C/NL/06/01

Application to import carnation variety FLORIGENE Moonaqua™ (123.8.12)

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DEFINITIONS & ABBREVIATIONS[1]

#:	Number
ALS:	Acetolactate Synthase
Agrobacterium:	Means "*A. tumefaciens* AGL0"
***Ant*CHS:**	*Antirrhinum* chalcone synthase
ANZFA:	Australia New Zealand Food Authority (now FSANZ)
AQIS:	Australian Quarantine Inspection Services
AU:	Australia
BCH:	Biosafety Clearing House
BGGO, Bureau GGO, or GMO Office:	Bureau of Genetically Modified Organism, or Genetically Modified Organism Office
$CaCl_2$	Calcium Chloride
CaMV:	Cauliflower mosaic virus
cfu/g	Colony forming units
CHI:	chalcone isomerase
CHS:	chalcone synthase
DFR:	dihydroflavonol reductase
DHK:	dihydrokaempferol
DHM:	dihydromyricetin
DHQ:	dihydroquercitin
DIR:	Dealing involving intentional release
DNA:	Deoxyribonucleid Acid
EC:	European Community
ERA:	Environmental Risk Assessment
EU:	European Union
F3H:	flavanone 3-hydroxylase
F3'H:	flavonoid 3' hydroxylase
F3'5'H:	flavonoid 3'5' hydroxylase
FLS:	flavonol synthase
FSANZ:	Food Standards Australia New Zealand (formerly ANZFA)
g:	Gram
GM:	Genetically Modified
GMAC:	Genetic Manipulation Advisory Committee
GMHP:	Genetically Modified Higher Plant
GMO:	Genetically Modified Organims
GR:	General Release Licence
GTTAC:	Gene Technology Technical Advisory Committee
ha:	Hectare
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IOGTR	Interim Office of the Gene Technology Regulator
JP:	Japan
LB:	Left Border
n/a:	not applicable
MAC:	Cauliflower mosaic virus/Mas chimeric promoter
MAFF	The Ministry of Agriculture, Forestry and Fisheries of Japan
mg:	Milligrams
mg/g:	Milligrams per gram
MG/L	Milligram per liter
mL:	Milliliter
mm:	Millimeter
mRNA:	Messenger ribonucleic acid

ng/g:	Nanograms per gram
n/k:	not known
NRA:	National Registration Authority for Agricultural and Veterinary Chemicals
OECD:	Organisation for Economic Co-operation and Development
OGTR:	Office of the Gene Technology Regulator
PBR:	Plant Breeder's Rights
PCR:	Polymerase Chain Reaction
ppm:	Parts per million
RB:	Right Border
RHS:	Standard colour reference from the Royal Horticultural Society, London
S.D.	Standard deviation
SuRB:	Sulfonylurea resistance gene B
T-DNA	Transfer-Deoxyribonucleid Acid
TGA:	Therapeutic Goods Administration
***Ti*:**	Tumour-inducing
TLC:	Thin Layer Chromatography
™:	Trade Mark
UK:	United Kingdom
UPOV:	Union for the protection of plant varieties
US or USA:	United States of America
US EPA:	United States Environmental Protection Agency
US FDA:	United States Food and Drug Administration
WHO:	World Health Organisation
w/v:	Weight per volume
µg/g:	micorgrams per gram
µg/mL:	micrograms per milliliter

[1] *Note: any terms, words or abbreviation(s) not listed above can be taken to have the "common general meaning" as at the date of this submission.*

SECTION A
Technical information required in Annex IIIB of Directive 2001/18/EC

GENERAL INFORMATION

A.1 Name and address of the notifier (company or institute)

Florigene Pty. Limited, 1 Park Drive, Bundoora, VIC 3083, Australia (company).

A.2 Name, qualifications and experience of the responsible scientist(s)

The application is coordinated by Dr Steve Chandler.

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Dr Chandler has a BSc and PhD from Birmingham University, UK. Dr Chandler has 25 years experience in the areas of plant physiology, plant biochemistry, tissue culture, floriculture and the trial and evaluation and commercialisation of genetically modified floricultural crops.

A.3 Title of the project

Application to import carnation variety FLORIGENE Moonaqua™(123.8.12)

INFORMATION RELATING TO (A) THE RECIPIENT OR (B) (WHERE APPROPRIATE) THE PARENTAL PLANTS

A.4 Complete name

(a) Family name: Caryophyllaceae

(b) Genus: *Dianthus*

(c) Species: *caryophyllus*

(d) Sub-species: not applicable

(e) Cultivar/breeding line: Product is the cultivar FLORIGENE Moonaqua™(123.8.12)

(f) Common name: UK carnation, NL anjer, ESP clavel

A.5(a) Information concerning reproduction

(i) **Modes(s) of reproduction:** In wild *Dianthus* species, pollination is dependent on insects and is only achieved by Lepidoptera (butterflies, moths), because of the position of the nectaries deep in the flower. Apart from Lepidoptera no other insect can facilitate pollen transfer. *Dianthus* pollen cannot be spread by wind.

Carnations are vegetatively propagated by taking cuttings from plants when grown commercially, but the species does not spread vegetatively. The plants do not produce stolons, rhizomes, root-borne shoots, tubers etc and cuttings have to be struck under optimised conditions, as roots will not form on discarded material.

A review of the reproduction biology of *Dianthus* and carnation is provided at Attachment B5. Carnation is a highly intensively managed horticultural crop, from which flowers are harvested.

(ii) **Specific factors affecting reproduction, if any:** Abiotic factors which influence reproduction in wild *Dianthus* are seasonal. The factors relate to a summer flowering period and incidence of pollinating insects.

(iii) **Generation time:** Wild *Dianthus* species are annuals.

A.5(b) Sexual compatibility with other cultivated or wild plant species, including the distribution in Europe of the compatible species

Carnations are double-flowered cultivars and in the general trade, botanical and horticultural literature carnation cultivars are considered to belong to the species *Dianthus caryophyllus.* Details are provided in Attachment B5. The common name for *Dianthus caryophyllus* is carnation. However, the exact taxonomic and breeding history of carnation is not known and it is almost certain that carnation is a hybrid involving two or more *Dianthus* species, one of which is likely to be *Dianthus caryophyllus.*

Details of *Dianthus* distribution in Europe, and of compatibility to carnation, are provided in Attachment B5. Whilst there are wild *Dianthus* species in Europe, there is no compatibility between these plants and imported carnation flowers, as there is no potential for hybridization (Attachment B5). There are no reports exist of spontaneous hybridization between carnation cultivated in Europe and either wild *Dianthus* species or species of other genera.

The potential for gene dispersal to occur as a result of hybridization is vanishingly small, if not impossible because the following mutually dependent, very unlikely, events would all need to successfully occur;

- A lepidopteran-type insect would need to enter a room where the flowers were on display
- The insect would need to collect rarely present viable pollen from the flower
- The insect would then need transfer the pollen, which would need to remain viable, to any recipient *Dianthus* plants that might be present in the near vicinity.
- Potential recipients would need to be in flower and have flowers at the correct developmental stage
- The recipient plant would need to be compatible and fertile
- Seed set would have to occur
- Seed would need to be dispersed into a suitable environment
- Seed germination and successful plant establishment and maturation would have to occur.

Dr. Keith Hammett, an acknowledged expert on *Dianthus* breeding concluded ***"that the likelihood of gene dispersal from cut flowers of fully double carnations to be highly improbable, if not inconceivable"*** (refer to Attachment B5 of this application)**.**

Dianthus sylvestris

One wild *Dianthus* species, *Dianthus sylvestris*, is found from South East Spain to Greece and Northwards to the Swiss Jura and the Alps. The typical habitat for this species is rocky places (Polunin, 1980). There are also isolated pockets of garden escapes. For example, Clement and Foster (1994) describe a single population of *Dianthus sylvestris* in the UK, established on the rocks near Whitby harbour, in Yorkshire. However, despite the fact carnation has been grown and traded in Europe on a large scale for decades there are no reports of the existence of hybrids between carnation and *D. sylvestris* in the wild in Europe (Tutin et al., 1993).

