ASSESSMENT REPORT OF THE NETHERLANDS COMPETENT AUTHORITY IN ACCORDANCE WITH DIRECTIVE 2001/18/EC

NOTIFICATION C/NL/06/01

1. THE NOTIFICATION

The notification, submitted by Florigene Ltd, Melbourne, Australia, concerns placing on the market of imported cut flowers derived from genetically modified carnation (*Dianthus caryophyllus*) line 123.8.12 in accordance with Directive 2001/18/EC. The flowers of the carnation line have been modified with the *F3'5'H* and *dfr* genes, resulting in a modified flower color. Line 123.8.12 also contains an herbicide resistance gene (*SuRB),* used to facilitate selection *in vitro*. The commercial name of the product is Florigene Moonaqua™.

2. SCOPE OF THE NOTIFICATION

This notification concerns import, distribution and retailing of line 123.8.12 (Moonaqua™) in the cut flower market in the same way as any other carnation. This notification does not include cultivation, the use as feed or as food of line 123.8.12.

3. HISTORY

Carnation Moonaqua (line 123.8.12) is commercially grown for five years outside Europe. The transgenic variety Moonshadow (C/NL/97/13-1363A), which is constructed using the same vector pCGP1991, has already been approved for commercial production within the EU in 1998 and flowers are already imported into the EU for several years. In addition a similar transgenic variety Moondust has been previously approved for commercial production within the EU in 1997 (C/NL/96/14-11). The authorization for placing on the market, concerning import, distribution and retailing of another similar transgenic variety Moonlite (C/NL/04/02) is currently pending. The information provided in the notification for variety Moonlite, which was assessed positively by the Dutch Committee on Genetic Modification (COGEM) and the European Food Safety Agency (EFSA), is similar to that of the present notification for the variety Moonaqua (line 123.8.12).

4. PROCEDURE

The Netherlands competent authority (CA) received this dossier on October 13, 2006 under Directive 2001/18. The dossier has been assessed with reference to Article 13 of this directive.

Additional information

During the assessment period further information was requested on the following aspects:

Inconsistencies regarding the copy number of the RB

The applicant was asked to clarify the reported inconsistent results regarding the exact copy number of the RB as determined by Southern analysis. The applicant submitted additional information containing sequence data of the individual loci. After re-sequencing of the loci it was demonstrated that all three loci in line 123.8.12 contain a copy of the RB. Thus, the revised sequences agree with the original Southern blots.
Clock stopped October 31 (2006) till December 26 (2006).

Faint bands visible in some Southern blots

In some of the Southern blots additional bands seemed to be present only in the lanes containing genomic DNA of line 123.8.12 and not in the lanes containing genomic DNA of the non transgenic parental line when hybridized with *SuRB* and *F'3'5'H* DNA probes. The applicant subsequently submitted a higher definition scan of the autoradiogram of the blot hybridized with the *SuRB* probe showing that the additional bands are also visible in the lanes containing genomic DNA of the parental line. The applicant suggests that the hybridization is due to

endogenous ALS. Regarding the faint bands visible when hybridized with the *F'3'5'H* probe the applicant suggests that is an experimental artifact. The Netherlands CA accepts the clarification of the applicant.
Clock stopped October 31 (2006) till December 26 (2006).

Scientific advice
Based on the notification of October 13 (2006) and the additional information of December 26 (2006) the Dutch scientific advisory committee (COGEM) gave its advice on February 6 (2007) (CGM/070206-02). The COGEM concluded that the risks for the environment and human health associated with import of cut flowers of line 123.8.12 are negligible.

Public comments
The Summary Notification Information Format (SNIF) was initially published on the Joint Research Center (JRC) website on October 24 (2006). Public comments were received during 30 days, and originated from the Netherlands (1). The one public comment originating from a Dutch person is addressed by the Netherlands CA in this assessment report, and summarized below. No public comments originating from other member states were received during the abovementioned 30 days.

