

## **Import, distribution and retail of gm-carnation 'Moonshadow'**

### **COGEM advice CGM/090407-08**

*The present application by Florigene Ltd. of file C/NL/97/13, 'Renewal application to import carnation variety FLORIGENE® Moonshadow<sup>TM</sup>', concerns the renewal of the authorization for the genetically modified carnation variety 'Moonshadow'. In 1998, this variety was authorized for production, import, distribution and retail in Europe. The current application concerns the renewal of the authorization for import, distribution and retail. The applicant does not wish to renew the authorization for cultivation.*

*In 1997, COGEM concluded that the risks for human health and the environment resulting from import of cut flowers of 'Moonshadow' are negligible. Since 1997 no new information has become available that changes the outcome of the previous risk assessment. No adverse effects concerning handling of 'Moonshadow' have been reported.*

*Carnation does not have weedy characteristics and although carnation is grown for centuries it has never been found in the wild. The genetically modified carnation variety 'Moonshadow' has a modified flower color and is tolerant to sulfonylurea herbicides. These traits do not introduce a potential for weediness.*

*Carnation is not able to fertilize wild relatives spontaneously and, therefore, the risk of transfer of the introduced traits to related species is negligible. Formation of seed on cut flowers is highly improbable.*

*In rare cases petals of carnation are used in dishes or as garnishing. Although 'Moonshadow' will not be authorized for use as food, COGEM cannot exclude the possibility that in rare cases small quantities of 'Moonshadow' petals are used in dishes. Results of toxicity tests indicate that 'Moonshadow' does not cause detrimental effects. COGEM has no reason to assume that inadvertent consumption of the genetically modified carnation poses a risk to human health.*

*In view of the aforementioned, COGEM is of the opinion that the risks for the environment and human health resulting from import of cut flowers are negligible.*

### **Introduction**

The present application concerns the genetically modified carnation (*Dianthus caryophyllus*) variety 'Moonshadow' (1363A). 'Moonshadow' expresses the genes *dfr*, *f3'5'h* and *suRB* resulting in a modified flower color and tolerance to sulfonylurea herbicides.

The genetically modified carnation variety 'Moonshadow' was authorized in 1998 for production, import, distribution and retail in Europe. The current application concerns the

renewal of the authorization for import, distribution and retail. The applicant does not wish to renew the authorization for cultivation in Europe.

#### *Previous COGEM advices*

In 1997, COGEM advised positively on the application for production, import, distribution and retail of the genetically modified carnation variety 'Moonshadow' (1). In addition, COGEM issued positive advices on several other carnation varieties modified with genes that are identical or similar to the genes that have been introduced in 'Moonshadow' (2, 3, 4).

#### *History of production and import*

The genetically modified carnation variety 'Moonshadow' was authorized in 1998 for production, import, distribution and retail in Europe. 'Moonshadow' was grown in Europe from December 1998 until July 1999. From 2001 'Moonshadow' was imported from countries outside Europe, primarily from Ecuador. 'Moonshadow' has been grown in Colombia, Ecuador and Australia and has been imported into the United States of America, Canada and Japan.

Other genetically modified carnation varieties which contain the same or similar genes have been authorized in Europe. 'Moondust' was authorized for production, import, distribution and retail in 1997 and was grown in Europe between 1998 and 2000. 'Moonlite' was authorized for import, distribution and retail in 2007. The authorization for import of another genetically modified carnation variety 'Moonaqua' is currently under consideration in the European Union. COGEM and the EFSA GMO panel considered it unlikely that import of 'Moonaqua' cut flowers would have adverse effects on human health or the environment (3, 5).

#### **Carnation, aspects of the crop**

Carnations are considered to belong to the species *Dianthus caryophyllus* of the widely cultivated genus *Dianthus*. The non-horticultural single-flower form of *D. caryophyllus* (the 'clove pink') is native to southern Europe where it grows on walls, in rock crevices and on dry stony slopes around the Mediterranean coastal regions (6). *D. caryophyllus* has occasionally been found naturalized in the United Kingdom.

The nomenclature is somewhat confusing. Nowadays the common name of *D. caryophyllus* is carnation. However, some carnations are known as 'pinks' and the term carnation is sometimes used to indicate other *Dianthus* species. Moreover, some cultivated carnations are hybrids with *D. plumarius* (6). This application concerns a cultivated double-flowered carnation (*D. caryophyllus*) variety.

Carnations have been cultured for many hundreds of years and are presently amongst the most extensively grown cut flowers with more than ten billion carnations produced around the world each year. Carnations are sold as cut flowers, cuttings or plants. Cultivated carnation is not propagated by seed but vegetatively by cuttings and tissue culture. Propagation in the horticulture involves the use of so-called mother plants (6). Cuttings of these mother plants are used for the production of flowers for a period of two years. Carnation does not spread vegetatively spontaneously, and it does not produce vegetative organs like bulbs, stolons or rhizomes.

