Application for renewal of consent for marketing approval under directive 2001/18/EC

Event; FLORIGENE®Moonlite™

Decision authorisation number; C/NL/04/02
Number of consent; C/NL/04/02.abb1
Date of consent; July 11 2007

Unique identifier; original FLO-4Ø644-4, corrected to FLO-4Ø 644-6

Submitted to Bureau GGO by email on May 23 2016. Hard copy sent by courier on May 24 2016.

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Supplementary files (provided as separate files on CD)

1. Monitoring reports CNL0402 2008-2015.pdf
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6. Trial data.xlsx.
7. Copies of recent literature reviews

FOLDER with copies of literature cited.

1. Introduction

Consent to market transgenic carnation event FLO-4Ø644-6 (Florigene®Moonlite™) in the EU was granted by the NL competent authority on July 11 2007, following issue of a commission decision on May 23 2007. The marketing consent allows for import of cut flowers only and not cultivation in the EU. The purpose of this document is to formally request a renewal of the marketing application consent for this event. The renewal seeks a consent to market imported cut flowers only and not to extend the scope of the marketing approval to cultivation in the EU.

In accordance with article 17 of Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, this document is submitted to the competent authority which received the original notification no later than 9 months before the expiry of the consent.

Aside from the specific requirements laid out in paragraph 2 of article 17 of directive 2001/18/EC, we are unaware of any guidelines for the renewal of events approved under directive 2001/18/EC. We are aware of the EFSA guidelines for renewal of applications of genetically modified food and feed authorised under Regulation (EC) No 1829/2003 (EFSA, 2015a) and based on those recommendations have decided to make an updated bioinformatic analysis as part of this renewal request for FLO-4Ø644-6 (Florigene®Moonlite™).

1.1 Rationale for request for renewal

The rationale for the request for renewal is that we expect demand for Florigene®Moonlite™ to be sustained or increased. Renewal is desired in order to maintain supply to EU based customers that require the product on a long term basis.

As outlined elsewhere in this document, no information or observations have been collected on FLO-4Ø644-6 since it was granted marketing approval in the EU that suggests an increased risk of the product to human health or the environment.

FLO-4Ø644-6 (Florigene®Moonlite™) is now an established carnation variety in the EU market, with a trend to increasing sales year by year. Volumes imported into the EU, by month, are shown in figure 1. From marketing approval until the end of March 2016 13.00 million flowers of the variety have been imported into the EU, through a single Netherlands based importer. In the last full calendar year (2015) 2.12 million flowers were imported.

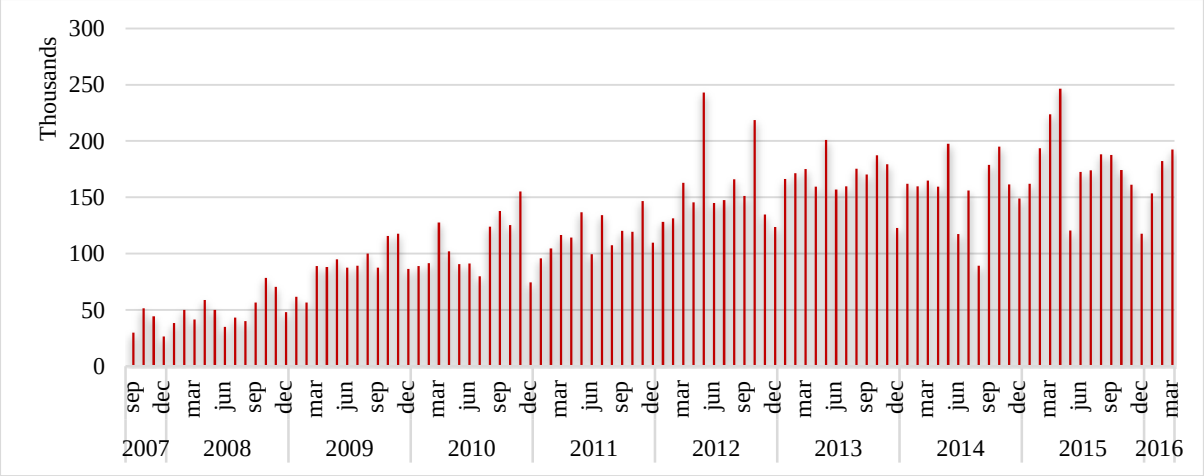

Figure 1. Monthly volume of flowers of transgenic carnation event Florigene®Moonlite™ imported into the EU from marketing approval to the end of March 2016.

1.2 Related approvals

Event FLO-40644-4

Table 1 lists the approvals for event FLO-4Ø644-6 that are registered with the Biosafety Clearing House. The event is also approved in Ecuador, USA and Canada.

Table 1. List of records in the Biosafety Clearing House database for event FLO-40644-6.

Country	*Record ID
Australia	7572
Colombia	7108
European Union	103151
Japan	8299
Malaysia	104636
Netherlands	108638

**https://bch.cbd.int/database*

No approvals for cultivation or import of FLO-4Ø644-6 have been cancelled or rescinded since the EU marketing consent was issued in 2007.

Other transgenic carnation events in the EU

Five other transgenic carnation events have been approved in the EU, or are currently under review, since marketing approval was given for FLO-40644-6. These are listed in table 2. The 5 events are closely related to FLO-40644-6 because the modified phenotype is the same; expression of resistance to sulphonylurea type herbicides for selection *in vitro* and accumulation of delphinidin-based anthocyanins in flower petals (table 2) to confer novel flower colour. Scientific opinions provided by EFSA on dossiers C/NL/09/01, C/NL/09/02, C/NL/13/01 and C/NL/13/02 all conclude that there is no scientific reason to consider that the import, distribution and retailing in the EU of the transgenic carnation cut flowers for ornamental use will cause any adverse effects on human health or the environment (EFSA, 2014a, 2014b, 2015b, 2016).

Table 2. Other transgenic carnation events which have been approved in the EU, or are currently under review, since marketing approval was given for FLO-40644-6.

Event			Marketing consent date	Anthocyanidin (mg/g fresh weight petal)	
Tradename	**Unique identifier**	**Dossier number**		**Delphinidin**	**Cyanidin**
FLORIGENE ®Moonlite™				**0.09**	**0.03**
FLORIGENE® Moonaqua™	FLO-4Ø689-6	C/NL/06/01	July 24 2009	0.07	0.02
FLORIGENE® Moonberry™	IFD-25958-3	C/NL/09/01	July 20 2015	0.54	0.10
FLORIGENE® Moonvelvet™	IFD-26407-2	C/NL/09/02	July 20 2015	2.87	0.37
FLORIGENE® Moontea™	SHD-27531-4	C/NL/13/01	Application in process	1.18	0.51
FLORIGENE® Moonvista™	FLO-4Ø685-2	C/NL/13/02	Application in process	1.8	0.02

For commercial reasons, two transgenic carnation events have been withdrawn from the EU market since the marketing consent for FLO-40644-6 was issued. These two events are;

- FLORIGENE®Moonshadow™ (FLO-11363-1)
- FLORIGENE®Moondust™ (FLO-07442-4)

2. Information required under paragraph 2, article 17 of directive 2001/18/EC

2.1 A copy of the consent to the placing on the market of the GMO

A copy of the consent to the placing on the market is provided in appendix 1.

2.2 A report on the results of the monitoring

Monitoring reports have been submitted on an annual basis from July 2008. Each report has covered the period July to June (the next report will be lodged in July 2016). From July 2011 the monitoring report combined two events; FLO-40644-6 and FLO-40689-6 **(**FLORIGENE®Moonaqua™).
The supplementary file **1. Monitoring reports CNL0402 2008-2015.pdf** provides a single file with all monitoring reports bookmarked by year for ease of navigation.

General monitoring has been used for FLO-40644-6. In summary, the monitoring actions that have been carried out, and the observations that have been made are;

Questionnaire feedback from importer
These questionnaires have been provided by the importer each year. The importer has reported every year that they were not aware of any illegal growing and that neither their staff nor consumers have reported any adverse effects of handling the flowers.

Expert monitoring group
Since 2008 an expert group, comprising breeders and research experts has been established. Each year members of the group have been asked to report on whether they have become

aware of any illegal propagation of transgenic carnation in Europe, or of the incidence of any wild carnation populations. Responses from at least some of the group have been obtained each year including information on survey work carried out by botanical experts. There was no evidence of the establishment of transgenic carnation in the wild, or of introgression to wild *Dianthus* species in any survey, in any year. The survey work was largely confined to the Netherlands, Greece and the Swiss Alps. No reports of illegal propagation were made.

Mailing list

With the exception of the year 2009 herbaria, European botanical and plant conservation groups, national plant protection authorities, Italian phytosanitary agencies, national botanic survey networks, plant protection services, botanical gardens and individual scientists have been contacted by mail and email to request information on any reports of the identification of wild populations of carnation. In 2011 brochures with descriptions of the transgenic varieties were included with the mail outs. From 2012, an emphasis was placed on Spain, Italy and France and communications have been made in the languages of these countries as well as English and Bulgarian. From 2008 to 2015 1,184 contacts were made. Some responses identified recent wild populations of *Dianthus caryophyllus*. In all cases where it was possible to confirm the nature of the samples, collections were of the 5- petal unimproved *Dianthus caryophyllus*, **and not carnation.** Seed of wild *Dianthus caryophyllus* was also obtained, through a contact in France.