Public comments on the notification C/NL/06/01 and reaction of the Netherlands CA
Public comments which were addressed by the Netherlands CA were submitted by:
- Ms. Bos, Lelystad, The Netherlands;

1. Ms. Bos notes that carnation may form roots or may be propagated by stem cuttings. Therefore it can not be ruled out that the material will be propagated to plants by third parties.
   Answer: The Committee on Genetic Modification (COGEM) has reviewed this aspect in their advice CGM/070206-02. Carnation is not able to spread vegetatively and cut flowers are not able to form roots. Although the abovementioned aspect can not be ruled out, carnation has no weedy characteristics and the traits (blue pigmentation and herbicide tolerance) do not alter the biology of carnation. It is therefore highly unlikely that the genetically modified carnation line 123.8.12 will spread in the environment.

2. Ms. Bos states that the presence of CaMV 35S promoter in carnation line 123.8.12 may cause 'genetic pollution'.
   Answer: The scope of the notification is import only, so no cultivation will take place in the EU. Furthermore, both COGEM (CGM/050207-01 and CGM/070206-02) and EFSA (Question No EFSA-Q-2005-282) conclude in their opinions that carnation can only theoretically hybridize with wild relatives. Due to the intended use of cut flowers only, the likelihood of a successful hybridization with wild relatives is further drastically reduced. It is therefore highly unlikely, if not impossible, that CaMV 35S promoter sequences will be transferred to non-transgenic carnation.

3. Ms. Bos is of the opinion that all flower bunches sold to consumers should have attached a label mentioning that the flowers are transgenic.
   Answer: The Netherlands CA proposes the condition that the product will be labeled or accompanied by a document showing the words 'This product is a genetically modified organism' or 'This product is a genetically modified carnation', and the words 'not for human or animal consumption nor for cultivation'.

Confidentiality
The notification does not contain any information which the applicant regards as Confidential Business Information.

5. LIST OF DOCUMENTS

The dossier consists of:

- Technical information required according to Annex III B of Directive 2001/18/EC;
- Environmental risk assessment according to Annex II of Directive 2001/18/EC;
- Information according to Annex IV of Directive 2001/18/EC;
- Monitoring plan according to Annex VII of Directive 2001/18/EC;
- Summary notification format;
- Eighteen attachments.

6. PARENTAL OR RECIPIENT CROP

Carnation is a crop with a long history of safe use. Cultivation of carnation in the field is mainly conducted in Italy and Spain. In northern European countries as Germany, France and the Netherlands carnation is grown in greenhouses, due to the less favourable climate. Within Europe wild carnation is only found in the Mediterranean area in Italy, Greece, Sicily, Sardinia and Corsica.
Carnation, an annual plant, does not form vegetative reproductive structures such as stolons, rhizomes, root-borne shoots, tubers, etc. Carnation is semi-winter hardy and can not survive in areas where temperatures occur below - 5 °C .The genetic material of carnation can only be disseminated via pollen and seeds.
Carnation is highly domesticated by generations of breeding aimed at improvement of flower size and colour variation. As result of domestication, dissemination through pollination is much less effective in carnation than in wild *Dianthus* species. In general, production of viable pollen by carnation is much lower than that of wild *Dianthus* species.
In the unlikely event that pollination should occur, no seed set will occur in cut flowers as the process of seed development (at least 5 weeks) overruns the time cut flowers will remain in consumers hand before dying (at most three weeks).
Wild relatives which can give viable progeny after hybridisation with carnation are absent in large areas of Europe. The only possible hybridization partners are other cultivated carnations and in the Mediterranean area wild carnation. There has never been any evidence of hybridization between carnation and wild *Dianthus* species.
Carnation is not a weed. Despite hundreds of years of cultivation, and plantings in parks and gardens, it has not become a weed, or escaped from cultivation, anywhere in the world.

