

Import of genetically modified carnation ‘Moonaqua’

COGEM advice CGM/070206-02

The present application by Florigene Ltd. of file C/NL/06/01, ‘Import carnation variety Florigene MoonaquaTM (123.8.12)’ concerns the import, distribution and sale of a genetically modified carnation. The cut flowers are produced outside the EU member states. A similar transgenic carnation variety has previously been approved for commercial production within the EU in 1997.

Carnation does not have weedy characteristics and although carnation is grown for centuries it has never been found in the wild. The introduced traits (flower, color and herbicide tolerance) do not alter the other biological characteristics of the plant. Carnation is not able to outcross with wild relatives and therefore the risk of transfer of the introduced traits to related species is absent.

Petals of carnation are occasionally used as garnishing. However, the notification does not concern food purposes. Moreover, the genetically modified carnation variety has a history of safe use and results of toxicity tests indicate that detrimental effects are absent. 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 commercial import, distribution and sale of a genetically modified carnation (*Dianthus caryophyllus*) variety with a modified flower colour. The cultivar involved (‘Moonaqua’) also contains a herbicide tolerance gene.

‘Moonaqua’ is admitted and commercially grown since five years outside Europe. A similar transgenic carnation variety ‘Florigene Moondust’ has previously been approved for commercial production within the EU in 1997 (C/NL/96/14-11).

Carnations, like roses or chrysanthemums, do not produce a blue pigment called delphinidin since these plants lack part of the anthocyanin biosynthetic pathway. Consequently, it is impossible to create blue carnations by traditional breeding methods. However, genetic modification offers a means to introduce a violet or blue colour in these plants. This can be achieved by insertion of the *dfr* and *F3'5'H* gene originating from *Penunia X hybrida* and *Viola sp.* respectively, in the genome of carnation varieties with white flowers. These genes produce enzymes which convert certain flavonoids into delphinidin. Accumulation of delphinidin in petals results in different shades of violet of the flowers depending on the level of accumulation.

Previous COGEM advices

In the past COGEM has advised positively on similar applications involving carnation varieties ('Moondust' and 'Moonlite') with a modified flower colour.^{8, 9, 10, 12} These applications involved the same or similar genes and vector DNA. 'Moondust' was admitted to the European market in December 1997 and was cultivated between 1998 and 2000 in the Netherlands and Spain and sold in Europe. The application for 'Moonlite' concerned only import of cut flowers and is currently under consideration in the European Union.

Furthermore, COGEM advised positively on an application for production of carnation with an increased shelf life in the EU in 1997.¹¹

Dianthus, Aspects of the crop

Carnations are considered to belong to the species *Dianthus carophyllus* of the widely cultivated genus *Dianthus*. However, the exact origin is obscure. Most likely cultivated carnation stems from a hybrid involving *D. carophyllus* and another *Dianthus* species.^{1,7} The non-horticultural single-flower form of *D. carophyllus* (the 'clove pink') is a rare wild species of the coastal areas of Southern Europe.^{2,3}

The nomenclature is somewhat confusing. Nowadays the common name of *D. carophyllus* 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. plumaris*. This application concerns a cultivated double-flowered carnation (*D. carophyllus*) variety.

Carnations have been cultured for many hundreds of years and are presently amongst the most extensively grown cut flowers with more than 10 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. 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 colour variation. Carnation is semi-winter hardy, has no weedy characteristics and after decades of cultivation it is not able to establish itself in the wild.²

Wild *Dianthus* species occur worldwide.^{1,2} They are common wildflowers in the United States and Canada. In Europe *Dianthus* species are found in mountainous areas like the

alpine region, mainly in the Balkan and the Mediterranean area. In the Netherlands, even some rare *Dianthus* species occur: *D. deltoides* (steenanjel; Maiden pink), *D. armeria* (ruige anjer; Deptford pink), *D. superbus*, (pracht anjer; Large pink) and *D. carthusianum* (Kartuizer anjer; Charterhouse pink).⁴ The species *D. barbatus* (duizendschoon; Sweet William) is commonly grown as a garden plant and has established itself in the wild.⁴

Pollination of *Dianthus* in nature occurs exclusively by insects (Lepidoptera species). 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 proterandrous, 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.

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. However, spontaneous hybridisation between cultivated carnation and wild *Dianthus* species has never been reported, despite decades of cultivation in gardens and parks. No records of hybrids exist in the scientific literature or floras.

Molecular characterisation

Origin and function of the introduced genes

The genetically modified carnation line was produced by transformation with *Agrobacterium tumefaciens* using a disabled *Ti* plasmid. For the production of violet or delphinidin derived pigment the *dfr* and *F3'5'H* genes were inserted in the carnation genome. Accumulation of these pigments in the petals results in a violet flower colour.

As a selection marker in the transformation process a herbicide tolerance (*suRB*) gene was introduced in the transgenic line. This herbicide tolerance has no agronomic relevance.