Literature review

From 2010 a literature search was undertaken on an annual basis to identify any new, or previously unidentified, scientific reports on any aspects of *Dianthus* biology or distribution in Europe. This was primarily undertaken to identify any reports of carnation in the wild. Since 2010 226 reports were identified and summarised in monitoring reports and though the information added to baseline knowledge of *Dianthus* biology, medicinal value and traditional uses **none of these reports identified carnation in the wild** nor evidence of introgression to wild *Dianthus* species. In 2015, the Office of the Gene Technology Regulator (Australia) updated their reference document "The Biology of *Dianthus caryophyllus* L. (Carnation)"[1].

Publications of direct relevance to FLO-40644-6 were Sun et al. (2010), Kim et al. (2010) and Li et al. (2012). In these studies event-specific qualitative and quantitative PCR assays were developed for the identification and quantification of Florigene®Moonlite™ (all work was carried out independently of Suntory or Florigene). Zhu et al. (2011) confirmed the suitability of ANS as an endogenous control in PCR-based method for identification of transgenic carnation varieties. Chandler et al. (2013) reviewed the biosafety of transgenic carnation and included experimental data for Florigene®Moonlite™. Tanaka and Brugliera (2013) provided a detailed description of transgenic carnation varieties, including Florigene®Moonlite™. Anderson and Walker (2013) studied the acceptability of transgenic carnation (including Florigene®Moonlite™) in the floristry industry in the US.

Database review

From 2011, annual database and website review was added to the general monitoring process. 149 sites have been examined (most on an annual basis), all of which are actively maintained European based floras, vegetation checklists and on-line herbaria. Sites are in multiple languages. None of these reports identified carnation populations in the wild, though useful information was gained on the location and form of wild *Dianthus caryophyllus* populations,

[1] *http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/biology-documents*

largely in France. In all cases where it was possible to confirm the nature of the records, these collections were of the 5- petal unimproved *Dianthus caryophyllus*, and not carnation.

Website
The Florigene website has been in place continuously since 2007 (http://www.florigene.com). No information on possible wild populations of Florigene®Moonlite™ has been sent to the website during the period from the public, distributors or retailers.

2.3 Any other new information which has become available with regard to the risks of the product to human health and/or the environment

No information has become available since the consent was issued that shows or implies a change in the risks of FLO-4Ø644-6 (Florigene®Moonlite™) to human health and/or the environment. New information that has become available since the consent is;

- Annual monitoring (section 2.2) and surveys at the production sites (section 3.2) indicates there has been no escape into the environment.
- Monitoring of the scientific literature published since consent has not identified any relevant reports (sections 2.2 and 3.5).
- Up to date bioinformatics analysis has not identified any significant homologies to toxins or allergens. Details are provided in appendix 2.
- The event is phenotypically stable and trials carried out to compare to the parental variety show no significant morphological changes since the event was approved. Details are provided in section 3.3 and 3.4.
- Literature reviews of potential allergenicity and safety of delphinidin have been carried out since the consent. Details are provided in section 3.5.

2.4 As appropriate, a proposal for amending or complementing the conditions of the original consent, *inter alia* the conditions concerning future monitoring

Changes to the conditions of the original consent.
Two amendments are requested;

1. Unique identifier. We request the unique identifier is amended to FLO-40644-6. The original unique identifier stated in the marketing consent (FLO-40644-4) was incorrectly calculated. This error has been corrected in the records of the biosafety clearing house, as shown in table 1.
2. The original consent application was lodged by Florigene Limited, Melbourne, Australia and this company was identified in the marketing application. Florigene Limited is no longer a registered company, having been purchased by Suntory Limited, Osaka, Japan. We request that the company named in the renewal of the marketing application be nominated as;
   Suntory Flowers Limited
   4-17-5 Shiba, Minato-ku,
   Tokyo 108-0014 Japan

Future monitoring
We propose that a general monitoring scheme is continued with no changes.
The detection method for the event was approved by the JRC;
http://gmo-crl.jrc.ec.europa.eu/docs-valid-2001-18/CRL_Report_Flor_Moonlite_v2.pdf

3. Additional information

Additional information related to FLO-4Ø644-6 (Florigene®Moonlite™) is provided in this section to support the assertion that no information has become available since the consent was issued that shows or implies a change in the risks of FLO-4Ø644-6 (Florigene®Moonlite™) to human health and/or the environment.

3.1 Boinformatic analysis

An up to date bioinformatic analysis of the sequence information provided with the original application for marketing approval of FLO-40644-6 is provided at appendix 2.
In summary;

- Blastn and tblastx analysis of a carnation genome database indicates that flanking sequences of the two insertion loci of FLO-40644-6 are located within two different scaffold sequences from the carnation genome. Lack of any biologically significant similarities when databases at http://blast.ncbi.nlm.nih.gov/Blast.cgi were searched using blastn suggest the insertion sites are within non-coding regions of the scaffolds.
- Database searches (blastp searches at http://blast.ncbi.nlm.nih.gov/Blast.cgi and allergen search at FARRP allergen protein database) have confirmed that the two ORF sequences identified at the flanking region of locus 1 of FLO-40644-6 do not have homology to toxins or allergens and substantially confirm the derivation of the ORF sequences from the region of locus 1 spanning the partial TetA sequence integration.
- Database searches (blastp searches at http://blast.ncbi.nlm.nih.gov/Blast.cgi and allergen search at FARRP allergen protein database) have confirmed that the inserted genes do not have homology to toxins or allergens.

3.2 Botanical surveys at production sites

From July 2007 until the end of March 2016 approximately 30.2 million flowers of FLO-40644-6 have been produced in Ecuador and 27.5 million flowers in Colombia. Aside from a small amount of production in Australia, these are the two sites of production and the composting areas (figure 2) have been inspected regularly (table 3).

Table 3. Dates of visits to composting areas of transgenic carnation production sites. The yellow cells indicate visits to Colombia only and the blue cells to Colombia and Ecuador. 21 inspections have been carried out in Colombia and 6 in Ecuador.

	J	F	M	A	M	J	J	A	S	O	N	D
2008												
2009												
2010												
2011												
2012												
2013												
2014												
2015												
2016												

Figure 2. Composting areas in Ecuador (upper photograph) and Colombia (lower photograph). Photographs taken in April 2016.

At each site visit the composting areas have been inspected for the possible establishment of wild populations of transgenic carnation, including FLO-40644-6. Composting areas were selected as these were considered the most likely places in which a wild population might establish.

On no inspection has a wild population of carnation been identified. In Colombia the plants growing near carnation and rose compost heaps have been identified (Chandler et al., 2008). 100 species from 35 families were identified across 8 sample sites. The dominant family at farm locations was the Asteraceae, followed by the Poaceae. The species found at the most sites in the farm environments were *Pennisetum clandestinum* (kikuyu grass), *Taraxacum officinale* (dandelion) and *Poa annua* (annual bluegrass). No carnation (*Dianthus caryophyllus*) plants were found in any transect. The only Caryophyllaceae species found were *Arenaria lanuginosa* (Michx.) Rohrb., *Silene gallica* L., *Spergula arvensis* L. and *Stellaria media* (L.) Cirillo. Of these 4 species, only *Stellaria media* was identified at the composting area itself.

3.3 Phenotypic stability

Event FLO-40644-6 is phenotypically stable. The flower colour has been consistent since the event was approved for marketing in the EU and there has been a consistently low (figure 3) frequency of "off-types" (production of pink flowers instead of the normal violet) at the production sites.

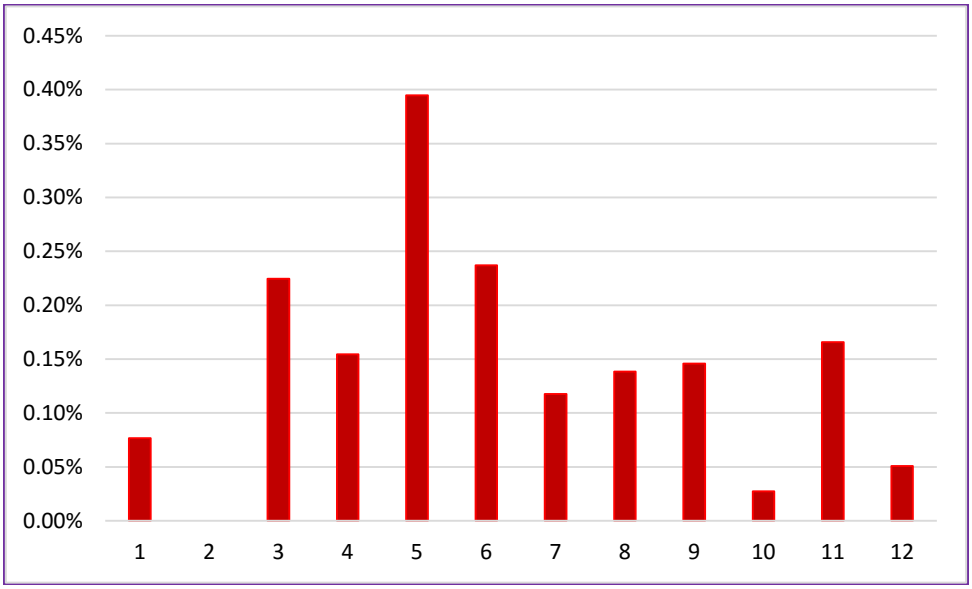

Figure 3. Frequency of production of "off-types" in FLO-40644-6 grown in Colombia. Data is measured from a sample of at least 1,300 flowers on each of 12 samples made since January 2011. Sample 2 was 0%.