Summarised, carnation does not have any characteristic which might pose a risk to the environment or human health.

7. DESCRIPTION OF THE PRODUCT

The genetically modified carnation (*Dianthus caryophyllus* L.) line 123.8.12 exhibits a modified flower color (violet) resulting from expression of the *dfr* and *F3'5'H* genes. Gene expression enables the biosynthesis of delphinidin pigment in the petals. Line 123.8.12 also contains the herbicide tolerance gene *SuRB* (also known as *ALS)* used to facilitate selection *in vitro*. Expression of this gene confers tolerance to sulfonylurea herbicides.

8. MOLECULAR CHARACTERISATION

The Netherlands CA is of the opinion that the provided information regarding the molecular characterization of line 123.8.12 is sufficient to assess possible hazards for human health and the environment.

Modification
Carnation line 123.8.12 was obtained by *Agrobacterium tumefaciens* mediated transformation, by co-cultivating cells with strain AGL0 which contain vector pCGP1991. The same vector is used for the construction of line Florigene Moonshadow™ (C/NL/97/13). This carnation event was admitted to the EU market for cultivation and import in 1998.

Plasmid pCGP1991 contains the following elements in the insert:

Genetic element	Size (kbp)	Origin and function in plant
LB	0.9	T-DNA border from *Agrobacterium tumefaciens*
35S promoter	0.2	Constitutive promoter in plants from *Cauliflower mosaic virus* (CaMV)
Cab 5'utr	0.1	5'untranslated region (UTR) of the Chlorophyll a/b binding protein from *Petunia x hybrida*
SuRB	4.0	Encodes acetolactate synthase resistant to chlorsulfuron. Gene with own terminator from *Nicotiana tabacum*
***Dfr* genomic clone**	5.0	Encodes dihydroflavonol-4-reductase protein with its own promoter and terminator from *Petunia x hybrida*, a key enzyme in the anthocyanin biosynthesis pathway
CHS promoter	1.2	Petal specific promoter from a gene encoding chalcone synthase from *Antirrhinum majus*.
***F3'5'H* cDNA**	1.8	Encodes flavonoid 3'5'-hydroxylase protein from *Viola* sp.; a key enzyme in the anthocyanin biosynthesis pathway
D8 terminator	0.8	Terminator sequence from *Petunia x hybrida*
RB	1.8	T-DNA border from *Agrobacterium tumefaciens*

Plasmid pCGP1991 contains the antibiotic resistance marker tetracycline on the vector backbone.

F3'5'H and *dfr*
The genes *F3'5'H* encoding flavonoid 3'5'hydrolase and *dfr* encoding dihydroflavonol 4-reductase (DFR) are both derived from *Viola* and *Petunia*, respectively. Simultaneous expression of both genes in carnation results in a modified flavonoid synthesis in flowers, and subsequent formation of the blue pigment delphinidin. Carnation lacks part of the anthocyanin biosynthetic pathway involved in the production of delphinidin, *i.e.* carnation lacks the flavonoid 3'5' hydrolase en DFR enzyme activities. Expression of both inserted genes, in combination with endogenous genes, results in a modified flower color (violet in stead of white).

SuRB
The *SuRB* gene from *Nicotiana tabacum* encodes a mutated acetolactate synthase. Expression of the mutation confers tolerance to sulfonylurea herbicides. According to the applicant, this tolerance was only included to allow selection *in vitro*.

Molecular characterization
Inserts
The full sequence of the transformation vector pCGP1991 is part of the notification and the function of all genes (or parts of genes) encoded by pCGP1991 is known.
Genomic DNA isolated from the transgenic line Moonaqua (123.8.12) and non-transformed lines were compared using Southern blot analysis to identify integrated sequences and copy number of the introduced genes. It is established that three loci of the inserted sequences are present in the carnation genome.
All three loci have been cloned and sequenced. Locus 1 (14433 bp) contains a complete copy of the T-DNA insert. Locus 2 (5140 bp) contains a partial D8 terminator, the RB, the CHS promoter, the *F3'5'H* gene, a full copy of the D8 terminator and a second copy of the RB. Locus 3 (1741 bp) contains a partial F3'5'H gene, a full copy of the D8 terminator, a polylinker and the RB.