- *Clement E.J and Foster, M.C. (1994) Alien plants of the British Isle, Botanical Society of the British Isles, London, 590 pages*
- *Polunin, G. (1980). Flowers of Greece and the Balkans, Oxford University Press, 592 pages.*
- *Tutin, T.G. and Walters, S.M., (1993). Dianthus L. In: Tutin, T.G., Heywood, V.H., Burges, N.A., Chaler, A.O., Valentine, D.H., Edmondson, J.R., Moore, D.M., Walters, S.M., Webb, D.A. (Eds). Flora Europea, Second Edition, Vol 1. Cambridge University Press*

A.6 Survivability

(a) Ability to form structures for survival or dormancy: The survival structures carnation can produce are seeds and pollen, though it is impossible for imported carnation flowers to form seed. Pollen dispersal from imported carnation flowers is also extremely unlikely, as outlined in Attachment B5 of this proposal. Under optimal conditions carnation breeders may store *Dianthus* seeds for 1-3 years.

(b) Specific factors affecting survivability, if any: Imported carnation flowers will not survive more than 3 weeks in the hands of the consumer. During this time seed set is impossible. Discarded carnation flowers have no vegetative propagation ability.

In cultivation, carnation requires careful irrigation, insect and disease control, and heavy fertilization. Typical crop cycles are 12 - 30 months. Older crops may die as a result of fungal infections - particularly *Fusarium.* Carnation does not spread vegetatively and are not winter hardy in areas with average minimum temperatures lower than -5°C.

A.7 Dissemination

(a) Ways and extent (for example an estimation of how viable pollen and/or seeds decline with distance) of dissemination: Genetic material from cultivated carnation plants could only theoretically be disseminated through seed or insect pollination or vegetative propagation. The assessment at Attachment B5 of this application provides a detailed overview of each of these routes. NONE of these avenues are realistic avenues for gene dispersal in the case of the carnation flowers imported into Europe.

(b) Specific factors affecting dissemination, if any: Not applicable

A.8 Geographical distribution of the plant

The carnation is a cultivated plant and is not found in the wild. Wild *Dianthus caryophyllus* is very rare and has only been reported in specific coastal regions of Corsica, Sardinia, France and Italy. Other *Dianthus* species are found in Europe, and their distribution is described as part of the environmental risk assessment (see Attachment B5 of this application).Varieties of “carnation” have been reported very occasionally to have naturalized in the British Isles. These reports are summarized in the table below. These accounts refer to garden plant varieties of pinks and carnation, which are not the same as cut-flower varieties and predate the development of modern cut-flower varieties by several centuries... Clement and Foster (1994) refer to carnation in the wild as "cultivars of this species (*caryophyllus*), native *D. gratianapolitans* and *D. plumarius*”.

Summary of reports of naturalization of Dianthus caryophyllus and hybrids in British floras

Reference	***Dianthus* species**	**Comments by authors**
Maby, 1996	*D. caryophyllus*	Probably introduced by Normans and naturalized in old walls in Rochester castle and Beaulieu abbey
Perry and Ellis, 1994	*D. caryophyllus*	"an escape of European origin" (no further detail)
Walters, 1993	*D. caryophyllus X D. barbatus*	Single report from the year 1717
Clement and Foster, 1994	*D. caryophyllus*	Known for more than 3 centuries on the walls of Rochester castle
Clapham et.al.,1987	*D. caryophyllus*	Occasionally naturalized on old garden walls
Preston et.al, 2002	*D. caryophyllus*	"*D. caryophyllus* has been cultivated in Britain since the 16th century and is very common in gardens. It was first recorded in the wild in 1778, when it was discovered on the walls of Rochester Castle (E. Kent)" "Some historical records may be referable to hybrids with other species, which are also commonly grown." "Not known with any certainty as a wild plant; recorded as doubtfully native in S. Europe."
	D. caryophyllus X D. gratianopolitans	A single record in 1980.
	D. caryophyllus X D. plumarius	A tufted perennial herb which "occurs as a casual garden throw-out on rubbish tips and roadside verges"
Stace, 1997	*D. caryophyllus*	Introduced. Occasionally naturalized on old garden walls
	D. caryophyllus X D. gratianopolitans, D. caryophyllus X D. gratianopolitans X D. plumarius	Parentages of escaped garden pinks, some of which are occasionally naturalized on old walls.

- *Clapham, A.R., Tutin, T.G. and Moore, D.M (1987) Flora of the British Isles, 3rd ed. Cambridge University Press, 688 pages.*
- *Clement E.J and Foster, M.C. (1994) Alien plants of the British Isle, Botanical Society of the British Isles, London, 590 pages*
- *Maby, R. (1996) Flora Brittannica. Sinclair-Stevens, 48p.*
- *Perry, A.R and Ellis, R.G (1994) The common ground of wild and cultivated plants. National museum of Wales, Cardiff, 166 pages*
- *Preston, C.D., Pearman, D.A. and Dines, T.A.(2002). New Atlas of the British Isles. Oxford University Press, 910 pages.*
- *Stace, C. (1997) New flora of the British Isles 2nd ed. Cambridge University Press,1130 pages*
- *Walter, M (1993). Wild and garden plants. Harper Collins, 200 pages.*

A.9 In the case of plant species not normally grown in the member state(s), description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts

Not applicable. Carnation flowers are routinely imported into the EU from Africa, South America and the Middle East, and are also widely grown in Europe. Approximately 8 billion carnation flowers are distributed within the EU per annum.

A.10 Other potential interactions, relevant to the GMO, of the plant with organisms in the ecosystem where it is usually grown, or elsewhere, including information on toxic effects on humans, animals and other organisms

This application is for the import of carnation flowers, which will be imported and distributed through commercial avenues of the floricultural industry in Europe. After and during use there are no opportunities for gene dispersal, and the flowers will not be cultivated in Europe.
The environmental risk analysis assessment carried out for the product (Section B) indicates there are no potential toxic effects associated with importation of the GMO. Attachments B2, B3 and B4 provide toxicity assay data. Attachment B1 provides a detailed review of the potential toxicity of the selectable marker gene.
Bioinformatic analysis of the coding sequences of the inserted genes indicates no homologies to known toxins or allergens (Attachments A8 and A9).

INFORMATION RELATING TO THE GENETIC MODIFICATION

A.11 Description of the methods used for the genetic modification

Genetic material was inserted into carnation by *Agrobacterium*-mediated transformation using disarmed *Agrobacterium tumefaciens* strain AGL0 carrying the transformation vector pCGP1991, developed by Florigene Pty. Limited, Bundoora, Australia. FLORIGENE Moonaqua™ (123.8.12) was produced in the Florigene Europe laboratories in The Netherlands.
Agrobacterium was killed during the transformation process using the antibiotic ticarcillin. Lack of residual *Agrobacterium* was confirmed by the absence of PCR-detectable *Agrobacterium* sequences (Refer to Attachment A1).

A.12 Nature and source of the vector used

The transformation vector is pCGP1991, developed by Florigene Pty. Limited, Bundoora, Australia. A map of pCGP1991 and details of its construction are shown in Attachment A2. The full sequence of the transformation vector is shown in Attachment A3. The transformation vector pCGP1991 contains no sequences of unknown origin and contains no known transposable elements or related sequence elements (Attachment A3). The transformation vector pCGP1991 was used to produce the transgenic carnation variety FLORIGENE Moonshadow™, which was approved for commercial production within the EU in 1998 (C/NL/97/13-1363A).

Overview of construction of transformation vector pCGP1991

The transformation vector pCGP1991 contains three genes for transfer to plants, namely *F3'5'H*, *DFR* and *ALS*, with their respective controlling elements (promoters and terminators). pCGP1971 is a pBluescript-based plasmid (Stratagene Cloning Systems, La Jolla, USA) containing a chimeric gene comprised of: a snapdragon *CHS* promoter sequence (Sommer and Saedler, 1986), a cDNA fragment containing the entire coding region of *F3'5'H* from black pansy (Brugliera and Tanaka, 2003), and a petunia phospholipid transfer protein homologue terminator sequence (Holton, 1992). The identity and function of the *F3'5'H* cDNA clone was confirmed by sequence comparison with a petunia *F3'5'H* clone (Holton *et al*, 1993) and complementation of a *F3'5'H* mutant in petunia (Brugliera and Tanaka, 2003). pCGP1472 is a pBluescript-based plasmid carrying the entire gene for *DFR* from petunia (promoter, coding region and terminator) (Beld *et al*, 1989). The transformation vector pCGP1991, was assembled by combining the chimeric gene from pCGP1971 with the *DFR* gene from pCGP1472 in a binary vector pWTT2132. pWTT2132 contains a chimeric gene comprised of: a CaMV 35S promoter sequence (Franck *et al*, 1980) ligated with the coding region and terminator sequence for *ALS* from the *SuRB* locus of tobacco (Lee *et al*, 1988).

