Import, distribution and retail of GM-carnation IFD-25958-3

COGEM advice CGM/090429-01

The present application by Florigene Pty. Ltd. of file C/NL/09/01 'Application to import carnation line IFD-25958-3' concerns the authorization for import, distribution and retail in Europe.

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 IFD-25958-3 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. 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 aforementioned, COGEM is of the opinion that the risks for the environment and human health resulting from import of cut flowers of the genetically modified carnation variety IFD-25958-3 are negligible.

Introduction

The present application concerns the genetically modified (GM-) carnation (*Dianthus caryophyllus*) IFD-25958-3. In this GM-carnation variety the endogenous *dfr* gene is silenced and the *dfr* gene from petunia, the *f3'5'h* gene from viola and the *als (suRB)* gene from tobacco are expressed. As a result IFD-25985-3 has a modified flower color and is tolerant to sulfonylurea herbicides.

Previous COGEM advices

COGEM issued positive advices on several other GM-carnation varieties with a modified flower color.[1,2,3,4,5] Some of these carnation varieties were authorized in Europe. 'Moonshadow' was authorized for production, import, distribution and retail in 1998, and was grown in Europe from December 1998 until July 1999. Recently, COGEM issued a positive advice concerning the renewal of the authorization for import, distribution and retail of 'Moonshadow'.[5] In 1997 'Moondust' was authorized for production, import, distribution and retail, and 'Moondust' 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.

Carnation, 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 the Mediterranean coastal regions.[6] *D. caryophyllus* has occasionally been found 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.

Cross-pollination of wild *D. caryophyllus* depends on lepidopteran insects.[7] The domesticated carnation produces little pollen with reduced viability[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* (steenanjer; maiden pink), *D. armeria* (ruige anjer; Deptford pink), *D. superbus*, (prachtanjer; 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 protrandous, 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 haven 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 GM-carnation variety IFD-25958-3 was produced by *Agrobacterium tumefaciens* mediated transformation using the disarmed *A. tumefaciens* strain AGL0 and the transformation vector pCGP3366.

The insertion cassette 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)
- 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*
- *dfr* genomic clone with its terminator and promoter, derived from *Petunia* x *hybrida* and encoding the dihydroflavonol 4-reductase protein
- 35S constitutive promoter, derived from CaMV
- *dfr* hairpin consisting of two copies of a segment of the *dfr* gene from carnation in opposite orientation that are separated by an intron from the petunia *dfr* gene
- 35S terminator, derived from CaMV
- 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 or purple carnations by traditional breeding methods.

Introduction of the *f3'5'h* gene in IFD-25958-3 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 dihydromyricetin (DHM).[11] The dihydroflavonol 4-reductase (DFR) enzyme of carnation can use both DHK and DHM as substrates. The orange/red pigment pelargonidin is produced if DHK is converted and the blue pigment delphinidin is produced if DHM is converted.

Not all DFR enzymes use DHK and DHM equally efficient. The DFR enzyme of petunia cannot efficiently use DHK as a substrate.[11] In the GM-carnation variety IFD-25958-3 the endogenous *dfr* gene is silenced and the *dfr* gene from petunia is introduced. As a result, the DHK substrate is no longer efficiently used as a substrate. As the DFR enzyme from petunia preferentially uses DHM, delphinidin is the predominant pigment in the flowers of IFD-25958-3.

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 mainly confined to the petals.

Properties of the introduced gene conferring herbicide tolerance

The *suRB* gene has been introduced in the GM-carnation variety IFD-25958-3 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. The absence of the tetracycline resistance gene was also confirmed by PCR analysis.

In addition, hybridizations with six different probes (*Nt*ALS, *Vh*F3'5'H, tCaMV35S, *Ph*DFR, LB and RB) show that IFD-25958-3 contains one insert with one copy of the different elements. The insert and 150 bp of its flanking regions were sequenced. The junctions between the insert and its flanking regions were analyzed for the presence of putative open reading frames. Twelve putative open reading frames were translated *in silico* into amino acids and analyzed for homology to known allergens or toxins. No similarity was detected.

Recently, COGEM reconsidered the elements of the molecular characterization which are needed for commercial releases of genetically modified crops.[13] The molecular characterization of IFD-25958-3 fulfils the requirements of COGEM.

Advice

This application concerns the import of cut flowers of the GM-carnation variety IFD-25958-3.

In the application a General Surveillance plan to observe and register adverse effects of the import of GM-carnation variety IFD-25958-3 was provided. COGEM considers the General Surveillance plan provided sufficient for import, distribution and retail of the GM-carnation variety IFD-25958-3.

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 has been cultivated for 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 a vase are pollinated by butterflies. 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. Although, theoretically, it is possible that cut flowers in the vase are visited by butterflies, it is unlikely that they will distribute viable pollen. 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 compared IFD-25958-3 to its parental line and reported that IFD-25958-3 has more, but shorter, filaments. Only 25% of the anthers in IFD-25958-3 was viable. In view of the general characteristics of carnation and the data provided on IFD-25958-3 the possibility of hybridisation of IFD-25928-3 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 GM-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 modified flower color nor the herbicide tolerance 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 GM-carnation variety to species in unmanaged environments is insignificant.

In rare cases small amounts of carnation petals are used in dishes and as garnishing.[14,15,16] This notification concerns import and distribution of cut flowers and not food purposes. Therefore, retailers will not be allowed to sell the petals of the GM-carnation variety IFD-25958-3 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. 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 GM-carnation variety IFD-25958-3 expresses the *dfr, f3'5'h* and *als* (*suRB*) genes. The *dfr* and *f'3'5'h* genes have been introduced to produce delphinidin. Delphinidin is also produced in fruits like blueberries. In addition, the applicant provides evidence that the ALS protein encoded by the *als (suRB)* gene is not allergenic or toxic.

In view of the above, 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.

Summarizing, the application involves import of cut flowers. The GM-carnation variety IFD-25958-3 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 there are no indications that the GM-carnation variety IFD-25958-3 poses a threat to the health of consumers.

In view of the above mentioned, COGEM is of the opinion that the proposed import of cut flowers of the GM-carnation variety IFD-25958-3 poses a negligible risk to human health or the environment.

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