Viable anther number and style length, which are important morphological characters related to any change in propensity for gene flow, are also stable (table 4).

Table 4. Measurement of style length, petal number and anther number in FLO-40644-6 grown in Colombia. At each time point 10 replicate flowers were analysed.

	Style length	**Petal number***	**Viable anther number**
Jan 2012	1.5 ± 0.2	41 ± 5	0.1 ± 0.2
July 2012	1.7 ± 0.2	42 ± 4	0
Dec 2012	1.6 ± 0.2	44 ± 3	0.5 ± 0.7
Apr 2013	1.6 ± 0.1	44 ± 5	0
Aug 2013	1.5 ± 0.2	42 ± 6	0.1 ± 0.3
Dec 2013	1.6 ± 0.4	42 ± 4	0
June 2014	1.8 ± 0.3	43 ± 3	0.2 ± 0.4
Feb 2015	1.4 ± 0.2	41 ± 5	0.1 ± 0.3
Oct 2015	1.6 ± 0.2	41 ± 5	0
Jan 2016	1.9 ± 0.3	39 ± 4	0
Apr 2016	2.0 ± 0.1	42 ± 4	0.1 ± 0.3

**Number of petals with pigment visible*

As was noted in the initial application for marketing approval for FLO-40644-6, this variety produced a low number of intact anthers when grown in the Netherlands or Australia. The

event also produces a very low number of viable anthers when grown in Colombia (tables 4 and 5).

3.4 Comparison to parental variety

A comparison to the parental variety (the variety used for transformation) was included in the marketing application in 2004 and in support of the present application for renewal of marketing approval for event 40644-6, a comparative trial of FLO-40644-6 and Cream Cinderella (the parent variety) was planted in Colombia. This trial was designed over three replicate blocks (each character measured with 9, 10 or 12 replicates per block) to allow a two way ANOVA separating the effect of planting location from variety. The trial was planted in May 2015 and harvested from late November to mid-December 2015. Results are summarised in table 5 and raw data is provided in the supplementary file **6. Trial data.xlsx**.

Table 5. Summary of means, P-values and statistical significance (P<0.05) for 18 characters measured in a comparative trial of FLO-40644-6 and its parental variety.

Character	Means		P-values		Statistical significance	
	Parent	FLO-40644-6	Variety	Block	Variety	Block
Thickness of 5th node (mm)	9.0	8.5	0.007458712	0.00283716	Yes	Yes
Length of 5th node (mm)	105.9	106.6	0.700540218	0.869864567	No	No
Leaf length (mm)	64.1	55.5	1.4116E-05	0.113383635	Yes	No
Calyx diameter (mm)	22.1	22.0	0.792783761	0.15627277	No	No
Lobes per calyx	6	6	N/A	N/A	No	No
Style number	4.1	3.8	0.005681083	0.304428843	Yes	No
Style length (mm)	19	20	0.338924272	0.572839395	No	No
Calyx height (mm)	33.9	31.6	0.000192058	0.127245521	Yes	No
Plant height (cm)	115	128	0.000171015	0.070364121	Yes	No
Corolla height (mm)	29.4	30.6	0.210583469	0.609802306	No	No
Stamen number	5.0	3.8	0.007416366	1.259244689	Yes	No
Stamen length (mm)	15.8	16.2	0.704474811	0.456142496	No	No
Flower diameter (mm)	80.9	78.7	0.108997399	0.051021034	No	No
Petal number*	54	51	0.063173282	0.651674304	No	No
Petal width (mm)	40	40	0.512011963	0.075123517	No	No
Stem length (cm)	95	103	6.88041E-06	0.276960979	Yes	No
Number of viable anthers	0.5	0.3	0.145968108	0.211563972	No	No
Total number anthers	3.1	2.1	0.022237946	0.191207578	Yes	No

**including small, unpigmented petals*

There was a statistically significant effect of block for only one character (thickness of fifth node). Statistically significant differences noted between the parent and FLO-40644-6 are highlighted in orange in the second last column on the left in table 5. In no case where there was a statistically significant difference could that difference suggest an increased risk of the FLO-40644-6 to human health or the environment. This is because the characters were either biologically irrelevant to gene flow (node thickness, leaf length, calyx height, plant height, stem length) or the character may be related to gene flow probability but was lower in FLO-40644-6 (style number, stamen number, total number of anthers).

The trial data shown in the original application for event FLO-40644-6 was carried out in 2000 under greenhouse conditions in the Netherlands. In that trial a significant difference between FLO-40644-6 and the control was only measured for corolla height (greater in FLO-

40644-6) and number of intact (viable) anthers (lower in FLO-40644-6). Though a comparison between the 2000 data and that obtained in 2015 are not possible because the climatic and growing conditions during the trials are different, corolla height was also greater in the control than in FLO-40644-6 in the Colombia trial and number of intact anthers lower (table 5).

3.5 Literature review

The initial application for FLO-40644-6 was made in 2004, with additional information provided to EFSA in 2006 and at both those times literature reviews were provided. Since that time three more comprehensive and up to date literature reviews have been carried out for other lines of transgenic carnation. These lines have a comparable phenotype to FLO-40644-6 (table 2) in that the modified phenotype is the same; expression of resistance to sulphonylurea type herbicides for selection *in vitro* and accumulation of delphinidin-based anthocyanins in flower petals to confer novel flower colour. For cross reference, copies of the three literature reviews, which were carried out in 2013 and 2014, are provided in the supplementary file **7. Copies of recent literature reviews.doc**. The literature reviews cover;

- An updated assessment of the probability of gene flow.
- An updated review of the biosafety of the ALS gene.
- A literature search on information relevant for the safety of GM carnation to humans, including the safety of delphinidin and potential allergenicity.

The literature reviews (supplementary file 7) were an important part of the risk assessment process which has concluded that the release of transgenic carnation modified for express of delphinidin-related anthocyanins in flowers does not pose a risk to human health or the environment.

Elsewhere in this application for renewal we have referred to research reports of direct relevance to FLO-40644-6 (section 2.2), publication of a carnation genome (Yagi et al., 2013) and a review in 2015 of the biology of carnation made by the Australia regulatory agency, OGTR[2]. Literature review has also been carried out and reported on an annual basis as part of the monitoring process (refer to 2.2). The literature reviews (supplementary file 7) were an important part of the risk assessment process which has concluded that the release of transgenic carnation modified for express of delphinidin-related anthocyanins in flowers does not pose a risk to human health or the environment.

On May 3 and May 4 2016 a citation and academic literature database search was carried out to determine whether any new scientific reports have been published relating to the issues covered in the literature reviews provided in supplementary file **7. Copies of recent literature reviews.doc**. No new reports (appendix 3) were identified to suggest that FLO-40644-6 poses any greater a risk to human health or the environment than was evident at the time of the marketing approval. The reports which were found have added to relevant baseline knowledge and so are listed and briefly summarised in appendix 3.

4. Literature cited

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Nakamura N, Suzuki T, Tanaka Y, Sato S, Shirasawa K, Isobe S, Miyamura Y, Watanabe A, Nakayama S, Kishida Y, Kohara M, Tabata S, 2013. Sequence analysis of the genome of carnation (*Dianthus caryophyllus* L.) DNA Res. doi:10.1093/dnares/dst053, 2013.

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Appendix 1. Copy of marketing approval

VROM

Directoraat-Generaal Milieu
Directie Stoffen, Afvalstoffen, Straling
Afdeling Straling, Nucleaire en Bioveiligheid

RIVM/SEC/Bureau GGO
Anthonie van Leeuwenhoeklaan 9
Postbus 1
3720 BA Bilthoven

Telefoon 030 274 27 93
Fax 030 274 44 01
bggo@rivm.nl
www.vrom.nl/ggo-vergunningverlening

Florigene
dr. S.F. Chandler
1 Park Drive
VIC 3083 Bundoora
Australië

Consent to placing on the market
Notification: C/NL/04/02

Datum	Kenmerk	Bijlagen
18-07-2007	C/NL/04/02.abb1	- C/NL/04/02.b - C/NL/04/02.pub2

Uw brief	Uw kenmerk	Afschrift aan
30-07-2004		

Dear mr. Chandler,

In accordance with Article 18 of Council Directive 2001/18/EC of 17 April 2001, I herewith send you a consent to market genetically modified organisms.

