Import, distribution and retail of GM carnation FLO-40685-1

COGEM advice CGM/131217-01

The application by Suntory Holdings Ltd. of file C/NL/13/02 concerns the authorisation for import, distribution and retail of genetically modified (GM) carnation variety FLO-40685-1.

Carnation does not have weedy characteristics and although carnation has been cultivated for centuries, there are no reports of establishment of cut flower carnation varieties in the wild. The GM carnation variety FLO-40685-1 has a modified flower colour and is tolerant to sulfonylurea herbicides. These traits are not associated with a potential for weediness.

Carnation is not able to fertilise wild relatives spontaneously and, therefore, the risk of transfer of the introduced genes to related species is negligible. Production of seed on cut flowers is highly improbable.

The molecular characterization of FLO-40685-1 meets the criteria of COGEM. COGEM considers the General Surveillance plan adequate for import of FLO-40685-1.

The application does not concern an authorization for use as food. COGEM is of the opinion that there are no reasons to assume that a rare case of incidental consumption will pose a risk to human health.

In view of the above, COGEM is of the opinion that the risks to the environment and human health resulting from import of cut flowers of GM carnation variety FLO-40685-1 are negligible.

Introduction

The present application concerns the import, distribution and retail of cut flowers of the genetically modified carnation variety FLO-40685-1. This GM variety expresses the *dfr*, *f3'5'h* and *suRB* genes resulting in a modified flower colour and tolerance to sulfonylurea herbicides.

Previous COGEM advisory reports on other GM carnation varieties

COGEM issued several positive opinions on similar carnation varieties with an altered flower colour. [1,2,3,4,5,6,7] The GM carnation variety *'Moonshadow'* was authorised for production, import, distribution and retail in 1998 and cultivated in Europe from December 1998 until July 1999. In 2009, COGEM advised positively on the renewal of the authorisation for import, distribution and retail of this GM carnation.[7] In 1997, COGEM issued a positive advice on carnation variety *'Moondust'*. The line was authorised for production, import, distribution and retail, and grown in Europe between 1998 and 2000. The authorisations for both lines have been withdrawn recently upon request of the applicant.

In 2005 and 2007, COGEM advised positively on the GM carnation varieties *'Moonlite'* and *'Moonaqua'*.[4,5] Both lines were authorised for import, distribution and retail in the European Union in 2007 and 2009 respectively.[8] In July 2013, COGEM issued a positive opinion on GM carnation variety SHD-27531-4.[9] For GM carnation lines *'Moonaqua,' 'Moonshadow'* and SHD-27531-4,

the same transformation vector (pCGP1991) was used as in the current application of GM carnation FLO-40685-1.

Aspects of the crop

Carnation belongs 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 in Mediterranean regions from the coast up to more than 200 km from the coast.[10] Occasionally, *D. caryophyllus* has been found naturalised on old castle walls in the United Kingdom.[11]

Wild *Dianthus* species occur worldwide.[12] In Europe, *Dianthus* species are found in mountainous areas in the alpine region, the Balkan and the Mediterranean area.[13,14,15] In the Netherlands, some rare *Dianthus* species occur: *D. deltoides* (Steenanjer; maiden pink), *D. armeria* (Ruige anjer; Deptford pink), *D. superbus*, (Prachtanjer; large pink) and *D. carthusianorum* (Kartuizer anjer; Carthusian pink).[16] The species *D. barbatus* (Duizendschoon; sweet William) is commonly grown as a garden plant and has established itself in the wild.[16]

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 varieties.[13,12,17] However, spontaneous hybridisation between cultivated carnation and wild *Dianthus* species has never been reported, despite decades of cultivation in gardens and parks.

The nomenclature of *Dianthus* 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. This application concerns a cultivated double-flowered carnation variety.