Southern blot analysis demonstrates that the integration patterns of the introduced genes remain stable and unchanged in the nuclear genome. The genetically modified carnation has been vegetatively propagated since 1999 and approximately 6.75 million flowers have been produced since the start of commercial production in 2001. During the production period only a very limited number of off-types (white streaks) were found.

Flanking sequences
The flanking sequences of both ends of the three loci are sequenced (150 bp). No novel ORFs (larger than 50 amino acids starting with a methionine) were found at the junctions insert/plant, excluding the formation of novel putative chimeric proteins. Bioinformatic analysis of the inserted DNA has shown that carnation line 123.8.12 does not contain DNA sequences with homology to known toxins or allergens.

Absence of tetracycline resistance gene (tetA)
Southern blot analysis was conducted to demonstrate the absence of backbone vector sequences. The results conclusively prove the absence of any backbone vector sequences, including *tetA* sequences encoding a resistance gene to the antibiotic tetracycline.

Gene expression
Northern analysis conducted on RNA isolated from petal leaves showed that all three genes are expressed in Moonaqua whereas no signals could be detected in parental line FE123. The low expression of the *Dfr* gene is consistent with the relatively low levels of delphinidin and thus the pale flower colour observed.
Except for flowers, delphinidin production has not been observed in other tissues of the transgenic plant, such as stems, nodes, leaves and roots. Due to the petal specific promoter (CHS), production of delphinidin is confined to the petals. Moreover, the biochemical pathway leading to anthocyanin biosynthesis is induced to coincide with flower development.
The concentration of delphinidin and other anthocyanins was determined in flower samples of line 123.8.12 and of the non-transformed recipient strain by HPLC. The delphinidin concentration amounts 0.07 mg/g fresh weight petal. The cyanidin concentration amounts 0.02 mg/g fresh weight petal.

9. ENVIRONMENTAL RISK ASSESSMENT

The Netherlands CA is of the opinion that the provided information regarding the environmental safety of line 123.8.12 is sufficient to assess possible hazards for human health and the environment.

The environmental risk assessment of the carnation with a modified flower colour was restricted to issues that are relevant within the scope of the notification: import, distribution and retailing of cut flowers. In this respect, only the probability of gene dispersal and weediness were assessed. Furthermore, the potential risks to consumers due to incidental consumption were assessed.

Selective advantage and potential for increased weediness or persistence
Dfr and F3'5'H genes
There is no reason to assume that carnation plants from spilled or discarded carnation exhibit an increased potential to survive, as a result of the modified colour of flowers by expression of the *dfr* and *F3'5'H* genes. The gene products of the *dfr* and *F3'5'H* genes are involved in the biosynthesis of the pigment delphinidin in petals. Accumulation of these pigments in petals results in a violet to blue flower colour. This accumulation results in a modified flower colour and does not alter the biological characteristics of carnation. Therefore it is highly unlikely that the genetically modified carnation line 123.8.12 exhibits a selective advantage over non-modified carnation, based on the presence of the *dfr* and *F3'5'H* gene.

SuRB gene
Carnation is not considered to be a weed in Europe. Carnation plants resistant to sulfonylurea herbicides can only exhibit a selective advantage after application of such herbicide. However, sulfonylurea herbicides are not designed/registered for use with ornamentals. Sulfonylureas are not effective against grasses, the major weeds of concern in the flower industry. The notifier prohibits use of sulfonylureas on their crops by their contract growers. The herbicide is not generally used for widescale control of weeds outside agriculture.