Yours Sincerely,

dr. ir. M.M.C. Gielkens
Bureau GGO

VROM

Directoraat-Generaal Milieubeheer
Directie Stoffen, Afvalstoffen, Straling

Rijnstraat 8
Postbus 30945
2500 GX Den Haag
Interne postcode 645

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beschikking
DGM/SAS C/NL/04/02

De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

in overeenstemming met de Minister van Landbouw, Natuur en Voedselkwaliteit,

gelezen de aanvraag van Florigene Ltd., te Melbourne, Australië, van 30 augustus 2004 en de aanvullende informatie ontvangen op 7 december 2004 en 27 december 2004, om een vergunning als bedoeld in artikel 23 van het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen (Stb. 1993, 435) (hierna: Besluit ggo), en

gelet op de beschikking van de Commissie van de Europese Gemeenschappen (hierna: de Commissie) van 23 mei 2007, betreffende het in de handel brengen van een anjer (*Dianthus caryophyllus* L., lijn 123.2.38), genetisch gemodificeerd met het oog op bloemkleur, overeenkomstig Richtlijn 2001/18/EG (hierna: de richtlijn) van het Europees Parlement en de Raad (2007/364/EG)[1],

BESLUIT:

Artikel 1
Vergunning

1. De Minister van VROM, in overeenstemming met de Minister van LNV, verleent een vergunning, als bedoeld in artikel 23 van het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen (Stb. 1993, 435), aan Florigene Ltd., om het in artikel 2 gespecificeerde product, waarvan aanvraag is gedaan door Florigene Ltd. (referentienr. C/NL/04/02), overeenkomstig deze beschikking in de handel te brengen.

2. Overeenkomstig artikel 19, lid 3, van de richtlijn worden aan de vergunning de voorschriften verbonden zoals beschreven in de artikelen 3 en 4.

Artikel 2
Product

1. De genetisch gemodificeerde organismen die als product in de handel worden gebracht (hierna: het product) zijn snijbloemen van een anjer (*Dianthus caryophyllus* L.) met een gewijzigde bloemkleur, afgeleid van een celcultuurlijn van *Dianthus caryophyllus* L. en gemodificeerd met *Agrobacterium*

[1] PB L 138 van 30.5.2007, blz. 50.

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tumefaciens, stam AGL0, met behulp van de vector pCGP1470, waaruit lijn 123.2.38 is ontstaan. Het product bevat de volgende DNA -sequenties in drie cassettes:

(a) Cassette 1

De promoter van een leeuwenbekgen dat codeert voor chalconsynthase, cDNA voor flavonoïd-3'5'-hydroxylase (F3'5'H) uit petunia en de terminator van het petuniagen dat codeert voor een fosfolipide-transporteiwit-homoloog.

(b) Cassette 2:

De constitutieve promoter Mac, cDNA voor dihydroflavonol-4-reductase (DFR) uit petunia en de terminator van het gen uit *Agrobacterium tumefaciens* dat codeert voor mannopinesynthase (Mas).

Gelijktijdige expressie van beide genen in anjers leidt tot een gewijzigde flavonoïd-synthese in de bloemen met als gevolg de vorming van het blauwe pigment delfinidine.

(c) Cassette 3:

De 35S-promoter van het bloemkoolmozaïekvirus, een niet-vertaalde regio van het cDNA dat correspondeert met het petuniagen dat codeert voor bindingseiwit 5 van chlorofyl a/b en het gen *SuRB* (*als*) dat codeert voor een mutant acetolactaatsynthase-eiwit (ALS), dat tolerantie voor sulfonylureum geeft, afgeleid van *Nicotiana tabacum* (tabak), met inbegrip van de terminator daarvan.

Dit gen is gebruikt voor in vitro selectie.

2. De vergunning geldt voor nakomelingen die zijn verkregen door ongeslachtelijke voortplanting van de genetisch gemodificeerde anjer (*Dianthus caryophyllus* L., lijn 123.2.38).

Artikel 3
Voorschriften voor het in de handel brengen

Het product mag alleen voor sierbloemen worden gebruikt, niet voor de teelt, en mag met inachtneming van de volgende voorwaarden in de handel worden gebracht:

(a) de vergunning heeft een geldigheidsduur van tien jaar, ingaande op de datum waarop de vergunning wordt verleend;

(b) de eenduidige identificatiecode van het product is FLO-4Ø644-4;

(c) onverminderd artikel 25 van de richtlijn wordt de methodologie voor de detectie en identificatie van het product, met inbegrip van experimentele gegevens waarmee de specificiteit van de methodologie wordt aangetoond, zoals gecontroleerd door het communautair referentielaboratorium, ter beschikking van de bevoegde instanties en de inspectiediensten van de lidstaten en de communautaire controlelaboratoria gesteld;

(d) onverminderd artikel 25 van de richtlijn stelt de houder van de vergunning op verzoek positieve en negatieve controlemonsters van het product of het genetisch materiaal daarvan of referentiematerialen ter beschikking van de bevoegde instanties en de inspectiediensten van de lidstaten en de communautaire controlelaboratoria;

(e) op een etiket of in een bij het product gevoegd document worden de woorden „Dit product is een genetisch gemodificeerd organisme" of „Dit product is een genetisch gemodificeerde anjer" en de woorden „niet voor consumptie door mens of dier of voor de teelt" vermeld.

→

Artikel 4
Monitoring

1. Gedurende de gehele geldigheidsduur van de vergunning ziet de houder van de vergunning erop toe dat het in de aanvraag opgenomen monitoringplan, dat bestaat uit een algemeen plan van toezicht om na te gaan of de behandeling of het gebruik van de producten eventueel nadelige effecten heeft op de gezondheid van mens of dier of op het milieu, wordt ingevoerd en ten uitvoer wordt gebracht.

2. De houder van de vergunning stelt de exploitanten en gebruikers rechtstreeks in kennis van de veiligheid en de algemene kenmerken van het product en de voorwaarden ten aanzien van de monitoring, inclusief de beheersmaatregelen die in het geval van accidentele teelt moeten worden genomen.

3. De houder van de vergunning dient bij de Commissie en de bevoegde instanties van de lidstaten jaarlijks een verslag in over de resultaten van de monitoringactiviteiten.

4. Onverminderd artikel 20 van de richtlijn wordt het in de aanvraag opgenomen monitoringplan in het licht van de resultaten van de monitoringactiviteiten door de houder van de vergunning, indien nodig en voor zover de Commissie en de Minister van VROM hiermee instemmen, en/of door de Minister van VROM, voor zover de Commissie hiermee instemt, herzien. Voorstellen voor de herziening van een monitoringplan worden bij de bevoegde instanties van de lidstaten ingediend.

5. De houder van de vergunning is bij machte om aan de Commissie en de bevoegde instanties van de lidstaten aan te tonen dat:

   (a) de bestaande monitoringnetwerken, met inbegrip van de nationale botanische toezichtsnetwerken en gewasbeschermingsdiensten, zoals gespecificeerd in het in de aanvraag opgenomen monitoringplan, de informatie verzamelen die relevant is voor de monitoring van het product; en

   (b) deze bestaande monitoringnetwerken hebben toegezegd deze informatie vóór de datum van indiening van de monitoringverslagen bij de Commissie en de bevoegde instanties van de lidstaten overeenkomstig lid 3 aan de houder van de vergunning beschikbaar te stellen.

Artikel 5
Inwerkingtreding

Deze beschikking wordt van kracht overeenkomstig het bepaalde in artikel 20.3 van de Wet milieubeheer.

VAN TOEPASSING ZIJNDE REGELGEVING

1. Het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen[2]. Paragraaf 3 'Doelbewuste introductie in het milieu'.

2. Richtlijn 2001/18/EG van het Europees Parlement en de Raad van 12 maart 2001, inzake de doelbewuste introductie van genetisch gemodificeerde organismen in het milieu en tot intrekking van Richtlijn 90/220/EG van de Raad[3]. Deel C 'In de handel brengen van GGO's als product of in producten'.

[2] Stb. 1993, 435
[3] PB L 106 van 17-4-2001, blz. 1.

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DE GEVOLGDE PROCEDURE

(1) Florigene Ltd. heeft op 3 september 2004 bij de Minister van VROM een aanvraag ingediend voor een vergunning voor het in de handel brengen van een genetisch gemodificeerde anjer (*Dianthus caryophyllus* L., lijn 123.2.38) bedoeld in artikel 23 van het Besluit ggo.

(2) Florigene Ltd heeft, op verzoek van de Minister van VROM op 7 december 2004 en 27 december 2004 aanvullende informatie ingediend.

(3) De aanvraag en de aanvullende informatie zijn getoetst aan de vereisten van artikel 28 van het Besluit ggo en artikel 13, tweede lid, van de richtlijn en voldoen daaraan.

(4) De aanvraag is behandeld conform het bepaalde in paragraaf 3.3 van het Besluit ggo en deel C van de richtlijn.

(5) In overeenstemming met artikel 29 van het Besluit ggo is een beoordelingsrapport opgesteld. Het beoordelingsrapport vermeldt dat anjerlijn 123.2.38 onder voorwaarden in de handel kan worden gebracht. Dit beoordelingsrapport en een afschrift van de aanvraag zijn op 4 maart 2005 naar de aanvrager en de Commissie gezonden.