Detailed Description of Construction of pCGP1991

Construction of pCGP1472 Isolation of petunia *DFR* genomic clone A genomic library was made from *Petunia hybrida* cv. Old Glory Blue DNA in the vector 2001 (Holton, 1992). Approximately 200,000 pfu were plated out on NZY plates, lifts were taken onto NEN filters and the filters were hybridised with 400,000 cpm/mL of ^{32}P-dCTP labeled petunia *DFR* cDNA fragment (described in Brugliera *et al*., 1994). Hybridizing clones were purified, DNA isolated from each and mapped by restriction enzyme digestion. A 13 kb *Sac*I fragment of one of these clones was isolated and ligated with *Sac*I ends of pBluescriptII (Stratagene) to create pCGP1472. Finer restriction endonuclease mapping indicated that a 5 kb *Bgl*II fragment contained the entire *DFR*

gene.

Construction of pCGP1984. Plasmid pCGP1984 was constructed by cloning the petunia *DFR* gene from pCGP1472 into the binary vector pWTT2132.Plasmid pCGP1472 was digested with *Bgl*II to release the ~5.0kb fragment containing the entire petunia *DFR* gene. The resulting 5'-overhangs at each end of the fragment were repaired using DNA Polymerase I (Klenow fragment) according to standard protocols (Sambrook *et al*., 1989). The binary vector pWTT2132 (obtained from DNAP technology, Oakland, USA) was digested with *Kpn*I to linearize the vector. The resulting overhanging 3'-ends of the vector were removed with T4 DNA polymerase according to standard protocols (Sambrook *et al*., 1989). The 5.0kb fragment containing the *DFR* gene was ligated with the repaired *Kpn*I ends of the binary vector pWTT2132. Correct insertion of the 5.0kb *DFR* fragment in pWTT2132 was established by restriction endonuclease digestion of DNA isolated from tetracycline resistant *E. coli* transformants. The resulting plasmid was designated pCGP1984.

Construction of 1971. The plasmid pCGP1971 contained a chimeric gene consisting of:
(i) snapdragon (*Antirrhinum majus*) *CHS* promoter fragment which includes 1.2kb of sequence 5' of the translation initiation site fragment of a *CHS* gene of snapdragon (Sommer and Saedler, 1986),
(ii) the coding region of a *F3'5'H* cDNA clone isolated from *Viola sp*. ('black pansy') which consists of a 1.6kb *Kpn*I/*Xba*I fragment from pCGP1961 (Brugliera and Tanaka, 2003).
(iii) a terminator sequence from a petunia phospholipid transfer protein homologue (PLTP) which consists of a 0.7kb *Sma*I/*Xho*I fragment from pCGP13 Bam (Holton, 1992) and includes a 150bp untranslated region of the transcribed region of *PLTP* gene (i.e. 3' to the deduced stop codon of the *PLTP* coding sequence).

Final preparation pCGP1991. The plasmid pCGP1971 was digested with *Eco*RV/*Not*I to release a 3.5kb fragment containing the chimeric gene described above consisting of a snapdragon *CHS* promoter sequence, a black pansy *F3'5'H* cDNA fragment and a petunia phospholipid transfer protein terminator sequence. The resulting 5'-overhang was repaired using DNA Polymerase I (Klenow fragment) according to standard protocols (Sambrook *et al*., 1989). The plasmid pCGP1984 was digested with *Pst*I to linearize the vector. The resulting overhanging 3'-ends of the vector were removed with T4 DNA polymerase according to standard protocols (Sambrook *et al*., 1989). The 3.5kb fragment from pCGP1971 was ligated with the repaired *Kpn*I ends of the binary vector pCGP1984. Correct insertion of the fragment in pCGP1984 was established by restriction endonuclease digestion of DNA isolated from tetracycline resistant *E. coli* transformants. The resulting plasmid was designated as pCGP1991.

Transformation of *Escherichia coli* and *Agrobacterium tumefaciens*

Escherichia coli strains DH5α and XL1-Blue, used for routine manipulations, were transformed using the method of Inoue *et al*. (1990). The plasmid pCGP1991 was introduced into *Agrobacterium tumefaciens* strain AGL0 by adding 5 µg of plasmid DNA to 100 µL of competent *Agrobacterium tumefaciens* cells. Cells were prepared by inoculating a 50 mL mg/L (Garfinkel and Nester, 1980) culture and growing for 16 hours at 28°C. The cells were then pelleted and resuspended in 1 mL of 20 mM $CaCl_2$ before use. The DNA-*Agrobacterium* mixture was incubated in liquid nitrogen for 2 min and then allowed to thaw by incubation at 37°C for 5 min. The cells were then mixed with 1 mL of mg/L media and incubated with shaking for 4 hours at 28°C. Cells of *A. tumefaciens* carrying pCGP1991 were selected on MG/L agar plates (Sambrook *et al.*, 1989) containing 50µg/mL tetracycline. The presence of the plasmid was confirmed by southern analysis of DNA isolated from the tetracycline-resistant transformants. The transformation vector is able to transfer to dicotyledonous plants susceptible to the specific *Agrobacterium* strain in which it is carried. In the present application the disarmed *Agrobacterium tumefaciens* strain AGL0 was used (Lazo *et al*., 1991).

- *Beld, M., Martin, C., Hults, H., Stuitje, A.R. and A.G.M. Gerats. Flavonoid synthesis in Petunia hybrida: partial characterization of dihydroflavonol-4-reductase genes. Plant Molecular Biology 13: 491-502, 1989.*
- *Brugliera, F., Holton, T.A., Stevenson, T.W., Farcy, E., Lu, C-Y. and E.C. Cornish. Isolation and characterization of a cDNA clone corresponding to the Rt locus of Petunia hybrida. The Plant Journal 5: 81-92, 1994 .*
- *Brugliera, F. and Tanaka, Y. Genetic sequences and uses therefor. International PCT Application Number PCT/AU03/01111. Assignee: International Flower Developments, 2003.*
- *Franck, A., Guilley, H., Jonard, G., Richards, K. and L. Hirth. Nucleotide sequence of cauliflower mosaic virus DNA. Cell 21: 285-294, 1980.*
- *Garfinkel, D.J. and Nester, E.W., Agrobacterium tumefaciens mutants affected in crown gall tumorigenesis and octopine catabolism J. Bacteriol. 144:732-743, 1980.*
- *Holton, T.A. Isolation and characterization of petal-specific genes from Petunia hybrida. PhD Thesis, University of Melbourne, 1992.*
- *Holton, T.A., Brugliera, F., Lester, D., Tanaka, Y., Hyland, C., Menting, J.G.T., Lu, C-Y. and E.C. Cornish. Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366: 276-279, 1993.*
- *Inoue, H. Nojima, H. and Okayama, H. High efficiency transformation of Escherichia coli with plasmids. Gene, 96, 23-28,1990.*
- *Lazo, G.R., Stein, P.A. and R.A. Ludwig. A DNA transformation competent Arabidopsis genomic library in Agrobacterium. Bio/technology 9: 963-967, 1991.*
- *Lee, K.Y., Townsend, J., Tepperman, J., Black, M., Chui, C.F., Mazur, B., Dunsmuir, P. and J. Bedbrook. The molecular basis of sulfonylurea herbicide resistance in tobacco. The EMBO Journal 7: 1241-1248, 1988.*
- *Sambrook, J., Fritsch, E.F. and T. Maniatis. Molecular Cloning: A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory Press, USA, 1989.*
- *Sommer, H. and H. Saedler. Structure of the chalcone synthase gene of Antirrhinum majus. Molecular and General Genetics 202: 429-434, 1986.*

A.13 Size, source (name) of the donor organism(s) and intended function of each constituent fragment of the region intended for insertion

The donor organisms from which the DNA for construct pCGP1991 is derived are named in the table directly below:

Scientific name	Other Name
Agrobacterium tumefaciens	crown gall bacterium
Pseudomonas aeruginosa	pseudomonas
Viola sp.	pansy
Escherichia coli	"E.coli"
Petunia X hybrida	petunia
CaMV	Cauliflower Mosaic Virus
Nicotiana tabacum	tobacco
Antirrhinum majus	snapdragon

Maps illustrating the construction of plasmid pCGP1991 are provided in Attachment A2. The full sequence of the transformation transformation vector pCGP1991 is known (see Attachment A3) and the function of all genes (or parts of genes) encoded by pCGP1991 is known. Attachment A3 provides a list of the genetic elements comprising the transformation vector pCGP1991. The transformation vector pCGP1991 is 27,444 nucleotides in length.