Effects on non-target organisms
The environment in which the imported flowers will be used, the relatively small number of flowers imported, their dispersal across Europe, and the short longevity of the flowers are all factors that preclude any direct or indirect interaction between the genetically modified carnation

and non-target organism.
Therefore it is highly unlikely that non-target organisms will be affected as a result of import of cut flowers of line 123.8.12.

Effects on the soil ecosystem
Because the products are to be imported as cut flowers, no cultivation takes place. As the genetically modified carnation plants have similar production requirements as other carnations, any impact is no different to that of conventional carnation. Flowers imported to the EU will eventually be discarded in domestic and commercial waste, but the volume of the flowers and the fact that the products will be widely dispersed mean the organic mass is negligible. In addition, the compounds responsible for the colouration of the flowers are natural compounds which are widely present in the environment.
Therefore it is highly unlikely that any adverse effect on the soil ecosystem will occur as a result of imported or discarded genetically modified carnation.

Toxicity and allergenicity
Delphinidin and cyanidin
Carnation has been used safely by humans for ornamental purposes for centuries. The modification in line 123.8.12 (production of delphinidin) is novel for carnation, but there are many flowers and other ornamental species that produce delphinidin. Delphinidin is also present in many common foods. Toxicity studies of delphinidins and anthocyanins indicate very low levels of toxicity. Humans are commonly exposed to and ingest delphinidins in fruits and vegetables at similar or greater concentrations than are found in genetically modified carnation, without adverse effects.

DFR and HF1 proteins
Possible negative effects on human and animal health as a result of incidental consumption of petal leaves of carnation, for example as garnishing for food, were considered. The proteins for modified flower colour expressed in genetically modified carnation (DFR and F3'5'H) are similar to those found in purple-coloured fruits and vegetables that are commonly consumed, and in ornamental flowers. No homology was found between the inserted genes and known toxins or allergens.
Two toxicity tests were performed, namely an Ames mutagenicity test and an acute toxicity test conducted in mice. No indication of toxicity was found.
Reports of allergenicity to carnations are rare and there are no reports of allergenicity to genetically modified carnation. The transgenic carnation line 123.8.12 has been in commercial production for several years and over 6.5 million cut flowers have been grown and distributed to the general public without having any allergenic effect been reported.

SuRB protein
ALS enzymes are widely distributed among bacteria, yeast and higher plants. The *SuRB* gene codes for an alternative form of the acetolacetate synthase enzyme. This enzyme is not a known toxin or allergen and related enzymes are expressed in a variety of edible plants (e.g. soy bean and rice).
No homology was found between the *SuRB* gene and known toxins or allergens. An acute toxicity study with a carnation line 123.8.12 was performed with mice. No indication of toxicity was found.

Based on the nature of the inserted genes, the results of abovementioned toxicity tests and the history of safe use, it is concluded that it is highly unlikely that the genetically modification in carnation line 123.8.12 will cause an adverse effect on the human health with respect to incidental human consumption or allergenicity, as compared to conventionally bred carnation.

Change in agricultural practice
Since the notification covers only import, distribution and retailing of the genetically modified carnation, possible adverse environmental effects by changes in agricultural practice are not considered of importance for the risk analysis.

Conclusion
The Netherlands CA concludes that the provided information is sufficient and is of the opinion that in the context of its intended use, carnation Moonaqua, line 123.8.12, is unlikely to have adverse effects on human and animal health or the environment.

10. DETECTION METHOD

The applicant has provided a detection method that is specific for line 123.8.12, as is obligatory under the 2001/18/EC. The Netherlands CA considers the detection method as being sufficient. The detection method is not yet verified by the Community Reference Laboratory.

11. UNIQUE IDENTIFIER

The unique identifier for the carnation line is FLO-040689-6.