(6) Conform artikel 14, vierde lid, van de richtlijn is het beoordelingsrapport op 16 maart 2005 door de Commissie doorgezonden aan de lidstaten van de Europese Gemeenschap (hierna: de lidstaten).

(7) Conform de standaardprocedure als bedoeld in artikel 15 van de richtlijn kan een bevoegde instantie of de Commissie binnen 60 dagen na de verspreiding van het beoordelingsrapport om nadere informatie verzoeken, opmerkingen maken, of met redenen omklede bezwaren maken tegen het in de handel brengen van het /de betrokken GGO's. Er zijn opmerkingen, bezwaren en verzoeken om informatie van een aantal lidstaten ontvangen. De Minister heeft vervolgens 45 dagen de tijd gehad om te proberen tot overeenstemming te komen met de Commissie en de lidstaten. Dit is niet voor alle bezwaren gelukt.

(8) In overeenstemming met artikel 28 van de richtlijn heeft de Commissie advies gevraagd over de aanvraag aan de EFSA.

(9) De EFSA is in haar op 17 mei 2006 vastgestelde (en op 27 juni 2006 gepubliceerde) advies op grond van alle ingediende gegevens tot de conclusie gekomen dat het onwaarschijnlijk is dat snijbloemen van de genetisch gemodificeerde anjer (*Dianthus caryophyllus* L., lijn 123.2.38) in de context van het voorgestelde gebruik als sierbloemen nadelige effecten op de gezondheid van mens of dier of op het milieu zullen hebben. De EFSA concludeerde tevens dat de omvang van het door de houder van de vergunning ingediende monitoringplan in overstemming met het voorgenomen gebruik van de anjer is.

(10) Uit onderzoek van alle in het licht van de richtlijn ingediende bezwaren, de in de aanvraag ingediende informatie en het advies van de EFSA zijn geen redenen naar voren gekomen om aan te nemen dat het in de handel brengen van snijbloemen van de genetisch gemodificeerde anjer (*Dianthus caryophyllus* L., lijn 123.2.38) in de context van het voorgestelde gebruik als sierbloemen nadelige effecten op de gezondheid van mens of dier of op het milieu zal hebben.

(11) De Commissie heeft derhalve in overeenstemming met artikel 19 van de richtlijn een positief ontwerpbesluit, betreffende het in de handel brengen van *Dianthus caryophyllus* L., lijn 123.2.38, opgesteld. Het ontwerpbesluit is op 18 september 2006 besproken en ter stemming gebracht in het artikel 30 comité van de richtlijn. De stemming leidde noch tot een gekwalificeerde meerderheid voor noch tot een gekwalificeerde meerderheid tegen het ontwerpbesluit.

(12) Conform de artikelen 5 en 7 van Besluit 1999/468/EG is het ontwerpbesluit voor de Milieuraad van 20 februari 2007 geagendeerd. De stemming tijdens de Milieuraad leidde wederom niet tot een gekwalificeerde meerderheid voor of tegen het ontwerpbesluit.

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(13) Conform Besluit 1999/468/EG neemt de Commissie in een dergelijk geval het besluit zoals dat is voorgelegd aan de Milieuraad over. Op 30 mei 2007 is de positieve beschikking van de Commissie met nr. 2007/364/EG gepubliceerd in het publicatieblad van de Europese Unie (PB L 138/50-52).

(14) Deze beschikking van de Commissie is gericht aan de Minister van VROM en de Minister van LNV, om vergunning te verlenen aan Florigene Ltd. om *(Dianthus caryophyllus* L., lijn 123.2.38), in de handel te brengen.

(15) De beschikking vermeldt welke voorschriften op basis van artikel 19, derde lid, van de richtlijn aan de vergunning moeten worden verbonden.

KENNISGEVING EN BEZWAAR

Dit besluit wordt bekend gemaakt door kennisgeving ervan in de Staatscourant en in de Volkskrant.
Op grond van de Algemene wet bestuursrecht kan door belanghebbenden in de zin van artikel 1:2 Algemene wet bestuursrecht, binnen zes weken na de datum van verzending van dit besluit een bezwaarschrift worden ingediend. Het bezwaarschrift moet zijn gemotiveerd, gedagtekend en voorzien van naam, adres en woonplaats van de indiener. Het bezwaarschrift moet worden ingediend bij de Minister van VROM, p/a Bureau Genetisch Gemodificeerde Organismen, Postbus 1, 3720 BA Bilthoven.
Tevens kan de indiener van het bezwaarschrift gedurende de termijn dat bezwaar kan worden gemaakt een verzoek tot het treffen van een voorlopige voorziening als bedoeld in artikel 8:81 van de Algemene wet bestuursrecht indienen bij de voorzitter van de Afdeling bestuursrechtspraak van de Raad van State, Postbus 20019, 2500 EA Den Haag. Tegen de inhoud van deze beschikking kon reeds in de Europese besluitvormingsprocedure worden geageerd.

Den Haag, 11-07-2007

De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

dr. Jacqueline Cramer

Kennisgeving Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen

Introductie in het milieu door het in de handel brengen van genetisch gemodificeerde organismen.

Beschikking op de vergunningaanvraag van Florigene Ltd.

Op 11 juli 2007 is door De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer, in overeenstemming met de Minister van Landbouw, Natuur en Voedselkwaliteit, vergunning verleend, met kenmerk DGM/SAS C/NL/04/02, voor het in de handel brengen van genetisch gemodificeerde organismen krachtens artikel 23 van het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen (hierna: Besluit ggo) aan Florigene Ltd, gevestigd in Bundoora, in Australië. De beschikking is op 17 juli 2007 aan Florigene Ltd verzonden.
Op 30-07-2004 had Florigene Ltd een daartoe strekkende aanvraag ingediend. De genetisch gemodificeerde organismen die als product in de handel worden gebracht ten behoeve van import zijn snijbloemen van een anjer (*Dianthus caryophyllus* L.) met een gewijzigde bloemkleur, afgeleid van een celcultuurlijn van *Dianthus caryophyllus* L. en gemodificeerd met *Agrobacterium tumefaciens*, stam AGL0, met behulp van de vector pCGP1470, waaruit lijn 123.2.38 is ontstaan.

Procedure

Voor de behandeling van de aanvraag van Florigene Ltd is de procedure doorlopen als beschreven in paragraaf 3.3 van het Besluit ggo en deel C van de Richtlijn 2001/18/EG van het Europees Parlement en de Raad van de Europese Unie van 12 maart 2001 inzake de doelbewuste introductie van genetisch gemodificeerde organismen in het milieu.
Op 30 mei 2007 is de beschikking van de Europese Commissie met nr. 2007/364/EG gepubliceerd in het publicatieblad van de Europese Unie (PB L 138/50-52). Deze beschikking is gericht aan de Minister van VROM en de Minister van LNV om vergunning te verlenen aan Florigene Ltd om *Dianthus caryophyllus*, lijn 123.2.38, in de handel te brengen. Tegen de inhoud van deze beschikking kon in de Europese besluitvormingsprocedure worden geageerd.

Inzage beschikking
De beschikking en de overige relevante stukken liggen vanaf 19-07-2007 op werkdagen ter inzage in de Bibliotheek van het Ministerie van VROM, Rijnstraat 8 te Den Haag (op werkdagen geopend van 9.00 uur tot 16.00 uur en na telefonische afspraak (070-3393000) tot 20.30 uur. Op woensdag gesloten).

Bezwaar
Op grond van de Algemene wet bestuursrecht kan door belanghebbenden in de zin van artikel 1:2 Algemene wet bestuursrecht, binnen zes weken na de datum van verzending van dit besluit een bezwaarschrift worden ingediend. Het bezwaarschrift moet zijn gemotiveerd, gedagtekend en voorzien van naam, adres en woonplaats van de indiener. Het bezwaarschrift moet worden ingediend bij de Minister van VROM, p/a Bureau Genetisch Gemodificeerde Organismen, Postbus 1, 3720 BA Bilthoven.
Tevens kan de indiener van het bezwaarschrift gedurende de termijn dat bezwaar kan worden gemaakt een verzoek tot het treffen van een voorlopige voorziening als bedoeld in artikel 8:81 van de Algemene wet bestuursrecht indienen bij de voorzitter van de Afdeling bestuursrechtspraak van de Raad van State, Postbus 20019, 2500 EA Den Haag.

Appendix 2. Bioinformatic analysis

1. Introduction

The application dossier for FLO-40644-6 contained a blastp analysis of homology of the inserted genes to toxins and allergens. In addition, as part of the review process for the application for marketing consent of FLO-40644-6, information was provided to EFSA on the number of sequence inserts, sequence of inserts, sequence of theoretical ORFs and sequence of flanking genomic DNA flanking inserts. In 2006, these sequences were subjected to blastp analysis to identify any homology to toxins or allergens.

The purpose of the present work was to repeat the blastp analysis against up to date databases. With the publication of a carnation genome in 2013 (Yagi et al., 2013), we are now also in a position to identify the probable location of the inserts within the carnation genome.

2. Bioinformatic analysis of flanking sequences

2.1 Methods

The nucleotide sequence of the flanking regions of the two inserts present in FLO-40644-6 are shown in table 1.