Carnation is highly domesticated by generations of breeding aimed at improvement of flower size and color variation. Carnation is semi-winter hardy (6), has no weedy characteristics and after decades of cultivation it is not able to establish itself in the wild. Carnation produces little pollen with reduced viability (7). In the wild, cross-pollination of carnation depends on lepidopteran insects (7). Breeding has increased the number of petals present in carnation cultivars. As a result the reproductive tissues of the flower have become enclosed, restricting access to insect pollinators (7).

### **Aspects of wild *Dianthus* species**

Wild *Dianthus* species occur worldwide (8). In Europe, *Dianthus* species are found in mountainous areas like the alpine region, mainly in the Balkan and the Mediterranean area (7). In the Netherlands, some rare *Dianthus* species occur: *D. deltoides* (steenanker; maiden pink), *D. armeria* (ruige anjer; Deptford pink), *D. superbis*, (prachtanker; large pink) and *D. carthusianorum* (Kartuizer anjer; Carthusian pink) (9). The species *D. barbatus* (duizendschoon; sweet William) is commonly grown as a garden plant and has established itself in the wild (9).

Pollination of *Dianthus* in nature occurs exclusively by lepidopteran insects. The nectaries are at the base of the flowers and only insects with a proboscis longer than 2.5 cm can reach them. The number of insects visiting the carnation flower is further limited due to the fact that carnation cultivars have a long distance between the edge of the petals and the nectary, causing difficulty for insects to extract the nectar. *Dianthus* species are protandrous, which means that the anthers and pollen mature before the pistils. Pollen shedding takes place at the opening of the flower. As the flower ages the anthers fall off and the styles become receptive (7).

It is theoretically possible for carnation to cross-hybridise with other *Dianthus* species and interspecific crossings have been made manually by breeders to introduce new traits into carnation (7, 8, 10). However, spontaneous hybridisation between cultivated carnation and wild *Dianthus* species has never been reported, despite decades of cultivation in gardens and parks.

## Molecular characterisation

### *Origin and function of the introduced genes*

The genetically modified carnation variety 'Moonshadow' was produced by *Agrobacterium tumefaciens* mediated transformation using the disarmed *A. tumefaciens* strain AGL0 and the transformation vector pCGP1991.

The T-DNA region of the transformation vector pCGP1991 contained the following sequences:

- left border region, derived from the Ti plasmid of *A. tumefaciens*
- 35S constitutive promoter, derived from *Cauliflower mosaic virus* (CaMV)
- 5'untranslated region of the chlorophyll a/b binding protein, derived from *Petunia x hybrida* cDNA
- *suRB* gene and its terminator, derived from *Nicotiana tabacum* and encoding acetolactate synthase (ALS)
- *dfr* genomic clone with its terminator and promoter, derived from *Petunia x hybrida* and encoding the dihydroflavonol 4-reductase protein
- petal specific promoter, derived from the chalcone synthase (CHS) gene from *Antirrhinum majus* (snapdragon)
- *f3'5'h* cDNA, derived from *Viola 'hortensis'* and encoding the flavonoid 3'5'hydroxylase protein
- D8 terminator, derived from a putative phospholipid transfer protein homologue ('D8') from *Petunia x hybrida*
- right border region, derived from the Ti plasmid of *A. tumefaciens*

### *Properties of the introduced genes resulting in a modified flower color*

Carnations cannot produce the blue pigment delphinidin because part of the anthocyanin biosynthetic pathway is absent. Therefore, it is impossible to produce blue carnations by traditional breeding methods.

Introduction of the *dfr* and *f3'5'h* genes in carnation enables the production of the blue pigment delphinidin. The *f3'5'h* gene encodes the flavonoid 3'5' hydroxylase (F3'5'H) enzyme which converts dihydrokaempferol (DHK) to dihydroquercetin (DHQ) and then to dihydromyricetin (DHM) (11). Both products can be used as substrates by the dihydroflavonol 4-reductase (DFR) enzyme from petunia. The pink/red pigment cyanidin is produced if DHQ is converted and the blue pigment delphinidin is produced if DHM is converted. Delphinidin is the predominant pigment in the flowers of carnations genetically modified with the *dfr* and *f3'5'h* genes because the DFR enzyme from petunia preferentially uses DHM. The *f3'5'h* gene is under control of a petal specific promoter

and the substrates on which the F3'5'H enzyme acts are typically only present in flower petal tissue. Therefore, the production of delphinidin is confined to the petals.

#### *Properties of the introduced gene conferring herbicide tolerance*

The *suRB* gene has been introduced in the genetically modified carnation variety 'Moonshadow' to allow the selection of genetically modified plants in the transformation process. The *suRB* gene encodes a mutant acetolactate synthase (ALS) protein which confers tolerance to ALS inhibiting (sulfonylurea) herbicides.