INFORMATION RELATINGTO THE GENETICALLY MODIFIED PLANT

A.14 Description of the trait(s) and characteristics which have been modified

The product line of the present application consists of imported flowers from carnation plants that have a modified flower colour and are herbicide resistant. The transgenic line is transformed with the use of a disarmed *Ti*-plasmid of *Agrobacterium tumefaciens*.

Flower colour is generally the result of the relative concentration and type of two pigment classes - carotenoids and flavonoids. Carotenoids are responsible for yellow through orange colours however most plants do not contain carotenoid pigments. Anthocyanins are flavonoid based coloured pigments. There are three groups of anthocyanins, the delphinidins that generally produce blue flower colour, cyanidins that produce red or pink flower colour, and pelargonidins that produce

orange or brick red flower colour. Non-genetically modified carnations lack the part of the anthocyanin biosynthetic pathway that is responsible for the production of delphinidin, as they lack a gene encoding the enzyme flavonoid 3’5’ hydroxylase (F3'5'H) that converts dihydrokaempferol (DHK) to dihydroquercetin (DHQ) and then to dihydromyricetin (DHM). In the genetically modified carnation line FLORIGENE Moonaqua™ a gene encoding DFR has also been introduced, as the particular non-genetically modified starting material used lacked DFR activity. The enzyme DFR can use either DHQ or DHM as substrate. Delphinidin is thus produced as a result of the combined expression of the introduced genes *DFR* and *F3’5’H* together with endogenous genes in the anthocyanin biosynthetic pathway. The production of delphinidin results in a change in flower colour. The flower product of this application is a shade of lavender, as shown in the photograph below, compared to the white flower colour of the control line that was transformed to generate the transgenic.

Recipient variety (FE 123)

FLORIGENE Moonaqua™ (123.8.12)

The genes that have been inserted are:

- The **petunia *DFR* gene**, coding for dihydroflavonol 4-reductase (DFR), derived from *Petunia* X *hybrida*. The petunia DFR enzyme is only capable of using dihydroquercetin and dihydromyricetin as substrate, not dihydrokaempferol. It preferentially uses DHM over DHQ. This ensures that most of the anthocyanidin produced is delphinidin. The *DFR* gene is driven by its own promoter.
- The **pansy *F3’5’H* cDNA**, coding for flavonoid 3' 5' hydroxylase (F3’5’H), derived from *Viola sp.* F3’5’H acts by converting the dihydroflavonols dihydrokaempferol and/or dihydroquercetin into the dihydroflavonol dihydromyricetin. The cDNA for F3’5’H encodes

the enzyme F3’5’H allowing transgenic plants normally lacking this enzyme to produce violet or blue delphinidin derived pigments.

- ***ALS* gene *(SuRB),*** coding for a mutant acetolactate synthase protein (ALS), derived from *Nicotiana tabacum.* Expression of the mutation confers resistance to sulfonylurea herbicides. The gene is included to allow selection of transgenic shoots *in vitro.*

Anthocyanin profile of flowers

The concentration of delphinidin and other anthocyanidins was determined in flower samples by HPLC. For HPLC analysis freeze-dried petals were extracted with 10 times volume (to wet weight of the petals) 50% acetonitrile containing 0.1 % trifluoroacetic acid. This technique, described in Fukui et.al. (2003), is designed to extract all flavonoids, including the less water soluble isoflavones, flavanones and flavonols (Markham, 1982). Data represents a single assay of bulked petal samples, and is expressed as mg/g fresh weight petal. Roots and stems have not been assayed. There were no anthocyanins in the recipient variety, as it is a white flowered variety. Anthocyanins were produced in flowers from the transgenic line, because of the addition of the anthocyanin biosynthesis genes. There was a predominance of delphinidin over cyanidin (see table immediately below) because of the activity of flavonoid 3’ 5’-hydroxylase (F3’5’H), and a dihydroflavonol 4-reductase (DFR) whose activity is specific to the dihydroflavonol which is a substrate for delphinidin production. The delphinidin in the transgenic flowers of FLORIGENE Moonaqua™ (123.8.12) is likely to be glycosylated, as is the case for two other varieties of genetically modified carnation that have been analyzed in detail using HPLC (Fukui et.al., 2003).

Delphinidin concentration in transgenic carnation line **FLORIGENE Moonaqua™**

	Concentration (mg/g FW)		**Delphinidin**
Name	**Delphinidin**	**Cyanidin**	**(%)**
Recipient	0	0	0
FLORIGENE Moonaqua™	0.07	0.02	74

FLORIGENE Moonaqua™ also produced cyanidin. This is probably due to endogenous flavonoid 3’-hydroxylase activity and/or the activity of the F3’5’H which adds 2 hydroxyl groups to the substrate in a sequential fashion. The presence of cyanidin in flowers of FLORIGENE Moonaqua™ (123.8.12) is not a novel trait in carnation. Non-transgenic pink and red carnation varieties contain this anthocyanidin, and the anthocyanins derived from it. Cyanidin and its derivatives are commonly found in a number of plants including *Petunia* (Ando *et al*., 1999), carnation (Bloor, 1998), rose (Biolley and Jay, 1993), apple (Lancaster, 1992), sunflower seeds (Mazza and Gao, 1993), chrysanthemum (Schwinn *et al*., 1993; Andersen *et al*., 2000), *Vicia villosa* (Catalano *et al*.,

1998) and *Vitis* (Cachio *et al*., 1992). The cyanidin concentration of 0.02 mg/g FW in FLORIGENE Moonaqua™ (123.8.12*)* is 28 – 228 times lower than in the representative carnation varieties shown in the table below.

Cyanidin concentration in representative non-genetically modified carnation varieties. Measurements made by Florigene Pty. Ltd.

Variety	Colour	Cyanidin as % total anthocyanidin	Anthocyanidin (mg/g fresh weight petal)	
			Total	Cyanidin
Artisan	Pink/purple	99.6	0.57	0.57
Blacky	Deep red	99.7	4.57	4.56
Château	Red	99.6	1.89	1.88
Fire Queen	Red	99.5	0.91	0.91
Lavender Picolla	Pink/purple	99.5	0.62	0.62
Orchid Tessino	Pink/purple	100	0.60	0.60
Purple Spectro	Mauve	99.8	1.47	1.47
Vega	Pink/purple	99.4	2.77	2.75

- *Andersen et al., Food Chemistry, 69: 33-36, 2000*
- *Ando et al., Biochemical systematics and ecology, 27: 623-650, 1999*
- *Biolley and Jay, Journal of Experimental Botany, 44: 1725-1734, 1993*
- *Bloor, Phytochemistry, 49: 225-228, 1998*
- *Cachio et al., American J of Ecology and Viticulture, 43: 244-248, 1992*
- *Catalano et al., J. Agricultural and Food Chemistry, 49: 4568-4570, 1998*
- *Fukui et.al. ,Phytochemistry 63 15–23*
- *Lancaster, Critical reviews in Plant Sciences, 10(6): 487-502, 1992*
- *Markham, K.R (1982). Techniques of flavonoid identification. Academic press, London*
- *Mazza and Gao, Phytochemistry, 35: 237-239, 1993*
- *Schwinn et al., Phytochemistry, 35: 145-150, 1993*

A.15 Information on the sequences actually inserted/deleted

(a) Size and structure of the insert and methods used for its characterization, including information on any parts of the vector introduced in the GMHP or any carrier or foreign DNA remaining in the GMHP

The T-DNA between the left and right borders of pCGP1991 remains in the GMO, and is present as three loci. Through Southern Blot analysis it has been shown that no DNA outside the T-DNA borders is present in the GMO. Full details of Southern blot analysis are presented at Attachments A4 and A5.