Table 1. Nucleotide sequences of flanking regions

Region	Nucleotide sequence
Moonlite Locus 1 5' flank	CCTCGCCTTTTGTGGGCTCAATAGAGGCATTGGGGGTAATAAG TTATGTAAAGCGGAAATATGAAATATCGGTTTTTCTAACATTG AGAATTATTATCCCTATGTAACATTGCGAAAGTGAATTGATAC TCCTATTTTTGAGATTTGTAAG
Moonlite Locus 1 3' flank	CGCCAGTGAATGTGTACTCCGTAATTTGTAAGCATATAAGAAA AAACCGGAATATATCATTTAATTTTTGGTTCGCATTTGCGTATC ATTTTAAAGAGCTACAACTGTATGGATTCTTTGTTGAAACTGA TTAAAAAACAGTCAAAATATA
Moonlite Locus 2 5' flank	CTTTGTGTAGTTTGAAGTCAAAGATGATGATGATGATGATGAT TATACGTAGTAGTTGTCAATTAACGCCAGGGTTTTACGAATGG AAACTATTTTGTATTGTGATAATGTGAAGGTGACATGTCAAAT ATGCTACATCTCTTTCATTTT
Moonlite Locus 2 3' flank	GCGAGCTTTCTAATTGATAAAAATTACATTAAAATGAAAGAGA TGTAGCATATTTGACATGTCACCTTCACATTATCACAATACAA AATAGTTTCCATTCGTAAAACAACACATCATCACTCAAATTAT ATGCTCCGCAAGTTTTAGTCA

Genomic DNA (gDNA) sequences were subjected to;

a) A nucleotide homology search for identifying a query nucleotide sequence and for finding similar sequences in nucleotide databases by finding local regions of similarity (blastn).
b) A protein homology search using a query nucleotide sequence (blastx).

A provisional complete sequence of the cultivated carnation has been published (Yagi et al., 2013). The genome is publicly available at http://carnation.kazusa.or.jp, from where BLAST searches may be made. This database, in addition to that located at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) were used for sequence analysis. In

the case of the blastx analysis of the NCBI database the search was performed with and without the entrez query "Dianthus". Location of databases accessed and date of access are listed in section 2.2.

2.2 Results and discussion

2.2.1. blastn results. Outputs are summarised in table 2. For each of the three databases all flanking sequences (table 1) were searched using default search parameters.

Table 2. Summary of blastn search dates and results

Database	Date	Algorithm	Reference file
http://blast.ncbi.nlm.nih.gov/Blast.cgi Nucleotide collection (nr/nt)	April 29 2016	blastn	None. No significant similarity was found for any of the four query sequences
http://blast.ncbi.nlm.nih.gov/Blast.cgi Expressed sequence tags (est)	April 29 2016	blastn	None. No significant similarity was found for any of the four query sequences
http://carnation.kazusa.or.jp/ Carnation database	April 29 2016	blastn	**2. flanking region carnation database blastn.doc**

For all 4 query sequences, highly significant E-value scores and identity scores were observed against nuclear DNA scaffold sequences within the carnation nuclear genome database (table 3 and reference file **2. flanking region carnation database blastn.doc**). For both loci the same scaffold sequence was identified using either the 3' or 5' flanking sequences as a query (Table 3). This is to be expected if the T-DNA is inserted within the scaffold sequence.

Table 3. Summary of BLASTn results

Query sequence		Database sequence producing highest significant alignment		
Locus	**Flank**	**E-value**	**Identity similarity***	**Identity**
1	5'	4e-77	146/146	Scaffold 3618
	3'	2e-75	143/143	Scaffold 3618
2	5'	6e-36	77/77	Scaffold 8
	3'	1e-65	125/125	Scaffold 8

**Number of identical nucleotides over the query sequence*

2.2.2. blastx results. Outputs are summarised in table 4. In the case of the blastx analysis of the non –redundant protein sequence database the search was performed with and without the entrez query "Dianthus".

Table 4. Summary of blastx search dates and results

Database	Date	Algorithm	Reference file
http://blast.ncbi.nlm.nih.gov/Blast.cgi Non –redundant protein sequence	April 29 2016	blastx	None. No significant similarity was found for any of the four query sequences
http://blast.ncbi.nlm.nih.gov/Blast.cgi Non –redundant protein sequence, entrez query; Dianthus	April 29 2016	blastx	None. Either no significant similarity was found or E-value of most significant alignment was > 1.0.
http://carnation.kazusa.or.jp/ Carnation database	April 29 2016	tblastx	**3. flanking region carnation database tblastx.doc**

As expected, when the blastx database search was carried out with the carnation database the two insertion loci were identified to have occurred in the same scaffold sequences as identified with the blastn analysis (refer to reference file **3. flanking region carnation database tblastx.doc**). Hits were obtained from the search of the NCBI database with the entrez query term "Dianthus", but all E-value scores exceeded 1.0 and none were considered biologically significant.

2.3 Conclusions

The blastn analysis of a carnation genome database indicates that flanking sequences are located within two different scaffold sequences from the carnation genome (Yagi et al., 2013). Lack of any biologically significant similarities when databases at http://blast.ncbi.nlm.nih.gov/Blast.cgi were searched suggest the insertion sites are within non-coding regions of the scaffolds.

3. Bioinformatic analysis of ORFs

3.1 Methods

Two ORFs straddling the flanking regions were identified when the marketing application for FLO-40644-6 was compiled and reviewed. The sequence of these ORFs is shown in table 5.

Table 5. Amino acid sequence of ORFs

ORF	Sequence
ORF1	MIYSGFFLYAYKLRSTHSLAAHQPDLDRRTPPLHGDLCGFYNNVERVGMDCRR CPLLALPAGAASRALERRRATSRSLIVETIGTSLSMISCQT
OFR 2	MLTNYGVHIHWRLTSLTSIVGPLLFTAIYAASITTWNGWAWIAGAALYLLCLPA LRRGLWSGAGQRADR

Similarity to known toxic proteins

The two ORFs were subjected to a BLAST (blastp) homology search used for identifying similar sequences at (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997). The ORFs were searched twice;

- Default search parameters were used to search the non-redundant protein database. An E-value of less than 1.0 was used as a cut-off to identify ORF sequences with potential biological significance. Only the best 50 hits (if more than 50 hits were

generated) are shown for the output of each query where an E-value of less than 1.0 was generated. Where the number of hits was greater than 50, all hits were screened to confirm all relevant hits were displayed for risk assessment. This blastp analysis was carried out on April 29 2016.

- The blastp was repeated using the SwissProt protein database located at http://blast.ncbi.nlm.nih.gov/Blast.cgi and the Entrez query 'toxin'. An E-value of less than 1.0 was used as a cut-off to identify sequences with potential biological significance. This blastp analysis was carried out on April 29 2016.

Similarity to known allergenic proteins

The deduced amino acid sequences of all ORFs were analysed for the presence of known allergenic sequences in the FARRP allergen protein database located at http://www.allergenonline.org/databasefasta.shtml (version 16, Jan 27 2016). The amino acid sequences of the two ORFs were searched using the sliding 80mer window method and the 8mer exact match method.

3.2 Results and discussion

3.2.1 blastp results. The search outputs are provided in the reference file **4. ORF blastp output.pdf**. Please use the bookmarks provided in that reference file to navigate between the outputs for each ORF and database. Though there were some hits to sequences related to toxins when the SwissProt protein database and the entrez query 'toxin' was searched (highlighted in green in reference file **4. ORF blastp output.pdf**) the E-values were no less than 0.017 and were in some cases were greater than 1.0. The hits were therefore considered to be of no biological significance.

Significant E-values were found when the nr database was searched. For ORF2 there was a high level similarity to part of the TetA protein from various cloning vectors This is not surprising given the sequence of ORF 2 spans the partial TetA sequence present in locus 1. ORF1 showed similarity to putative membrane protein and also a tetracycline resistance protein. Both ORF1 and ORF2 were generated from the 3'flanking sequence of Moonlite locus 1.

3.2.2. FARRP allergen protein search results. Using the sliding 80mer window method no matches of greater than 35% identity were found and using the 8mer exact match method no sequences were found with an exact 8mer match. As no allergen proteins were identified in the blastp database searches we conclude neither ORF has homology to an allergen.

3.3 Conclusions

The database searches have confirmed that the two ORF sequences do not have homology to toxins or allergens and substantially confirm the derivation of the ORF sequences from the region of locus 1 spanning the partial TetA sequence integration.

4. Bioinformatic analysis of inserted genes

4.1 Methods

The sequence of the three inserted genes are shown in table 6. To search for similarity for known toxic proteins and known allergens the same search methods outlined in section 3.1 were used. The blastp analysis was carried out on April 30 2016.

4.2 Results and discussion

4.2.1 blastp results. The search outputs are provided in the reference file **5. Inserted gene blastp output.pdf**. Please use the bookmarks provided in that reference file to navigate between the outputs for each gene and database combination.