ALS inhibiting herbicides bind to the ALS enzyme which is required for the production of branched chain amino acids (valine, leucine and isoleucine) (12). This results in the production of reduced quantities of branched chain amino acids and a shortage of these amino acids. This shortage leads to rapid inhibition of cell division and subsequently to plant death (12). The *suRB* gene encodes an ALS protein that is insensitive to sulfonylurea herbicides thus conferring tolerance to these herbicides.

#### *Molecular analysis*

The applicant demonstrated by Southern blot hybridization that vector backbone sequences (including the tetracycline resistance gene) are not present in 'Moonshadow'. The absence of the tetracycline resistance gene was also confirmed by PCR analysis.

The applicant states that three integration loci are present in 'Moonshadow'. These three loci have been sequenced and schematics have been supplied for each of the integration loci. Locus 1 (18,566 basepairs) consists of three fragments of the insertion cassette that are linked by short stretches of non T-DNA (20 basepairs between fragment one and two and 41 basepairs between fragment two and three). Fragment one consists of the truncated *dfp* promoter, the *f3'5'h* expression cassette and the right border region. Fragment two consists of the left border region, the *suRB* expression cassette and the *dfp* expression cassette with a truncated promoter. The third fragment consists of the right border region, the *f3'5'h* expression cassette and a truncated *dfp* promoter.

Locus 2 (26,229 basepairs) consists of a fragment of the insertion cassette that contains the right border region, the *f3'5'h* expression cassette, the *dfp* expression cassette, plus the terminator and part of the coding region of the *suRB* expression cassette. This fragment is linked to a complete copy of the insertion cassette.

Locus 3 (31,603 basepairs) consists of two complete copies of the insertion cassette linked to a fragment of the insertion cassette that includes the right border region plus the terminator and part of the coding region of the *f3'5'h* expression cassette.

All three integration loci and 150 basepairs of their flanking regions have been sequenced. The junctions between the integration loci and their flanking regions were

analyzed for the presence of putative open reading frames. In addition, all junctions between the inserted fragments were analyzed for the presence of putative open reading frames. Sixty-one putative open reading frames were identified and translated *in silico* into amino acids. The deduced amino acid sequences were analyzed for homology to known allergens or toxins. This analysis resulted in the identification of some putative homologous sequences. The external regions of the allergenic proteins and toxins that had been identified, were determined by using hydrophilicity plots. The regions of the deduced amino acid sequences that were homologous to known allergenic proteins or toxins did not correspond to the external regions of the allergenic proteins and toxins. Therefore, the applicant concludes that the deduced amino acid sequences are not homologous to antigenic regions of known allergenic proteins or to known protein toxin domains.

### **Advice**

This application concerns the import of cut flowers of the genetically modified carnation variety 'Moonshadow'. In 1997, COGEM concluded that import of cut flowers of 'Moonshadow' posed a negligible risk to human health and the environment. Since our previous advice no new information has become available that changes the outcome of the previous risk assessment.

The genetically modified carnation variety 'Moonshadow' has been authorized in Europe for production, import, distribution and retail since 1998. 'Moonshadow' was grown in Europe from December 1998 to July 1999. From 2001 'Moonshadow' was imported from countries outside Europe, primarily from Ecuador. 'Moonshadow' has been grown in Colombia, Ecuador and Australia and has been imported into the United States of America, Canada and Japan. No adverse effects concerning handling of 'Moonshadow' have been reported.

In the current application a General Surveillance plan to observe and register adverse effects of the import of 'Moonshadow' was provided. COGEM considers the provided General Surveillance plan sufficient for import, distribution and retail of the genetically modified carnation variety 'Moonshadow'.

Carnation is not able to spread vegetatively and cut flowers are not able to form roots. This excludes the possibility that the imported material will give rise to plants and establish itself in the wild. Nevertheless, carnation can be propagated by stem cuttings, a method used both by professionals in the flower industry and amateur gardeners. Therefore, it cannot be completely ruled out that buyers will propagate the material to

plant in their gardens. However, carnation has no weedy characteristics (7). Although carnation is cultivated since decades, it has never been found growing in the wild. The introduced traits (modified flower color and herbicide tolerance) do not introduce a potential for weediness.

Formation of seed on cut flowers is highly improbable. Carnation is pollinated exclusively by butterflies or moths. Outcrossing during production or transport is unlikely as flowers are cut before opening and transported refrigerated. Theoretically, it is possible that cut flowers in the vase are visited by butterflies and become pollinated. Carnation plants require five to six weeks for seed development while the vase life of carnation flowers is only three to four weeks. Therefore, it is improbable that cut flowers will produce seed.