12. TRACEABILITY AND LABELLING

The notifier proposes to label flowers of the transgenic variety Moonaqua (line 123.8.12) similar to those of variety Moondust (C/NL/96/14) which are already imported into and sold in the EU. The notifier states that Florigene will place a label inside every box that is shipped to the EU. The proposed wording of the label is as follows: “These flowers are genetically modified to alter the flower colour and are only produced for use as an ornamental product”.
The Netherlands CA proposes to change the wording as is laid down in the conditions of the draft decision on the genetically modified carnation Moonlight (line 123.2.38) (C/NL/04/02). The proposal for this condition is formulated below under item 13.

13. MONITORING AND GENERAL SURVEILLANCE

Specific monitoring
Since the environmental risk analysis does not identify any potential risks, the notifier has not included a specific monitoring plan. The Netherlands CA accepts this reasoning.

General surveillance
The intended use of the placing on the market of this product is import, distribution and retailing. Therefore the general surveillance plan addresses escapes of the genetically modified carnation (or its traits) to the environment, and unforeseen effects on human health by handling the product. Amongst others, the following monitoring activities will be undertaken:

1. Florigene will maintain exact records of all imports into Europe;
2. Importers will be asked to monitor their markets for any suppliers selling flowers resembling the Florigene product and which may be sold outside of the regular distribution and retail channels;
3. On a 6 monthly basis the European importers will be asked in questionnaire format for feedback;
4. The Florigene website will provide a link at which European consumers will be invited to comment on Florigene products with all Florigene contact details;
5. After release, taxonomists and botanists with interest in *Dianthus* biology will be asked to alert Florigene in case of any unusual hybrids that they might find during survey work.

The Netherlands considers this general surveillance plan as sufficient.

14. ADVICE OF THE NETHERLANDS COMPETENT AUTHORITY FOR DIRECTIVE 2001/18/EC

Based on the notification, including all requested additional information, and the above mentioned considerations, the Netherlands competent authority concludes that no reasons have emerged on the basis of which consent to the proposed placing on the market should be withheld.

The Netherlands competent authority therefore proposes to consent to the placing on the market of the product as described below, for which a notification has been submitted on October 13, 2006, registered under number C/NL/06/01 under explicit specification of:

a) The consent will be granted to Florigene Ltd, Melbourne, Australia and concerns the placing on the market under part C of 2001/18/EC of the product consisting of cut flowers of carnation (*Dianthus caryophyllus* L.) genetically modified with the *dfr, F3'5'H* and *SuRB* genes for the purpose of import, distribution and retailing. The consent includes line 123.8.12, product name Florigene Moonaqua
b) The product may be put to ornamental use only. This consent excludes cultivation and excludes the use as feed or as food of line 123.8.12.
c) The unique identification code of the product will be FLO-040689-6.
d) The period of validity of the consent shall be 10 years starting from the date on which the consent is issued.
e) The words 'This product is a genetically modified organism' or 'This product is a genetically modified carnation', and the words 'not for human or animal consumption nor for cultivation' shall appear either on a label or in a document accompanying the product.
f) The consent holder shall, whenever requested to do so, make positive and negative control samples of the product, or its genetic material, or reference materials available to the competent authorities and to inspection services of Member States as well as the Community control laboratories.
g) Throughout the period of validity of the consent, the consent holder shall ensure that the monitoring plan, contained in the notification and consisting of a general surveillance plan to check for any adverse effects on human and animal health or the environment arising from handling or use of the product, is put in place and implemented.
h) The consent holder shall directly inform the operators and users concerning the safety and general characteristics of the product and of the conditions as to monitoring, including the appropriate management to be taken in case of accidental cultivation.
i) The consent holder shall submit to the Commission and to the competent authorities of the Member States annual reports on the results of the monitoring activities.
j) The decision shall apply from the date on which the detection method specific to carnation line 123.8.12 is verified by the Community Reference Laboratory.

The Hague, 27-02-2007

The State Secretary of Housing, Spatial Planning and the Environment,

drs. P.L.B.A. Van Geel