Import, distribution and retail of cut flowers with modified flower colour (GM carnation SHD-27531-4)

COGEM advisory report CGM/130711-01

The application by Suntory Holdings Ltd. of file C/NL/13/01 concerns the authorisation for import, distribution and retail of genetically modified (GM) carnation variety SHD-27531-4.

COGEM has previously advised on this application and concluded that the risk assessment could not be completed due to an incomplete molecular characterisation. The applicant recently provided additional molecular analyses. COGEM considers the molecular characterisation adequate.

Carnation does not have weedy characteristics and although carnation is cultivated for centuries there are no reports of establishment of cut flower carnation varieties in the wild. The GM carnation variety SHD-27531-4 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. Formation of seed on cut flowers is highly improbable.

The application does not concern an authorisation 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 to the environment and human health resulting from import of cut flowers of GM carnation variety SHD-27531-4 are negligible.

Introduction

The present application concerns the genetically modified (GM) carnation variety SHD-27531-4. SHD-27531-4 expresses the *dfr*, *f3'5'h* and *suRB* genes resulting in a modified flower colour (*dfr*, *f3'5'h*) and tolerance to sulfonylurea herbicides (*suRB*).

COGEM has recently advised on this application and concluded that the risk assessment could not be completed due to an incomplete molecular characterisation.[1] The applicant recently provided additional molecular analyses. COGEM has been asked to advise about these analyses.

Previous COGEM advisory reports on other GM carnation varieties

COGEM issued positive advisory reports on several similar carnation varieties with an altered flower colour. [2,3,4,5,6,7,8] 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.[8] In 1997, COGEM issued a positive advisory report 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'.[5,6] Both lines were authorised for import, distribution and retail in the European Union in 2007 and 2009 respectively.[9] For GM carnation lines 'Moonaqua' and 'Moonshadow' the same transformation vector (pCGP1991) was used as for the current application of GM carnation SHD-27531-4.

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.

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 is 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.

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 after decades of cultivation it is not 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 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 industry carnation pollen could 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 SHD-27531-4 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 is 14,131 base pairs (bp) in length and contains the following sequences:

- left border region, derived from the Ti plasmid of *R. radiobacter*
- 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 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 a putative phospholipid transfer protein homologue ('D8') from *Petunia* x *hybrida*
- right border region, derived from the Ti plasmid of *R. radiobacter*

Molecular analysis

Data submitted previously

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 SHD-27531-4. The absence of the tetracycline resistance gene was confirmed by PCR analysis. In addition, Southern blot hybridisation showed that SHD-27531-4 contains one insert with one copy of the different elements at a single integration locus.

The insert and 150 bp of its flanking regions were sequenced. Bioinformatic analysis showed that both flanks are genomic carnation DNA and indicated that the integration of T-DNA into the host genome in transgenic carnation line SHD-27531-4 did not interrupt known coding sequences. The bioinformatic analysis on the presence of putative open reading frames (ORFs) spanning the junctions between the insert and its flanking regions was flawed as only the deduced amino acid sequences starting with a methionine were analysed. Therefore, COGEM concluded that the molecular characterisation was incomplete.

Additional data

The applicant performed additional bioinformatic analyses. Putative ORFs spanning the junctions between the insert and the flanking regions were identified (stop to stop codon) and translated *in silico* into amino acid sequences. Eleven of the deduced amino acid sequences were more than six amino acid residues in length. These were analysed for homology to known toxic or allergenic proteins.

A small part of the deduced amino acid sequence of two putative ORFs showed similarity (five amino acids) and identity (six amino acids) to a toxin. In addition, one of the deduced amino acid sequences displayed exact identity to a short subsequence (six amino acids) of an allergenic protein. The observed similarities correspond to short stretches of amino acid sequences. No similarities to other parts of the identified toxic or allergenic proteins were observed. This indicates that the vast majority of the deduced amino acid sequence is different from the amino acid sequence of the identified toxic or allergenic proteins. Therefore, it is unlikely that the deduced amino acid sequence will correspond to a protein with a function and 3-D structure similar to the identified toxic or allergenic proteins. COGEM is of the opinion that the observed similarities are most likely coincidental and considers it unlikely that the identified similarities are biologically relevant.

Based on the molecular analyses which have been provided by the applicant, COGEM is of the opinion that the molecular characterisation of SHD-27531-4 is adequate and does not give any reason to assume that SHD-27531-4 will pose a risk to the environment or human health.

Properties of the introduced genes resulting in a modified flower colour

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 SHD-27531-4 contain the pigments cyanidin (red)

and pelargonidin (brick-red), whereas the flowers of carnations that are genetically modified with the *dfr* and *f3'5'h* genes also contain the delphinidin (blue) pigment. 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 SHD-27531-4 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.

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. The applicant compared SHD-27531-4 to its non-GM parental line and observed that SHD-27531-4 produced significantly fewer filaments and viable anthers than the parental line, and significantly shorter filaments. In view of the general characteristics of carnation and the data provided on SHD-27531-4, the possibility of hybridisation with wild relatives is considered very unlikely. There has never been any evidence of spontaneous hybridisation between carnation and wild *Dianthus* species, despite the fact that carnation is cultivated worldwide for centuries.

The genetic modification 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 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.

COGEM considers the molecular characterisation adequate. The molecular analyses of the event do not give any reason to assume that a rare case of incidental consumption will pose a risk to human health.

General surveillance

A General Surveillance plan to observe and register adverse effects of the import of SHD-27531-4 was provided. In 2010, COGEM published a report on the principles that should be followed for general surveillance.[29] The General Surveillance plan of SHD-27531-4 provided by the applicant fulfils the requirements laid down by COGEM. COGEM considers the plan sufficient for import, distribution and retail of SHD-27531-4.

Conclusion

This application concerns the import of cut flowers of the GM carnation variety SHD-27531-4. 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 SHD-27531-4 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 SHD-27531-4 poses a negligible risk to human health or the environment.

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