Carnation can only theoretically hybridize with wild relatives. Theoretically, it is possible that cut flowers in the vase are visited by butterflies. Carnation produces only a few anthers and little pollen with a reduced viability. Pollen shedding only takes place at the opening of the flower. The applicant reported that ‘Moonshadow’ produces a significantly lower numbers of anthers. In 69 flowers only 2 intact anthers were found. No microspores were present on these anthers. In addition, the applicant states that attempts to germinate microspores from ‘Moonshadow’ were unsuccessful. In view of these facts, the possibility of hybridisation with wild relatives is considered unlikely. Most importantly, there has never been any evidence of spontaneous hybridisation between carnation and wild *Dianthus* species, despite the fact that carnation is cultivated worldwide for decades.

Moreover, the environmental risks linked to hybridization of this genetically modified carnation variety with wild relatives are comparable with those of conventional carnation. The genetic modification involves genes which play a role in the anthocyanin pathway. The resulting blue pigmentation does not alter the ecological characteristics of carnation. Neither the *f3'5'h* gene, the *dfr* gene nor the herbicide tolerance gene *suRB* offer selectable advantages in nature. Accordingly, gene flow to wild relatives will not pose an environmental risk.

Therefore, COGEM concludes that the risk of transfer of genetic traits from the transgenic carnation variety to species in unmanaged environments is insignificant.

In rare cases petals of carnation are used in dishes and as garnishing (13, 14, 15). The amount of petals that are used are low. This notification refers to the import and distribution of cut flowers and not to food purposes. Therefore, retailers will not be allowed to sell the petals of the genetically modified carnation for food purposes.

However, it can not be entirely excluded that individuals will use petals of bought flowers in dishes or to garnish their plates.

The introduced genes were isolated from other plant species. The transgenic proteins do not share homologies with known toxins or antigens. In addition, an acute toxicity test was carried out with mice using a dose of 2 grams of petals/ kg body weight to verify that 'Moonshadow' did not exert any toxic effects. No abnormalities were observed. In addition, delphinidin is present in fruits like blueberries. In conclusion, there is no reason to assume that the introduced genes or the production of delphinine will pose a threat to human health.

Summarizing, the application involves import of cut flowers. The genetically modified carnation has no weedy characteristics and is not able to establish itself in the wild, the risk of transfer of the introduced genes to wild relatives is negligible, and the genetically modified variety does not pose a threat to the health of consumers. In addition, no adverse effects concerning handling of 'Moonshadow' have been reported.

In view of the above mentioned, COGEM is of the opinion that the proposed import of cut flowers poses a negligible risk to human health or the environment.

## References

1. COGEM (1997). Advies marktaanvraag BGGO 97/13 (CGM/970714-02)
2. COGEM (2005). Import of cut flowers of the genetically modified carnation variety 'Florigene Moonlite' (CGM/050207-01)
3. COGEM (2007). Import of genetically modified carnation 'Moonaqua' (CGM/070206-02)
4. COGEM (1997). Marktaanvraag voor een kleurgemodificeerde (violette) anjer (*Dianthus caryophyllus*), van Florigene B.V.) (CGM/970513-05)
5. EFSA GMO Panel (2008). Notification for the placing on the market of the genetically modified carnation Moonaqua 123.8.12 with a modified color, for import of cut flowers for ornamental use, under Part C of Directive 2001/18/EC from Florigene. The EFSA Journal (2008) 662, 1-25
6. CABI (2007). Crop Protection Compendium, 2007 Edition. Wallingford, United Kingdom
7. OGTR (2005). The biology and ecology of *Dianthus caryophyllus* L. (Carnation). Australian Government. Department of health and ageing. Office of the Gene Technology Regulator. August 2005
8. Andersson-Kottö I and Gairdner AE (1931). Interspecific crosses in the genus *Dianthus*. Genetica 13: 77-112
9. Van der Meijden R. (2005). Heukels' Flora van Nederland. Wolters Noordhoff Groningen, the Netherlands



10. Nimura M, Kato J, Mii M and Morioka (2003). Unilateral compatibility and genotypic difference in crossability in interspecific hybridization between *Dianthus caryophyllus* L. and *Dianthus japonicus* Thunb. Theoretical and Applied Genetics 106: 1164-1170
11. Nakatsuka T, Abe Y, Kakizaki Y, Yamamura S and Nishihara M (2007). Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Plant Cell Reports 26: 1951-1959
12. Corbett CL and Tardiff FJ (2006). Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. Pest Management Science 62: 584-597
13. [http://www.gardenbed.com/source/24/2306\\_edt.asp](http://www.gardenbed.com/source/24/2306_edt.asp) (April 2009)
14. <http://jardinsdepareillas.over-blog.com/article-2893237.html> (April 2009)
15. <http://cuisine.elle.fr/elle/elle-a-table/recettes-de-cuisine/tartines-au-beurre-d-oeillet> (April 2009)