Cultivated carnations are almost certainly hybrids between two or more *Dianthus* species, one of which is most likely *D. caryophyllus*. Carnations have been cultured for hundreds of years and presently are 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 propagated vegetatively by cuttings and tissue culture. In horticulture propagation involves the use of mother plants.[18] 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.[18]

To improve flower size and generate colour variants, carnation is bred for many generations. As a result, carnation is highly domesticated. Carnation is semi-winter hardy, has no weedy characteristics and even after decades of cultivation has never shown to be able to establish itself in the wild.[10]

In nature, pollination of *D. caryophyllus* occurs exclusively by lepidopteran insects. The nectaries are at the base of the flowers and only insects with a proboscis (a tubular mouthpart) longer than 2.5 cm can reach them. *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.[13] *Dianthus* carnation pollen cannot be spread by wind. Any pollen produced is heavy and sticky and deeply buried in the flower.[19,20] Although the Netherlands has a large carnation handling industry, carnation pollen can not be detected in the atmosphere.[21]

The domesticated carnation produces little pollen with reduced viability.[13,22] 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.[13] Due to these factors, the chance of natural hybridisation of cultivated carnations with wild relatives is low. The likelihood of dissemination of genetic material through pollen or seeds is limited even further in the production of cut flowers because stems are cut before anthesis.[13]

Molecular characterisation

The GM carnation variety FLO-40685-1 was produced by *Rhizobium radiobacter* (previously known as *Agrobacterium tumefaciens*[23]) mediated transformation using the disarmed *R. radiobacter* strain AGL0 and the transformation vector pCGP1991.

The transferred DNA (T-DNA) region of the transformation vector pCGP1991 contains the following sequences:

- left border region, derived from the Ti plasmid of *R. radiobacter*
- 35S constitutive promoter, derived from *Cauliflower mosaic virus* (CaMV)
- Cab 5' utr, 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 promoter and terminator, 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 *Petunia* ('D8') encoding a phospholipid transfer protein
- right border region, derived from the Ti plasmid of *R. radiobacter*

Molecular analysis

The applicant demonstrated by Southern blot hybridisation that backbone sequences of the transformation vector (including the tetracycline resistance gene) are not present in GM carnation variety FLO-40685-1. The absence of the tetracycline resistance gene was confirmed by PCR analysis. In addition, Southern blot hybridisation showed that integration of the T-DNA has occurred at four loci.

The insertions in the four loci and 150 bp of the associated flanking regions were sequenced. Bioinformatic analysis showed that the integration of T-DNA into the host genome in transgenic carnation line FLO-40685-1 did not interrupt known coding sequences.

Sequence analysis was undertaken to identify all open reading frames (ORFs) present at the four insertions of FLO-40685-1. There was no size limitation and ORFs were analysed from stop to stop codon. A total of 64 putative open reading frames were identified across the insert-genome junctions and a total of 2996 putative open reading frames were identified within the four inserts.

The applicant compared the deduced amino acid sequences of all ORFs with known toxins, allergens and proteins (Blastp, SwissProt, FARRP, and WHO-IUIS databases). A small part (eight amino acids) of the deduced amino acid sequence of four putative ORFs showed similarity to a subtilisin (protease) allergen. Additional analysis showed that the homologous sequence was not associated with the allergenicity of this protein.

In some instances, identity scores were equal or exceeding 35% when the deduced amino acid sequences of the ORFs were compared in the AllergenOnline Database using the 80 amino acid sliding window approach. However, considering the low E-values and the fact that the overall identity is less than 50%, the applicant considers it unlikely that the ORFs represent peptides with allergenic potential. The only sequence similarity linked to toxicity was an exact 22 amino acids sequence in a 38 amino acid synthetic protein produced in *Escherichia coli* for use in a study on the stability of the diphtheria toxin. There is, however, no sequence similarity of this 22 amino acid sequence to any sequence in the diphtheria toxin itself. COGEM considers it unlikely that the identified similarities are biologically relevant.

Based on the molecular analyses provided by the applicant, COGEM is of the opinion that the molecular characterisation of FLO-40685-1 is adequate and does not give any reason to assume that this GM carnation variety will pose a risk to the environment or human health.

Properties of the introduced genes resulting in a modified flower colour

Normally, carnations cannot produce the blue pigment delphinidin because part of the anthocyanin biosynthetic pathway is absent. 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).[24] Both products can be used as substrates by the dihydroflavonol 4-reductase (DFR) enzyme from petunia. Conversion of DHQ results in the production of the red pigment cyanidin, while the conversion of DHM results in the blue pigment delphinidin. Flowers of the parental variety of FLO-40685-1 are cream coloured whereas the flowers of FLO-40685-1 are dark purple. The production of delphinidin is confined to petals, since 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.