No toxic or allergenic proteins were identified when translated nucleotide sequence of the genes contained in the T-DNA of the binary vector proteins were subjected to a BLAST (blastp) homology search of the non-redundant protein database. Highly significant E-values were found to sequence query results identifying similar proteins to the query proteins, largely from different plant species.
Though there were some hits to sequences related to toxins when the SwissProt protein database and the entrez query 'toxin' was searched (refer to reference file **5. Inserted gene blastp output.pdf**) the E-values were no less than 0.0004 and were in some cases greater than 1.0. The hits were therefore considered to be of no biological significance.
4.2.2. FARRP allergen protein search results. Using the sliding 80mer window method no matches of greater than 35% identity were found and using the 8mer exact match method no sequences were found with an exact 8mer match. As no allergen proteins were identified in the blastp database searches we conclude neither ORF has homology to an allergen. The three newly expressed proteins are ubiquitous, well-characterized proteins and are not known to be allergens.

4.3 Conclusions

The database searches have confirmed that the inserted genes do not have homology to toxins or allergens.

Table 6. Amino acid sequence of inserted genes

Protein	Sequence
*Nicotiana tabacum SuR*B (ALS)	MAAAAAAPSPSFSKTLSSSSSKSSTLLPRSTFPFPHHPHKTTPPPLHLTPTHIHSQ RRRFTISNVISTTQKVSETQKAETFVSRFAPDEPRKGSDVLVEALEREGVTDVF AYPGGASMEIHQALTRSSIIRNVLPRHEQGGVFAAEGYARATGFPGVCIATSGP GATNLVSGLADALLDSVPIVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYL VMDVEDIPRVVREAFFLARSGRPGPVLIDVPKDIQQQLVIPDWDQPMRLPGYM SRLPKLPNEMLLEQIVRLISESKKPVLYVGGGCSQSSEELRRFVELTGIPVASTL MGLGAFPTGDELSLSMLGMHGTVYANYAVDSSDLLLAFGVRFDDRVTGKLE AFASRAKIVHIDIDSAEIGKNKQPHVSICADIKLALQGLNSILESKEGKLKLDFS AWRQELTVQKVKYPLNFKTFGDAIPPQYAIQVLDELTNGSAIISTGVGQHQMW AAQYYKYRKPRQWLTSGGLGAMGFGLPAAIGAAVGRPDEVVVDIDGDGSFI MNVQELATIKVENLPVKIMLLNNQHLGMVVQWEDRFYKANRAHTYLGNPSN EAEIFPNMLKFAEACGVPAARVTHRDDLRAAIQKMLDTPGPYLLDVIVPHQEH VLPMIPSGGAFKDVITEGDGRSSY
Petunia hybrida DFR	MASEAVHAPSPPVAVPTVCVTGAAGFIGSWLVMRLLERGYNVHATVRDPENK KKVKHLLELPKADTNLTLWKADLTVEGSFDEAIQGCQGVFHVATPMDFESKD PENEVIKPTVRGMLSIIESCAKANTVKRLVFTSSAGTLDVQEQQKLFYDQTSWS DLDFIYAKKMTGWMYFVSKILAEKSAMEETKKKNIDFISIIPPLVVGPFITPTFPP SLITALSLITGNEAHYCIIKQGQYVHLDDLCEAHIFLYEHPKADGRFICSSHHAII YDVAKMVREKWPEYYVPTEFKGIDKDLPVVSFSSKKLTDMGFQFKYTLEDM YKGAIETCRQKQLLPFSTRSAADNGHNREAIAISAQNYASGKENAPVANHTEM LTNVEV
Petunia hybrida F3'5'H	MMLLTELGAATSIFLIAHIIISTLISKTTGRHLPPGPRGWPVIGALPLLGAMPHVS LAKMAKKYGAIMYLKVGTCGMAVASTPDAAKAFLKTLDINFSNRPPNAGAT HLAYNAQDMVFAHYGPRWKLLRKLSNLHMLGGKALENWANVRANELGHM LKSMSDMSREGQRVVVAEMLTFAMANMIGQVMLSKRVFVDKGVEVNEFKD MVVELMTIAGYFNIGDFIPCLAWMDLQGIEKRMKRLHKKFDALLTKMFDEHK ATTYERKGKPDFLDVVMENGDNSEGERLSTTNIKALLLNLFTAGTDTSSSAIE WALAEMMKNPAILKKAQAEMDQVIGRNRRLLESDIPNLPYLRAICKETFRKHP

	STPLNLPRISNEPCIVDGYYIPKNTRLSVNIWAIGRDPQVWENPLEFNPERFLSG RNSKIDPRGNDFELIPFGAGRRICAGTRMGIVMVEYILGTLVHSFDWKLPSEVIE LNMEEAFGLALQKAVPLEAMVTPRLQLDVYVP

5. Literature cited

Altschul Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402) located within the NCBI (The National Centre for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov)

Yagi M, Kosugi S, Hirakawa H, Ohmiya A, Tanase K, Harada T, Kishimoto K, Nakayama M, Ichimura K, Onozaki T, Yamaguchi H, Sasaki N, Miyahara, T, Nishizaki, Ozeki Y, Nakamura N, Suzuki T, Tanaka Y, Sato S, Shirasawa K, Isobe S, Miyamura Y, Watanabe A, Nakayama S, Kishida Y, Kohara M, Tabata S, 2013. Sequence analysis of the genome of carnation (*Dianthus caryophyllus* L.) DNA Res. doi:10.1093/dnares/dst053, 2013.

Appendix 3. Literature review updates

Afrin, S., Giampieri, F., Gasparrini, M., Forbes-Hernandez, T. Y., Varela-López, A., Quiles, J. L., Mezzetti, B. and Battino, M. (2016). Chemopreventive and therapeutic effects of edible berries: A focus on colon cancer prevention and treatment. Molecules 21;169. doi:10.3390/molecules21020169
A review of the *in vitro* and *in vivo* studies that have demonstrated that berry consumption (including berries with high levels of delphinidin) has therapeutic and preventive effects against colon cancer. Mechanisms of action are discussed.

Bishayee, A., Haskell, Y., Do, C., Siveen, K. S., Mohandas, N., Sethi, G., and Stoner, G. D. (2015). Potential benefits of edible berries in the management of aerodigestive and gastrointestinal tract cancers: preclinical and clinical evidence. Critical reviews in food science and nutrition DOI: 10.1080/10408398.2014.982243
A review of the potential chemopreventive and therapeutic potential of berry extracts on cancers including oesophageal, stomach, intestinal, oral and colorectal cancers.
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Cassidy, A., Rogers, G., Peterson, J. J., Dwyer, J. T., Lin, H., and Jacques, P. F. (2015). Higher dietary anthocyanin and flavonol intakes are associated with anti-inflammatory effects in a population of US adults. The American journal of clinical nutrition 102; 172-181.
Metadata analysis evidence is presented to suggest that a reduction in risk of certain chronic diseases can be associated with higher intakes of anthocyanins and flavonols. It is suggested an anti-inflammatory effect may be a key component of the beneficial effect.

Cerletti, C., Curtis, A., Bracone, F., Digesù, C., Morganti, A. G., Iacoviello, L.,Gaetano, G. and Donati, M. B. (2016). Dietary anthocyanins and health: data from FLORA and ATHENA EU projects. British Journal of Clinical Pharmacology doi: 10.1111/bcp.12943
A summary of results from two EU studies that suggest that dietary anthocyanin enrichment is beneficial against a number of health conditions. It is also proposed that anthocyanin supplementation can provide an anti-inflammatory effect.

Chakrabarti, M., and Ray, S. K. (2015). Direct transfection of miR-137 mimics is more effective than DNA demethylation of miR-137 promoter to augment anti-tumor mechanisms of delphinidin in human glioblastoma U87MG and LN18 cells. Gene 573; 141-152.
A study showing that delphinidin is a beneficial additive to treatments designed to inhibit human glioblastoma stem cells, responsible for brain tumours.

Chandra, S., and Rawat, D. S. (2015). Medicinal plants of the family Caryophyllaceae: a review of ethno-medicinal uses and pharmacological properties. Integrative Medicine Research 4; 123-131.
Dianthus species, including carnation were included in a review of edible plants. A number of positive health attributes were attributed to extracts from carnation.

Chandra, S., Rawat, D. S., Chandra, D. and Rastogi, J. (2016). Nativity, phytochemistry, ethnobotany and pharmacology of *Dianthus caryophyllus*. Research Journal of Medicinal Plant 10; 1-9.
Kaempferide triglycoside from carnation exhibits anticancer suppresses growth of colon cancer cell lines and inhibits growth of *Fusarium* wilt. Seed extract of *D. caryophyllus* exhibit potent antiviral activity.

de Castro, D. D. S. B., and Anderson, J. T. (2015). Anticancer properties of bioactive compounds of berry fruits-a review. British Journal of Medicine and Medical Research 6; 771. DOI: 10.9734/BJMMR/2015/15289
A review substantiating that dietary intake of berry fruits has a positive effect on human health and may diminish disease.

Deepika, S.D., Lakshmi, S.G., Sowmya, L.K., and Sulakshana, M. (2014). Edible flowers–a review article. International Journal of Advanced Research in Science and Technology 3; 51 – 57.
Dianthus is listed as a common edible flower.

