Import, distribution and retail of herbicide tolerant red-purple carnation

COGEM advisory report CGM/130528-02

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

Carnation does not have weedy characteristics and although carnation is cultivated for centuries carnation has never been found 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.

COGEM notes that the molecular characterisation, as presented by the applicant, does not meet COGEM guidelines. Specifically, the bioinformatic analysis of the putative proteins encoded by fusion ORFs spanning the junction between genomic carnation DNA and integrated T-DNA in transgenic carnation line SHD-27531-4 is incomplete. The applicant analysed these sequences from start codon to stop codon, while COGEM deems it necessary that the bioinformatic analysis is performed from stop codon to stop codon. As the dossier is incomplete the risk assessment cannot be completed.

Introduction

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

Previous COGEM advisory reports

COGEM issued positive advisory reports on several other modified carnation varieties with an altered flower colour.[1,2,3,4,5] 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 concerning the renewal of the authorisation for import, distribution and retail of this GM carnation.5 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 The authorisations for both lines have been withdrawn recently upon request of the applicant.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'.[2,3] Both lines were authorised for import, distribution and retail in the European Union in 2007 and 2009 respectively.[6] 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

Wild *Dianthus* species occur worldwide.[7] In Europe, *Dianthus* species are found in mountainous areas in the alpine region, the Balkan and the Mediterranean area.[8,9,10] 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).[11] The species *D. barbatus* (duizendschoon; sweet William) is commonly grown as a garden plant and has established itself in the wild.[11]

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.[8,7,12] However, spontaneous hybridisation between cultivated carnation and wild *Dianthus* species has never been reported, despite decades of cultivation in gardens and parks.

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.[13] Occasionally, *D. caryophyllus* has been found naturalised in the United Kingdom.

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. Moreover, some cultivated carnations are hybrids of *D. caryophyllus* with *D. plumarius*.[14] This application concerns a cultivated double-flowered carnation (*D. caryophyllus*) variety.

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.[14] 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 in the Netherlands.[13]

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 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.[8]
Dianthus carnation pollen cannot be spread by wind. Any pollen produced is heavy and sticky and buried deep in the flower.[15,16] Although the Netherlands has a large carnation industry carnation pollen could not be detected in the atmosphere.[17]

The domesticated carnation produces little pollen with reduced viability.[8,18] 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.[8] 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.[8]

Molecular characterisation
The GM carnation variety SHD-27531-4 was produced by *Rhizobium radiobacter* (previously known as *Agrobacterium tumefaciens*[19]) 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 was 14,131 base pairs (bp) in length and contained 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*

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)[20]. Both products can be used as substrates by the dihydroflavonol 4-reductase (DFR) enzyme from petunia. Conversion of DHQ results in the production of pink/red pigment cyanidin, while the conversion of DHM results in the blue pigment delphinidin. Delphinidin is the predominant pigment in the flowers of carnations that are genetically modified with the *dfr* and *f3'5'h* genes, because the DFR enzyme from petunia preferentially uses DHM. 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).[21] 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.[21] 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 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 presence of putative open reading frames (ORFs) spanning the junctions between the insert and its flanking regions was bioinformatically analysed. If an ORF was identified, the deduced amino acid sequence was further analysed by comparison with known toxic or allergenic protein sequences in databases. Three ORFs (referred to by the applicant as ORF1, 2 and 3) were identified and analysed for similarity with known allergens or toxins. COGEM deduces from the presented data that only amino acid sequences starting with a methionine were analysed.

Within the SwisProt and WHO-IUIS allergen databases one allergenic protein (Pol a 1.0101) had exact similarity with a short sub-sequence (six amino acids) of the deduced amino acid sequence of ORF1 (96 nucleotides in length). Hydrophilicity plots, performed by the applicant, revealed that the deduced amino acid group is most likely located at the internal region of the putative protein and not on external regions. Based on these plots, the applicant concluded that the

identified sequence does not correspond to the hydrophilic and thus antigenic regions of the allergenic proteins.
ORF3 (78 nucleotides in length) showed low sequence similarity (9 residues out of 14) to snake short neurotoxin 6. Short neurotoxin 6 is a non-enzymatic polypeptide which belongs to a polypeptide family characterised by three β-strand loops, a small globular hydrophobic core, four conserved disulphide bridges and a 60-62 amino acid residue.[22] According to the applicant, the sequence encoded by ORF3 is too short to produce a polypeptide similar to any of these sub-fragments and is very unlikely to be a neurotoxin. In summary, the applicant concludes that the deduced amino acid sequence, putatively encoded by the ORFs spanning the junctions between the genomic and insert DNA, does not show homology to known toxins or antigenic proteins.

In the view of COGEM the interpretation of the bioinformatic analysis as stated by the applicant is irrelevant since the sequences studied do not meet COGEM requirements. In 2008, COGEM reconsidered the elements of the molecular characterisation needed for commercial releases of genetically modified crops.[23] One of the assessment criteria is that during the analyses of theoretical fusion ORFs, the sequences have to be analysed from stop codon to stop codon. The applicant analysed sequences from start codon to stop codon. Therefore, the molecular characterisation of SHD-27531-4 does not meet the criteria laid down by COGEM for the molecular characterisation.

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 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.[8] Although carnation is cultivated for decades, it has never been found growing in the wild. 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. Theoretically, it is possible that cut flowers in the 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.

Theoretically, carnation can hybridise with wild relatives. It is possible that butterflies visit cut flowers in vases on window sills or cemeteries. In some European regions bouquets flowers (including carnation) are often brought to cemeteries. Wild carnation is found in south east France and Italy. Because in these regions cemeteries are frequently positioned adjacent to the natural environment, the transfer of viable transgenic pollen by butterflies to wild *D. caryophyllus* is highly unlikely but cannot be completely excluded in these geographical regions. 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. 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 centuries.

The environmental risks linked to hybridisation of this GM carnation variety with wild relatives are comparable with those of conventional carnations. The genetic modification involves genes playing 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* offers selectable advantages in nature. Accordingly, gene flow to wild relatives will not pose an environmental risk.

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.[24,25] 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.

As mentioned in the paragraph on the molecular characterisation of SHD-27531-4 , COGEM notes that molecular characterisation is flawed. Therefore, the risk assessment concerning incidental consumption cannot be completed.

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.[26] The General Surveillance plan of SHD-27531-4 provided by the applicant fulfils the requirements laid down by COGEM and therefore, 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 incomplete. COGEM deems it necessary that the applicant provides the missing data in order to

complete the risk assessment and confirm that the GM variety does not pose a threat to the health of incidental consumers.

Additional remark

The dossier contains typographic ambiguity. The applicant showed that the integration of T-DNA into the host genome in transgenic carnation line SHD-27531-4 has resulted in three novel ORFs. The results are presented in the Technical Dossier and in a raw bioinformatics output file. The ORF that shows partially similarity to a neurotoxin, is referred to as ORF3 in one of the attachments, while it is referred to as ORF2 in the raw bioinformatics output file. These discrepancies in the dossier can lead to unnecessary confusion in the risk assessment. COGEM points out that data presented by the applicant have to be unambiguous. The applicant is requested to correct ambiguities in the dossier.

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