A schematic of the arrangement of the inserted T-DNA is provided at Attachment A6 and the sequences of the three loci, including flanking sequences, is provided at Attachment A7.

A.15 Information on the sequences actually inserted/deleted

(b) In case of deletion, size and function of the deleted region(s);

Not applicable.

A.15 Information on the sequences actually inserted/deleted

(c) Copy number of the insert

Copy number is described as part of the Southern analysis (Attachments A4 and A5). The T-DNA is present at three integration loci, as summarized in the table below. The arrangement and sequence of T-DNA and flanking sequence at each loci is shown in attachments A6 and A7.

Estimated copy numbers of probes that span the region within the T-DNA in transgenic tissue of the line FLORIGENE Moonaqua™(123.8.12)

Probe	**Estimated Copy Number FLORIGENE Moonaqua™(123.8.12)**		
	Locus 1	**Locus 2**	**Locus 3**
LB	1	0	0
SuRB	1	0	0
F3'5'H	1	1	1
qDFR	1	0	0
RB	1	2	1

A.15 Information on the sequences actually inserted/deleted

(d) Location of the insert(s) in the plant cells (integrated in the chromosome, chloroplasts, mitochondria, or maintained in a non-integrated form) and methods for its determination

The insert is stably integrated into the nuclear genome. This is shown by the Southern blot analysis of the product, described in Attachments A4 and A5.

A.16 Information on the expression of the insert

(a) Information on the developmental expression of the insert during the lifecycle of the plant and methods used for its characterization;

All three inserted genes encode proteins that are biologically active enzymes. Concentration, *Vmax* and *Km* of these proteins have not been measured. Detection of delphinidin-type anthocyanins using TLC indicates expression of the introduced *F3'5'H* and *DFR* genes and enzymatic activity of the enzymes encoded by these genes. Successful isolation of transgenic shoots on selection medium indicates that the introduced *SuRB* encoding an herbicide-resistant ALS is also active. Proteins were thus not detected by direct means such as via gel electrophoresis (they are present in minor amounts and thus not readily detectable by this means). Each of the three introduced genes is a plant gene. Carnations normally carry DFR and ALS encoding genes.

Northern analysis. Northern analysis of expression of three introduced genes was carried out in FLORIGENE Moonaqua™™ (123.8.12) and the parental line it was derived from (FE 123).

In this study, total RNA was extracted from petals from flower petals (stage 9; mature flowers) using an RNAeasy Plant Mini Kit (QIAGEN). RNA samples (10 μg) were electrophoresed through 2.2 M formaldehyde/1.2% w/v agarose gels using running buffer containing 40 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was stained with ethidium bromide and visualised under UV-light. The ribosomal RNA was generally used as a guide in confirming that the RNA had not been degraded by intra- or extra- cellular ribonucleases. The RNA was transferred to nylon membrane filters (Roche) and treated as described by the manufacturer. The RNA blots were probed with DNA probes for *SuRB*, *DFR* and *F3'5'H* radiolabelled using ^{32}P-dCTP. Hybridization was for 16 hours at 42°C and the filters were then washed in a solution of 2 x SSC/ 1 % (w/v) SDS for 3 x 20 minutes and subsequently in 0.2 x SSC/1%SDS for 1 x 20 minutes at 65°C. Membranes were exposed to film overnight (SuRB and F3'5'H) and 7days (DFR). All probes were verified using positive controls (data not shown).

Results are shown in the autoradiogram overleaf.

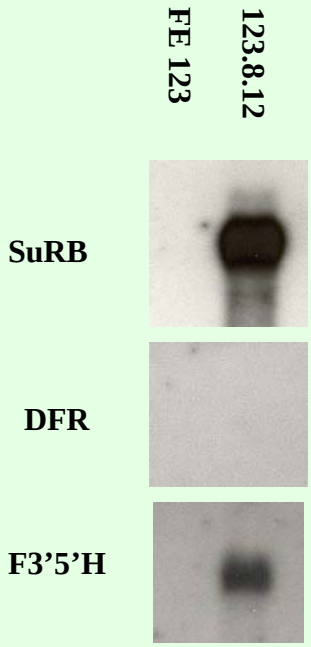
The *SuR*B(ALS) gene is under the direction of a CaMV 35S promoter which generates transcript in numerous plant tissues including the petal. A strong hybridization signal indicates the introduced ALS mRNA is present in petal tissue.

The petunia DFR gene used is under the direction of its own promoter which is relatively

weak and typically strongest early in flower development in petunia. In carnation DFR is typically stronger later in development when compared with petunia. The very low signal detected in FLORIGENE Moonaqua™ flowers is consistent with the relatively low levels of delphinidin and thus the pale flower colour observed. The level of DFR transcript may vary dependent on which flower stage is selected for analysis.

The pansy F3'5'H gene is under the control of an *Antirrhinum* CHS promoter which typically directs expression through most stages of flower development.

The parental line FE123 has no closely homologous ALS gene, is a DFR mutant and has no F3'5'H gene. Hence controls show no detectable transcript for all the probes used.



(b) Parts of the plant where the insert is expressed (for example roots, stem, pollen etc.)

Flowers of FLORIGENE Moonaqua™ (123.8.12) produce delphinidin whilst carnations which are not modified do not. The production of delphinidin results in a change in flower colour. The flower products of this application are lavender, compared to the white control line.

Delphinidin production has not been observed in other tissues of the transgenic flowers and plants, such as stems, nodes, leaves and roots. Production of delphinidin is confined to the petals as result of the use of petal specific promoters for some genes and because the biochemical pathway leading to anthocyanin biosynthesis is induced to coincide with flower development.

A.17 Information on how the genetically modified plant differs from the recipient plant in

(a) **Mode(s) and/or rate of reproduction:** Comparative trials of FLORIGENE Moonaqua™ (123.8.12) and the recipient variety used for transformation have been made, and morphological characteristics of FLORIGENE Moonaqua™(123.8.12) have been measured in detail for Plant Breeders Rights purposes. This data is provided at Attachment A11. In summary, transgenic line FLORIGENE Moonaqua™ produces smaller flowers than the parental line it is derived from, and these flowers have a significantly reduced reproductive capacity, measured by a much reduced number of anthers, styles and stamens. The styles and stamens are also significantly shorter in the transgenic line.

However, there is no avenue of reproduction from imported cut flowers, and the apparent reduced reproductive capacity in the transgenic variety therefore does not imply a reduced rate of reproduction.

(b) **Dissemination:** The environmental risk assessment at Section B provides an assessment of the potential of gene dispersal from imported carnation flowers. On the basis of this background information we conclude that the probability of unintentional gene dispersal from an imported carnation flower is nil, from either recipient or transgenic varieties. There are three theoretical avenues of gene dispersal;

1. Vegetative spread of the imported cut flowers, leading to the formation of wild clonal populations.
2. Formation and dispersal of seed from the imported cut flower as a result of self fertilization or fertilization with pollen from an external source.
3. Formation of seed by a recipient plant, fertilized by pollen dispersed from the imported cut flower.

The paragraphs below briefly outline why the probability of gene dispersal from a carnation flower by any of these routes is nil.

1. Vegetative spread. Carnation does not spread vegetatively, i.e. the plant does not produce organs such as stolons, rhizomes, root-borne shoots, tubers, bulbs, corms or runners. Roots will not form on discarded or old cut-flowers. Florigene Pty. Ltd. has experience of large scale production of carnation in Australia, Japan, Colombia and Ecuador and carnation has never been found growing wild, even in the immediate vicinity of carnation growing areas where waste material has been discarded or has been left for composting.