Properties of the introduced gene conferring herbicide tolerance
The *suRB* gene has been introduced in the GM carnation variety FLO-40685-1 to allow the selection of GM 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).[25] 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.[25] The *suRB* gene encodes an ALS protein that is insensitive to sulfonylurea herbicides thus conferring tolerance to these herbicides.

Theoretically, promiscuity of the enzymes encoded by the inserted genes could lead to the unintended conversion of substrates other than the intended substrates. However, based on the scientific literature and the molecular analyses of the event, there is no reason to assume that this will occur in FLO-40685-1.

Environmental risk assessment
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 by 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.[13] Although *D. caryophyllus* has been cultivated as an ornamental plant for centuries worldwide, there are only a few records of naturalised plants in some Mediterranean countries and on old castle walls in the United Kingdom.[11,26] There are no reports of establishment of cut flower carnation varieties in the wild, nor has carnation been reported as a weed, invasive species or pest species.[13] The introduced traits (modified flower colour 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. Although cut flowers in vases on window sills or

cemeteries could be visited by butterflies, it is improbable that cut flowers will produce seed because carnation plants require five to six weeks for seed development while the vase-life of carnation flowers is only three to four weeks.

Theoretically, carnation can hybridise with wild relatives. In some European regions bouquet flowers (including carnation) are brought to cemeteries. Wild carnation is found in southeast France and Italy. In these regions cemeteries are frequently positioned adjacent to the natural environment. If GM carnation would be brought to cemeteries close to the natural habitat of wild *D. caryophyllus*, the transfer of viable transgenic pollen by butterflies to wild *D. caryophyllus* cannot be completely excluded, although it is highly unlikely. The carnation flower opens out in the vase, increasing accessibility to the reproductive structures. Carnation produces only a few anthers and little pollen with a reduced viability. Pollen shedding only takes place at the opening of the flower. There has never been any evidence of spontaneous hybridisation between carnation and wild *Dianthus* species, despite the fact that carnation has been cultivated worldwide for centuries.

The genetic modification assessed here involves genes playing a role in the anthocyanin pathway resulting in an altered flower colour and a gene conferring tolerance to sulfonylurea herbicides. There is no evidence that the introduced traits will increase fitness in nature. Therefore, the environmental risks linked to (theoretical) hybridisation of this GM carnation variety with wild relatives are most likely comparable with those of conventional carnations.

In conclusion, COGEM is of the opinion that the risk of transfer of genetic traits from the transgenic carnation variety to species in natural environments is negligible.

Incidental consumption

In rare cases petals of carnation are used in dishes and as garnishing.[27,28] 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 GM carnation for food purposes. However, it cannot be entirely excluded that individuals will use petals of bought flowers in dishes or to garnish their plates. In general, people are advised against using flowers from flower shops or commercial growers for food purposes because these might contain residues from pesticides or other chemicals.

The molecular analyses of the event do not give any reason to assume that the inserted genes or their products have any toxic properties. Therefore, COGEM is of the opinion that incidental consumption will not pose a risk to human health.

General surveillance

A General Surveillance plan to observe and register adverse effects of the import of FLO-40685-1 was provided. In 2010, COGEM published a report on the principles that should be followed for general surveillance.[29] The General Surveillance plan of FLO-40685-1 provided by the applicant

fulfils the requirements laid down by COGEM. COGEM considers the plan sufficient for import, distribution and retail of FLO-40685-1.

Conclusion

This application concerns the import of cut flowers of the GM carnation variety FLO-40685-1. The GM carnation is not able to establish itself in the wild and has no weedy characteristics. The risk of transfer of the introduced genes to wild relatives is negligible. The molecular characterisation is adequate and there is no reason to assume that FLO-40685-1 will pose a risk to the environment or human health. In view of the above, COGEM is of the opinion that the proposed import of cut flowers of the GM-carnation variety FLO-40685-1 poses a negligible risk to human health or the environment.

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