Ezić, J., Kugić, A., Hadžić, M., Haverić, A., Bajrović, K., and Haverić, S. (2015). Analysis of delphinidin and luteolin genotoxicity in human lymphocyte culture. Journal of Health Sciences *5*; 41-45.
Delphinidin was determined to have no genotoxic effect on cultured human lymphocytes.

He, X., de Brum, P. A., Chukwudebe, A., Privalle, L., Reed, A., Wang, Y., Zhou, C.,Wang, C., Lu, J., Huang K. and Contri, D. (2016). Rat and poultry feeding studies with soybean meal produced from imidazolinone-tolerant (CV127) soybeans. Food and Chemical Toxicology 88; 48-56.
There was no effect of the inserted transgene on body weight, haematology or serum chemistry values.

Keravis, T., Favot, L., Abusnina, A. A., Anton, A., Justiniano, H., Soleti, R., Alibrahim, E., Simard, G., Andriantsitohaina, R. and Lugnier, C. (2015). Delphinidin inhibits tumor growth by acting on vegf signalling in endothelial cells. PloS one 10(12). e0145291.
doi:10.1371/journal.pone.0145291
Under some conditions, delphinidin is a promising compound to prevent pathologies associated with generation of vascular network in tumorigenesis.

Ko, H., Jeong, M. H., Jeon, H., Sung, G. J., So, Y., Kim, I.,Son, J., Lee, S. Yoon, H. and Choi, K. C. (2015). Delphinidin sensitizes prostate cancer cells to TRAIL-induced apoptosis, by inducing DR5 and causing caspase-mediated HDAC3 cleavage. Oncotarget 6; 9970 – 9984.
Delphinidin was shown to enhance the beneficial effects of chemical treatments designed to reduce the growth rate of prostate cancer by cells.

León-González, A. J., Sharif, T., Kayali, A., Abbas, M., Dandache, I., Etienne-Selloum, N., Kevers, C., Pincemail, J., Auger, C., Chabert, P., Alhosin, M. and Schini-Kerth, V. (2015). Delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside mediate the redox-sensitive caspase 3-related pro-apoptotic effect of blackcurrant juice on leukaemia Jurkat cells. Journal of Functional Foods 17; 847-856.
Delphinidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside were shown to promote programmed cell death in a model cancer cell growth system.

Lila, M. A., Burton-Freeman, B., Grace, M., and Kalt, W. (2016). Unraveling Anthocyanin bioavailability for human health. Annual Review of Food Science and Technology 10.1146/annurev-food-041715-033346.
A review covering aspects of the bioavailability of anthocyanin absorbed from foods.

Lim, W., Jeong, W., and Song, G. (2015). Delphinidin suppresses proliferation and migration of human ovarian clear cell carcinoma cells through blocking AKT and ERK1/2 MAPK signaling pathways. Molecular and cellular endocrinology 422; 172 – 181.
Delphinidin reduces the survival rates of breast cancer cells, induced cell death and inhibited cell migration.

Londoño, G.V., Villa, A.S., Rojas, C.D., Suárez, P.J., and Maya, C.W. (2015). Geles con acción espermicida a base de plantas, aplicación de la medicina tradicional en la anticoncepción (Herbal gels with spermicide effect, application of traditional medicine in contraception). Revista Cubana de Plantas Medicinales 20; 212-225.
Carnation extract was found to reduce sperm motility and viability and have a low cytotoxic effect on an HeLa cell line.

Lovell, C.R. (2012) Florists. In; Rustemeyer, T., Elsner, S.M. J and Maibach, H. (eds.), Kanerva's Occupational Dermatology, DOI 10.1007/978-3-642-02035-3_152, # Springer-Verlag, Berlin, Heidelberg.
Lists the many sources of irritants and allergens that can have negative dermatological effects in florists. One carnation citation, which has been covered in previous literature reviews, is listed.

Lu, B., Li, M., and Yin, R. (2015). Phytochemical content, health benefits, and toxicology of common edible flowers: A review (2000–2015). Critical reviews in food science and nutrition. DOI: 10.1080/10408398.2015.1078276
A review of edible flowers which does not include *Dianthus*.

Pal, H. C., Chamcheu, J. C., Adhami, V. M., Wood, G. S., Elmets, C. A., Mukhtar, H., and Afaq, F. (2015). Topical application of delphinidin reduces psoriasiform lesions in the flaky skin mouse model by inducing epidermal differentiation and inhibiting inflammation. British Journal of Dermatology 172; 354-364.
Delphinidin could be a promising agent for treatment of psoriasis and other skin disorders.

Pieroni, A., Nedelcheva, A., and Dogan, Y. (2015). Local knowledge of medicinal plants and wild food plants among Tatars and Romanians in Dobruja (South-East Romania). Genetic Resources and Crop Evolution 62; 605-620.
Carnation is listed as a medicinal plant in use in Romania.

Rahman, N., Jeon, M., and Kim, Y. S. (2016). Delphinidin, a major anthocyanin, inhibits 3T3-L1 pre-adipocyte differentiation through activation of Wnt/β-catenin signaling. BioFactors 42; 49 – 59.
A study which shows delphinidin effectively inhibits adipogenesis and so may be useful in the treatment of obesity.

Seitz, C., Ameres, S., Schlangen, K., Forkmann, G., and Halbwirth, H. (2015). Multiple evolution of flavonoid 3′, 5′-hydroxylase. Planta 242; 561-573.
A description of multiple evolutionary events leading to flavonoid 3'5'-hydroxylase from flavonoid 3'-hydroxylase.

Sogo, T., Kumamoto, T., Ishida, H., Hisanaga, A., Sakao, K., Terahara, N., Wada, K. and Hou, D. X. (2015). Comparison of the inhibitory effects of delphinidin and its glycosides on cell transformation. Planta medica 81; 26-31.
Evidence for the mechanism of action that is the cause of the weaker inhibitory effect of delphinidin glycosides compared to the aglycone delphinidin.

Song, S. E., Jo, H. J., Kim, Y. W., Cho, Y. J., Kim, J. R., and Park, S. Y. (2016). Delphinidin prevents high glucose-induced cell proliferation and collagen synthesis by inhibition of NOX-1 and mitochondrial superoxide in mesangial cells. Journal of Pharmacological Sciences 130; 235 – 243.
Delphinidin prevents high glucose-induced cell proliferation and collagen synthesis by inhibition of NOX-1 and mitochondrial superoxide in mesangial cells, the expansion of which is one symptom of damage due to diabetes.

Su, X., Xu, J., Rhodes, D., Shen, Y., Song, W., Katz, B., Tomich, J. and Wang, W. (2016). Identification and quantification of anthocyanins in transgenic purple tomato. Food chemistry 202; 184-188.
Petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin- 3-(trans-coumaroyl)-rutinoside-5-glucoside make up 86% of the total anthocyanins in transgenic purple tomato.

Tang, J., Oroudjev, E., Wilson, L. and Ayoub, G. (2015). Delphinidin and cyanidin exhibit anti-proliferative and apoptotic effects in MCF7 human breast cancer cells. Integrative cancer science and therapeutics 2; 82-86.
In cultured breast cancer cells delphinidin exhibits radical scavenging activity, inhibition of cell proliferation, and an increase in cancer cell death.

Thompson, K., Pederick, W., and Santhakumar, A. B. (2016). Anthocyanins in obesity-associated thrombogenesis: a review of the potential mechanism of action. Food and Function DOI: 10.1039/c6fo00154h
Anthocyanins exhibit anti-cholesterol accumulation and anti-blood clot properties. The review covers the potential use of anthocyanins in treating patients liable to thrombotic episodes.

Tsuda, T. (2016). Recent Progress in anti-obesity and anti-diabetes effect of berries. Antioxidants *5*;13. doi:10.3390/antiox5020013
A review of the potential health benefits of anthocyanins in diet in order to reduce obesity and diabetes.

Wallace, T. C., Slavin, M., and Frankenfeld, C. L. (2016). Systematic review of anthocyanins and markers of cardiovascular disease. Nutrients 8; 32. doi:10.3390/nu8010032
A review of metadata which concludes anthocyanin consumption improves LDL cholesterol levels and so may have benefits in prevention of cardiovascular disease.
No adverse effects of anthocyanins were found even at high levels of consumption.

Wightman, J. D., and Heuberger, R. A. (2015). Effect of grape and other berries on cardiovascular health. Journal of the Science of Food and Agriculture 95; 1584-1597.
A review of human clinical studies that concludes consumption of anthocyanins has a beneficial effect on cardiovascular health.

Yagi, M. (2015). Recent progress in genomic analysis of ornamental plants, with a focus on carnation. **The Horticulture Journal 84; 3–13.**
Discussion of comprehensive transcriptome sequences using NGS technology and whole-genome sequences generated for carnation, including the development of markers for fungal disease resistance and flower type.

Zhang, Y., Chu, G., Hu, Z., Gao,Q., Cui, B., Tian, S. Tian, S., Wang, B. and Chen, G. (2016). Genetically engineered anthocyanin pathway for high health-promoting pigment production in eggplant. Molecular Breeding 36; 1-14.
Transformation of a non-pigmented eggplant variety with a MYB transcription factor results in pigmented fruit in transgenic plants.