2. Formation of seed on a cut flower. For gene dispersal by seed formation to occur from a cut carnation flower, the following events would all need to occur successfully; arrival of viable pollen on the stigma of the carnation, pollen germination, pollen tube growth to the ovule of the carnation, fertilization, seed formation and seed dispersal. Notwithstanding the fact that successful pollination of a carnation flower in a vase is highly unlikely, no seed set could occur. This is because the process of seed development takes at least 5 weeks on a plant – where the growth of any developing embryo can be sustained. A cut flower will remain in consumer's hands for three weeks at most before dying.

3. Pollen dispersal from a cut flower leading to a successful hybridization event. There are several mutually exclusive facts that, in combination, indicate that potential pollen spread is not a feasible avenue for gene dispersal.

Firstly, the potential for pollen spread from a cut flower is only theoretically possible;

- In general, production of viable pollen by carnation is much lower than that of wild *Dianthus* species.
- Hybridization of *Dianthus* in nature is facilitated by insect pollination and is only effectively achieved by the *lepidoptera* (butterflies, moths). Pollen is not spread by wind.
- The only point in the chain where insects could be reasonably expected to access flowers is when on display or in consumers hands. The physical barrier of the multiple petals presents a significant obstacle to any potential pollinating insects in less open flowers.
- As a carnation flower opens out in the vase, any anthers are likely to have fallen from the stamens and any pollen would have significantly reduced viability.

The long history of cultivation and consumption of carnation flowers in Europe strongly supports the assertion that there is nil risk of gene dispersal because carnation is not a weed in Europe and 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. No hybrid between carnation and any other *Dianthus* species has ever been recorded in the wild.

(c) Survivability. Imported carnation flowers have no survival mechanisms.

A.18 Genetic stability of the insert and phenotypic stability of the GMHP

Carnations are vegetatively propagated and the genetic stability of carnation can be readily measured by the frequency of flower colour change. A particular variety (red, for example) may "sport" to white sectors in the flower. The primary factor affecting this appears to be the genetic background of the variety.

FLORIGENE Moonaqua™ (123.8.12) has been vegetatively propagated since February 1999. At July 2006, this represented approximately 10 generations of mother plants and approximately 200 collections of cuttings from these plants. Since 2001 plants have been in continuous commercial production in Ecuador and Colombia. By the end of July 2006 approximately 6.7 million flowers have been exported from the two production facilities in South America. Measured by flower colour, FLORIGENE Moonaqua™ (123.8.12) is genetically stable, as shown in the table immediately below.

Number of off-types observed in FLORIGENE Moonaqua™ (123.8.12) flowers in production beds in Colombia

Date of survey	Number of flowers assessed	Number of off-types	Off-type
June 2005	480	0	
September 2005	2,500	0	
June 2006	480	0	
June 2003	1,000	2	White streaks

A.19. Any change to the ability of the GMHP to transfer genetic material to other organisms

There is an extremely low risk of gene dispersal for imported carnation flowers. This is outlined in the environmental risk assessment at Section B, and in answer to question A.17. The imported flowers from the GMHP have a (theoretically) significantly reduced reproductive capacity, due to a reduced number of anthers, styles and stamens.

A.20 Information on any toxic, allergenic or other harmful effects on human health arising from the genetic modification

Carnation has no pathogenic characteristics, and the environmental risk assessment provided in Section B of this application indicates flowers from the GMHP poses no toxic, allergenic or other harmful risks to human health.

Bioinformatic analysis indicates no new ORF are generated at the flanking sequences of any of the three loci. BLAST analysis of the deduced amino acid sequences of the inserted DNA in FLORIGENE Moonaqua™ (123.8.12) showed no homologies to any known toxic or allergenic proteins found in the GenBank and SwissProt databases (see BLAST search analysis results in Attachments A8 and A9). A full analysis of the potential toxicity and allergenicity of the introduced selectable marker gene is provided at Attachment B1.

The transgenic carnation variety FLORIGENE Moonshadow™ has already been approved for commercial production within the EU (C/NL/97/13-1363A) and flowers are already exported into the EU. The potential toxicity issues that were discussed at the time that marketing permission was granted for that variety essentially apply to the present application, except that we are only seeking to import cut flowers.

Results of assays for potential toxicity are provided at Attachments B2, B3 and B4. There was no evidence for toxicity in any assay.

A.21 Information on the safety of the GMHP to animal health, particularly regarding any toxic, allergenic or other harmful effects arising from the genetic modification where the GMHP is intended to be used in animal foodstuffs

The imported cut flowers are intended for ornamental use only. They will not be used for animal foodstuffs.

A.22 Mechanism of interaction between the genetically modified plant and target organisms (if applicable)

Not applicable.

A.23 Potential changes in the interaction of the GMHP with non-target organisms resulting from the genetic modification

The flowers from the GMPH are intended to be used as an ornamental product, in the same way as other carnation flowers. There are no changes in this interaction as a result of the genetic modification.

A.24 Potential interactions with the abiotic environment

There are no potential effects on the abiotic environment. The flowers will be imported from outside of the EU. Flowers will be discarded after use but we have no reason to think their degradation will have any effect on the abiotic environment that could be different to non GM carnation flowers. Measurements were made of the microflora concentration in soil in which the transgenic carnation was grown (Attachment B4). There was no significant difference in the microflora profile of soil taken from around the roots of the recipient and transgenic plant.

A.25 Description of detection and identification techniques for the genetically modified plant

A PCR based identification method is described in detail in Section C of this application. This technique is suitable for use with flowers or vegetative parts of the imported cut flowers.

As the primary purpose of the application is to import flowers, the flowers themselves also provide simple means to identify the GMHP because they have a new colour that is not present in non-GMO carnations. The colour characteristics of the flowers will be emphasised as part of the marketing strategy.

As carnation normally never produces delphinidin-type anthocyanins, presence of these pigments can also be used to identify the genetically modified carnation, if flowers are available. Delphinidin can be identified using thin layer, paper or high pressure liquid chromatography.

A.26 Information about previous releases of the genetically modified plant, if applicable

Details are provided in Section F of this application. FLORIGENE Moonaqua™ (123.8.12) has been grown in trials in the EU. These greenhouse trials were carried out in the Netherlands in 1999 and 2000. FLORIGENE Moonaqua™ (123.8.12) has been grown and sold commercially elsewhere in the world for five years.

SECTION B

Environmental risk assessment required in Annex II of Directive 2001/18/EC

B1. Environmental risk assessments carried out on FLORIGENE Moonaqua™ (123.8.12)

There have been no risk assessments specific to FLORIGENE Moonaqua™ (123.8.12). However, millions of flowers have been traded and sold for several years and there is now a history of safe use of the product.

The transgenic carnation variety FLORIGENE Moonshadow™ , which was also developed using transformation vector pCGP1991, has already been approved for commercial production within the EU (C/NL/97/13-1363A) and flowers are already exported into the EU.

B2. Risk assessment (a) Disease to humans including allergenic or toxic effects

Toxicity of non-GM carnation

The toxicity of non-GMO *Dianthus*/carnation, in the context of human health, is summarized in Table 1 below.

Table 1. Evaluations of the potential toxicity of carnation

Citation	Plant	Summary of toxicity
Russell et al, 1997	*Dianthus* species	Causes only low toxicity if eaten. Skin irritation minor, or lasting only for a few minutes
UCSD, 2001	Carnation	Non-poisonous
UCD, 1998	Carnation	Minor toxicity. Ingestion of small amounts may not cause any symptoms at all. Exposure to sap or skin wound caused by plant material may cause skin rash or irritation
Lister, 2001	Carnation	Regarded as safe
US FDA, 1999	Carnation	Carnation is listed as a poisonous plant

At worst the non-GMO can be considered to have mild toxicity, but it is generally regarded as non-poisonous and safe. Carnation is not a food and there is no toxicology or nutritional data available for the recipient organism.

We do not believe the transgenic flower is likely to be used in the perfume or flavour industries;

- Carnation is not a traditional source of essential oils for the perfume industry.
- Other varieties of carnation would be equally as useful as a source of essential oils, if carnation was considered at all.
- Carnation is not a traditional source of flavor additives for the food industry.
- While the transgenic carnation produces delphinidin, and that is unique for this species, there are other natural sources of delphinidin for use as a food colorant. The main one of these is enocianina, prepared by the aqueous extraction of fresh, deseeded marc (grape residue after pressing for grape juice or wine) or fruit juice (grape, cranberry, chokeberry or elderberry). These sources are much less expensive than flowers, and have a higher concentration of the anthocyanin.

