soil microflora tests in the isolated field, no difference was observed in any of the microorganisms between the recipient organism and the recombinant plants. Consequently, it was considered that there is no difference in the production of harmful substances between the recipient organism and the recombinant plants.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

Appreciation of cut flowers, cultivation, storage, transportation, disposal and acts incidental to them.

(2) Emergency measures which should be taken to prevent Adverse Effect on Biological Diversity in case Adverse Effect on Biological Diversity could arise

Refer to the attached Plans of Emergency Measures.

(3) Information obtained from Use abroad

Similar to this recombinant plant, a purple-violet carnation with DFR and F3’5’H genes derived from petunia received approval for general release on September 25, 1995 in Australia. In Holland, approval for marketing in EC countries was issued in February 1997. Furthermore, marketing for general consumers started from December 1996 in Australia and from May 1998 in EC countries.

II. Item-by-item assessment of Adverse Effect on Biological Diversity

1. Dominance in competition

As a result of literature review, carnation cultivars are not known to become self-seeding. As a result of an investigation into various traits related to dominance in competition, statistically significant difference was observed only in the number of petals among parameters of morphological characteristics between the recombinant carnation and the non-recombinant control carnation. So, it is not considered that this difference raises the dominance of the recombinant plant in competition.

Based on the above, it was considered that there is no risk of adverse effect on biological diversity attributable to dominance in competition.

2. Production of harmful substances

As a result of literature review, production of harmful substances of carnation cultivars has not been reported. Also, no difference was observed in the plow-in test, succeeding crop test,

and soil microflora test between the recipient organism and the recombinant plants.
Based on the above, it was considered that there is no risk of adverse effect on biological diversity attributable to the production of harmful substances.

3. Hybridization

Carnation cultivars can be hybridized with wild relatives of genus *Dianthus*. Since there is a possibility of hybridization with four *Dianthus* species indigenous to Japan including *D. superbus* L., *D. kiusianus* Makino, *D. japonicus* Thunb., and *D. shinanensis* (Yatabe) Makino, there may be a possibility that nucleic acid transferred to the recombinant plant would be introgressed into these wild relatives.

Hybridization among plants of genus *Dianthus* can occur by insect pollination in nature, but the insects are limited to butterflies and moths. This is because only insects with a long proboscis (≥2.5 cm) can reach the nectary of *Dianthus* plants located at the base of the flower.

The distance from the edge of the petals to the nectary of carnation cultivars is especially long (4-5 cm), therefore even butterflies and moths with a long proboscis are unable to extract the nectar. Few flower-visiting insects other than butterflies and moths are observed for these cultivars.

In addition, through many years of cultivation, current carnation cultivars have significantly large flowers and many petals. As a result, their anther, stamen, and nectary are covered by petals in many cases. Thus, it is quite unlikely that insects which incidentally visit the flower are able to disperse the pollen. Furthermore, current carnation cultivars produce extremely little or no pollen, and if any, their fertility is extremely low. In addition, the longevity of the pollen is short being 1-2 days, and germination of the pollen cannot be observed at all on the 3rd day.

Therefore, there is virtually no possibility that the pollens of carnation cultivars are transferred through routes other than artificial hybridization. Similarly, there is virtually no possibility that the pollens of recombinant carnations are transferred through routes other than artificial hybridization, since there is no statistically significant difference between their growing characteristics and those of carnation cultivars except for the number of petals.

Consequently, it was considered that there is no risk to the maintenance of the species or individual population of *D. superbus* L., *D. kiusianus* Makino, *D. japonicus* Thunb., and *D. shinanensis* (Yatabe) Makino, which were identified as the species that may be subjected to the adverse effect attributable to hybridization.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

Regarding dominance in competition, it has not been reported that carnation cultivars become self-seeding, and there was no difference between the recipient organism and the recombinant plants in the traits related to dominance in competition. Regarding the production of harmful substances, there are no reports in literature showing that carnation cultivars produce harmful substances. Also, no difference was found between the recipient organism and the recombinant plants in the plow-in tests, succeeding crop tests, and soil microflora tests. Regarding hybridization, similarly to the recipient organism, the existence of pollen was

confirmed in the recombinant plant and their germinating capacity was maintained. But pollens of carnation cultivars do not disperse by wind, and flower-visiting insects are limited to special species because of the structure of the flower. Additionally, in order that the seeds set, artificial hybridization under strictly controlled growing conditions are required. Combined in these facts, it was considered that there is an extremely low possibility that the recombinant plant will hybridize with wild relatives through the dispersion of pollen in nature.

Consequently, it was judged that there is no risk of adverse effect on biological diversity in Japan attributable to the use of purple-violet carnation 123.2.2 for appreciation of cut flowers, cultivation, storage, transportation, disposal and acts incidental to them.