An overview of the sequences introduced is given below:

- *dfr* genomic clone, coding for dihydroflavonol 4-reductase hydroxylase, derived from *Petunia X hybrida*. The DFR enzyme uses dihydroquercetin and dihydromyricetin as a substrate to produce delphinidin;
- *F3'5'H* gene, flavonoid 3',5' hydroxylase, derived from *Viola*. *F3'5'H* converts dihydrokaempferol or dihydroquercetin into dihydroflavonol dihydromyricetin;
- *suRB* gene, coding for a mutant acetolactate synthetase protein (*ALS*), derived from *Nicotiana tabacum*. *ALS* confers tolerance to sulfonylurea herbicides;

- CHS-promoter, chalcone synthase petal specific promoter derived from *Anthirrhinium majus*;
- CaMV 35S promoter, 35S promoter of *Cauliflower mosaic virus*;
- LB, T-DNA border, derived from *Agrobacterium tumefaciens*;
- RB, T-DNA border, derived from *A. tumefaciens*;
- Cab 5'utr, 5'untranslated region (utr) of the Chlorophyll a/b binding protein gene, derived from *Petunia x hybrida*;
- D8 terminator; terminator sequence derived from *Petunia X hybrida*;

The inserted genes *dfr*, *F3'5'H* and *suRB* all originate from plants. The proteins encoded by these genes do not share homology with known toxins or antigens.

Molecular analysis

The COGEM notes that a tetracycline resistance gene is present in the backbone sequence of the vector used in the transformation process. This gene confers resistance to the antibiotic tetracycline and was used in the production and selection process of the vector DNA. Backbone sequences are presumed not to be inserted in the genome of the plant. The applicant has conclusively proven by Southern blot analysis that the tetracycline resistance gene is not present in the carnation variety 'Moonaqua'.

Insertion of foreign genes into the genome of an organism can lead to disruption of host genes and theoretically give rise to the formation of new open reading frames (ORFs) consisting of host and donor sequences. Therefore, applicants are required to analyse the border and bordering sequences of the insertion for the presence of chimer ORFs. The applicant characterised the T-DNA coding region and 150 bp of its flanking sequences. One full copy and two partial copies of the inserted sequences are present in the carnation genome. The applicant performed sequence analysis to determine whether new ORFs were formed due to these insertions. No ORFs larger than 50 amino acids were detected in the insert plant transition. However, it has to be noted that the applicant does not state whether the 150 bp of the flanking sequences are *Dianthus* genomic DNA. Theoretically it is possible that other DNA, originating from for instance chloroplasts, is co-integrated with the insert. COGEM assumes that the flanking sequences consist of plant genomic DNA. Also, COGEM concludes that no new ORFs are found.

Trials in greenhouses were carried out in the Netherlands in the summer of 1999 and 2000. The genetically modified carnation has been vegetatively propagated since 1999. Commercial production has started in 2001 and approximately 8.6 million flowers have been produced in South America. Hardly any colour changes were found and since 2003 only 2 flowers of 4,500 flowers were found with white streaks. To the opinion of the

experts of COGEM the genetic stability of the cultivar is sufficiently proven by the fact that during the production period hardly any phenotypic aberrations were found.

Advice

This application concerns the import of cut flowers of genetically modified carnation. This carnation variety is admitted and has already gone into commercial production in Colombia and Ecuador. A similar transgenic carnation variety, named 'Moondust' has already been approved for commercial production within the EU.

In the environmental risk assessment the probability of gene dispersal, weediness and potential risks to consumers due to incidental consumption has to be considered. The number of relevant issues is limited however, since this application involves the import of cut flowers.

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. It cannot be ruled out that buyers will propagate the material to plant in their gardens. However, carnation has no weedy characteristics² and the traits (blue pigmentation and herbicide tolerance) which are introduced in the genetically modified variety do not alter the biology of carnation to the opinion of COGEM. Although carnation is cultivated for decades worldwide, it has never been found growing in the wild.

Carnation can only theoretically hybridize with wild relatives. Carnation is pollinated exclusively by butterflies or moths. Outcrossing during production or transport is unlikely as flowers are cut before opening and transported refrigerated. It is possible, although unlikely, that cut flowers in the vase are visited by butterflies and become pollinated.

This situation is improbable though, since 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 provides data that the variety 'Moonaqua' produce significantly lower numbers of stamen, anthers and styles compared to the parental line. In view of these facts, the possibility of hybridisation with wild relatives is considered to be unlikely.

Formation of seed on cut flowers is highly improbable. Carnations plants require five to six weeks for seed development while the vase life of carnation flowers is only three to four weeks.

Most importantly, there has never been any evidence of 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 involving genes which play a role in the anthocyanin pathway resulting in blue pigmentation do not alter the biological characteristics of carnation. Neither the *F3'5'H* gene and *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.

Even though cut flowers are not a food, the risks of incidental consumption have to be considered. Petals of carnation are occasionally used as garnishing^{5,6}. In Europe they are mainly used (in low amounts) as garnishing in desserts.

The 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 to garnish their plates.

The introduced transgenic proteins all origin from plants and do not share homologies with known toxins or antigens. Delphinidin is present in fruits like blueberries. Thus, eventual expression of the introduced genes or the production of delphinine will not pose a threat to human health.

The applicant characterised the borders and 150 bp of the flanking sequence of the inserts. No ORFs larger than 50 amino acids were found at the six junctions of integrated and carnation genomic DNA. COGEM already analysed the possibility of the presence of chimeric ORFs and the potential risks to human health of potential consumers in a former advice on carnation and considered these risks to be negligible.¹²

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 nil, and the genetically modified variety does not pose a threat to the health of eventual consumers or the environment.

In view of the above mentioned, COGEM is of the opinion that the proposed import of cut flowers does not pose a significant risk to human health or the environment.

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