Allergenicity of non-GM carnation

Flowers are not listed with, tree nuts, legumes, fruit, shellfish, eggs, cows milk etc as a common source of food allergy and carnation pollen cannot be a source of hay fever type allergies, as the pollen is not wind-borne. Allergic reactions to carnation are rare. Florigene have close relationships with carnation growers in Australia, Colombia and Ecuador and none of these contacts have experienced allergy problems among their staff. We have found in the literature two reports of allergenicity in Spanish flower farm workers exposed to carnation (Sanchez-Guerrero *et al.*, 1999). In the first study an allergic response was obtained in susceptible workers using carnation extract. A later study indicated that spider mites associated with the carnation were the cause of the response (Cistero-Bahima *et al.*, 2000; Navarro *et al.*, 2001). In a second report Vidal and Polo (1998) carried out control experiments to eliminate the possibility that the allergy was caused by *Tetranychus urticae* (two spotted spider mite), demonstrating in some individuals that prolonged handling of fresh flowers may cause a mild allergic reaction. In the case reported by Vidal and Polo (1998) the subject was handling gypsophila and lily in addition to carnation.

We do not believe imported carnation flowers are likely to enter the food chain;

- It would make no economic sense to purchase flower for large scale use as an animal feed
- The flowers are likely to be purchased for the use they are intended, not for consumption as a food.
- The flowers are intended for home use, and once distributed will be widely spread, in small numbers, in individual hands.
- Used flowers are likely, as a matter of convenience, to be disposed of like other flowers used by the general public, and not fed to animals.

Carnation is not used as a food though there is a slight possibility that home consumers may decide to eat

flower petals, or garnish foods with flower petals. The possibility that someone would consume a large number of carnation flowers is negligible. In the event that this did occur, we do not believe the transgenic carnation poses any health risk and given the purported beneficial effect of anthocyanins to health, may even have a positive effect.

Theoretical potential for any change in toxicity or allergenicity in the GM carnation

The chemical components specific to the colour modified carnation are the DNA comprising the T-DNA of the introduced binary vector, the proteins (enzymes) translated from the introduced genes and the pigment delphinidin, produced by the activity of the enzymes. None of these new components are likely to be toxic or allergenic.

The DNA. We have no reason to believe the DNA *per se* could be a reasonable cause of any increase in toxicity or allergenicity.

The proteins. The proteins (enzymes) translated from the introduced genes are listed in Table 2.

Table 2. Introduced proteins expressed in Florigene Moonaqua™ (123.8.12)

Gene	Protein
SuR B	ALS (acetolactate synthase)
Hf1	F 3’5’H (flavonoid 3'5' hydroxylase)
dfr	DFR (dihydroflavonol reductase)

The proteins encoded by the three inserted genes are common plant proteins and are not toxic or allergenic, nor homologous to known toxic or allergenic proteins (Attachments A8 and A9). They would be degraded in the same way as any protein and the amino acids produced after digestion will be excreted or metabolized in the same way as any other plant protein. The *SuR*B gene is a mutation of an enzyme found in all plants (Attachment B1). As such, the amino acid sequence and protein is virtually the same as that of the enzyme found in all raw plant foods. A detailed review of the potential toxicity and allergenicity of the *SuR*B id given in Attachment B1. The F3'5'H protein is found in all plant foods producing delphinidin. This includes several raw foods containing high levels of delphinidin, normally consumed and handled by humans. The DFR protein is found in all plant foods containing delphinidin, as well as any foods containing the related anthocyanin pigments cyanidin and pelargonidin. Some foods, such as cherries and cranberries have high levels of anthocyanin, and so the enzyme is also present at a high concentration.

Delphinidin. Examples of widely grown ornamental plants that contain delphinidin-based pigments include *Agapanthus* (Bloor and Falshaw, 2000), cyclamen, *Hydrangea* (Takeda *et al*., 1990), verbena, *Petunia* (Ando *et al*., 1999), *Delphinium* (Kondo *et al*., 1991), *Lobelia* (Yoshitama, 1977) freesia,

pansy, and *Hyacinth* (Hosokawa *et al.*, 1995). These plant species are freely sold in Europe as both garden plants and/or cut flowers. The concentration of delphinidin in some common delphinidin containing species is shown in Table 3, and compared to Florigene Moonaqua™ (123.8.12). The levels of delphinidin detected in the transgenic carnation flowers are within the range seen in common, widely cultivated ornamental plants.

Table 3. Delphinidin concentration in some common delphinidin containing species. Data has been generated by Florigene's own analysis, with the exception of Hibiscus (Puckhaber et al., 2002).

	Delphinidin (mg/g FW)	**Delphinidin as % all anthocyanins**
FLORIGENE Moonaqua™ (123.8.12)	0.07	74
Agapanthus	0.12	82
Brachycome	0.75	83
Cineraria	0.96	71
Delphinium	0.52	98
Dampiera	1.64	100
Hibiscus species	1 - 10	Less than 50%
Iris	1.26	100
Rhododendrum	0.14	50
Lisianthus	2.8	90
Pansy	3.9	84
Wisteria	0.39	89

Delphinidin is not known to be a toxic compound, when consumed or when handled. There is no toxicity data in the Merck Index for the aglycone, the mono-glucoside or the 3'5'-glucoside of delphinidin. Anthocyanins have a low acute toxicity of ca. 20,000 mg/kg BW in rodents, and a very low order of toxicity (WHO, 2001) Delphinidin is found in many raw foods - such as red grapes, black currants, egg plant (aubergine), blueberry, elderberry, bilberry and Jalapeno pepper. Major health benefits attributed to anthocyanin consumption include improved cardiovascular health, antiviral capacity and treatment of infection (Broadhurst, 2001). The health properties of anthocyanins, including delphinidin, are further described in Sterling (2001) and Lila (2004).
The concentration of delphinidin we have measured in FLORIGENE Moonaqua™ (123.8.12) is approximately 0.1 mg delphinidin per gram fresh weight petal. This is approximately one-fiftieth the level in blueberry, for example, which may have up to 5 mg anthocyanin per gFW, 40% of which is delphinidin (Kalt *et al.*, 1999). We cannot rule out that petals from the transgenic carnation may be used by some individuals to garnish a meal, and that they may be deliberately consumed as part of this meal. However, it is reasonable to expect just a few petals would be used, which would be comparable to a very small portion of a fruit containing delphinidin.

Allergens are typically proteins. While there is no literature we are aware of reporting studies of the allergenicity of delphinidin, reactions to anthocyanin containing foods have been studied - most people are exposed to at least some anthocyanin in the course of their regular diet. A recent review states that there are no reports of allergic reaction to either grape skin extract or grape colour extract - both of which are widely used food colourants (Lucas *et al*., 2001), and contain delphinidin.

Toxicity and allergenicity of FLORIGENE Moonaqua™ (123.8.12)

There are no Open reading frames present in the flanking sequences.Bioinformatic analysis of the inserted DNA has shown that the transgenic plant FLORIGENE Moonaqua™ (123.8.12) does not contain DNA sequences with homology to known toxins or allergens.

Exposure to FLORIGENE Moonaqua™ (123.8.12) flowers has been highest at the farms, where flowers are produced (Figure 1 below) and treated in post harvest (Figure 2 below).

Figure 1. Harvest of transgenic carnation flowers, in Ecuador

Figure 2. Post harvest grading and sorting of carnations, Ecuador

The transgenic carnation line FLORIGENE Moonaqua™ (123.8.12) has been in commercial production for several years and we therefore have a direct test for allergenicity as a result of the large scale handling that has occurred. Specifically;

- In Ecuador 3.7 million flowers of FLORIGENE Moonaqua™ (123.8.12) have been produced and processed at the farm, and 3.2 million flowers shipped throughout the USA, Canada and Japan.
- In Colombia 4.0 million flowers of line FLORIGENE Moonaqua™ (123.8.12) have been produced and processed by the farm and 3.7 million flowers shipped throughout the USA, Canada and Japan

Zero allergenic or toxic effects have been reported at either the growing location, nor by any other person exposed to the flowers (carriers, wholesalers, retailers or consumers).

Direct measurement of the potential toxicity of FLORIGENE Moonaqua™ (123.8.12) in comparison with the parental variety the line was derived from have been made using an Ames test and an acute toxicity test. Results are shown in Attachments B2 (Ames test) and B3 (acute toxicity test) and indicate no toxic effects.

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B2.Risk assessment (b) Disease to animals and plants

Animal health

Imported cut flowers pose no risk of disease to animals. The conclusion from the review of toxicology and allergenicity provided at question B2(a) suggests that there is no potential for harm, but in any event there would be no opportunity for exposure of animals to significant numbers of flowers. This is because the distribution chain for flowers is separate to animal feed and because this application is for import of cut-flowers only. Lack of production sites in the EU completely rules out any potential for growers to deliver waste plant material as animal feed.

Aqueous extracts from both leaf and petal tissues of FLORIGENE Moonaqua™ (123.8.12) have been used in an acute toxicity assay was conducted in mice (Attachment B3) and there was no evidence of toxicity.

Plant health

Attachment B4 provides reports of experiments in which a plant bioassay was used to study potential phytotoxicity and possible effects on microflora in soil which had been used to grow the GMHP. While these tests are not directly relevant to imported cut flowers, the results indirectly support the contention that there is likely to be no impact on plant health.On the basis of a seed germination and growth assay, there was no evidence that there were any phytotoxic compounds leached into the soil by either the recipient plant or FLORIGENE Moonaqua™ (123.8.12).

B2.Risk assessment (c) Effects on dynamics of populations of species in the receiving environment

The product is imported cut flowers, and the receiving environment is the commercial environment of airports, warehouses, trucks, shops and the home. Discarded flowers will be dead, or soon dead, and have no ability to survive after use. Therefore, the primary consideration in terms of possible effects on populations of other organisms in the EU relates to the potential for gene dispersal from the imported cut flowers. This potential has been thoroughly assessed, and is outlined in Attachment B5. Our analysis of the potential of gene dispersal from carnation flowers imported into the EU has been independently reviewed by Dr. Keith Hammett (488c, Don Buck Road, Massey, Auckland 1008, and New Zealand). Dr. Hammett is acknowledged as an expert on *Dianthus* breeding. He has concluded ***"that the likelihood of gene dispersal from cut flowers of fully double carnations to be highly improbable, if not inconceivable".***

B2.Risk assessment (d) altered susceptibility to pathogens facilitating the dissemination of infectitious diseases and/or creating new reservoirs or vectors

Susceptibility to pathogens is monitored by the growers of the GMHP, who are located in Colombia and Ecuador. The most important pathogens in carnation growing are *Fusarium oxysporum, Fusarium roseum, Pseudomonas, Botrytis* sp and several leaf rusts. The most important insect pests are thrips and spider mites. Based on history of chemical control and the results of scouting in the field, there is no evidence that FLORIGENE Moonaqua ™(123.8.12) is any more susceptible to pathogens than other carnation varieties. Whilst the growers are not growing the recipient plant, the general resistance levels of the transgenic variety is within the normal range for carnation, excluding some varieties that have been bred for high *Fusarium* resistance. The performance of the transgenic variety is that

expected from specifications provided for the recipient variety by the breeder of that variety.
There is therefore no evidence of any increase in disease susceptibility that might lead to a change in pathogen levels, or the creation of new pest reservoirs. This risk is even less relevant in the case of imported cut flowers;

- Flowers are widely dispersed after arrival
- Flowers are dispersed into environments away from areas of commercial carnation production
- Flowers have a short longevity and will die a few weeks after import

There was no significant difference in the microflora profile of soil taken from around the roots of the either the recipient plant or FLORIGENE Moonaqua™ (123.8.12).Cut flowers imported into the EU are subject to pre- and post-entry inspection, and flower producers are very diligent in control of plant pathogens. There is therefore a low risk of introduction of pathogens with the flowers. Even if pathogens were to be imported, these pathogens are the same common fungal and bacterial diseases that infect all carnations.

B2.Risk assessment (e) potential compromise of plant protection treatments, for example by gene transfer

As outlined in answer to question B2(c) above there is no realistic potential for gene dispersal from the imported cut flowers. Therefore, the potential for dissemination of the herbicide resistance gene used as a selectable marker is negligible. However, as background information, we provide here information on herbicide use in carnation production.

<u>Herbicide use in carnation crop management</u>

Herbicides are not widely used in the carnation industry. A few herbicides (e.g. Treflan) may be recommended during production of some *Dianthus* bedding plants in a garden/ open situation (Lerner and Dana, 1994; Smith, 2001), but this is not the same situation as that faced by a professional carnation cut flower grower. An Italian company, Rocca Frutta, markets Oxadiazon for use in carnation, again in an open field situation. This herbicide has much wider efficacy than chlorsulfuron, which is designed for use in cereal crops against broad leaf weeds. Oxadiazon has an even lower toxicity than chlorsulfuron (Cornell, 2001).

<u>Potential for adoption of sulfonylurea in management of the GMHP</u>

There is a nil to negligible possibility that the presence of a sulfonylurea resistance gene in the genetically engineered carnation will lead to adoption of sulfonylurea herbicides by the carnation industry.

- Florigene prohibit use of sulfonylureas in our crops by our contract growers.
- Our current growers do not use herbicide for carnation production in any event - it is simply unnecessary. There is no significant weed problem within the crop, as laborers remove all large weeds within the crop and rapid canopy closure (carnation is planted at a density of 20 – 40 plants per square metre) prevents significant weed growth. The walkways between crops are well worn, preventing any weed growth and plants growing from weed seeds can be manually removed during flower collection, if necessary.
- Sulfonylureas (such as Glean®, Brush-off®) are not designed/ registered for use with ornamentals. Sulfonylureas are not effective against grasses, the major weeds of concern in the industry.
- Soil or soil substrate is usually treated with chemicals as a precaution against fungal disease. This also kills weeds in the initial planting area and new weeds can only be brought in on the wind or by people.

Literature cited

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- *Lerner, BR and Dana MN. Weed control for the garden and landscape. Purdue University Cooperative extension service, Department of Horticulture Rev7/94, 1994*
- *Smith, T. Weed management for outdoor cut flowers. University of Massachusetts Floriculture extension service, 2001*

B2.Risk assessment (f) potential effects on biogeochemistry, particularly carbon and nitrogen cycling

As the products are to be imported as cut flowers, no production related issues relating to fertilizer usage, water usage degradation of waste material and their possible impact on soils, waterways and the atmosphere are relevant. As the GMHPs have similar production requirements as other carnations, any potential impact is, in any event, no different to that of conventional carnation.

Flowers imported into the EU will eventually be discarded in domestic and commercial waste, but the volume of flowers and the fact that the products will be widely dispersed mean the organic mass is negligible.

B2.Risk assessment (g) socio-economic considerations

The socio-economic effects of the introduction of the GMHP will be positive.

Imports into the EU

Flowers from the two carnation varieties already approved for use in the EU have been imported and consumed for several years. Benefits have been associated with increased economic activity that has come with sales activity and our growers have also benefited from an increased exposure to the European market. A general observation is that the sales of the Florigene product have added to total flower sales – i.e. demand has been created by the availability of new colours in carnation. Flow on benefits to European based ancillary services such as graphic artists, floral designers, trade show organizers and advertisers will occur as a direct result of importers needing to market the products. On the basis of imports of 10 million flowers a year of FLORIGENE Moonaqua™ (123.8.12), we estimate approximately 2.5 – 5 million euros in new economic activity will be generated, and that three quarters to two-thirds of this value will remain in Europe.

Experience in other overseas markets

The experience in the US market has been positive.

- Sales of the Florigene product have created an increased demand for carnation flowers, as the availability of new colours has resulted in new uses for the flower, for weddings for example.
- Significant economic activity is generated in the areas of marketing, distribution, postage and advertising.

Other advantages of the introduction of our products have been to illustrate to the general public that there are non-food applications of GM technology. Genetic modification of flowers has been well received, and this is an important benefit for Europe, which is still the home of most of the world's flower breeders.

South America

The production of Florigene flowers in Colombia and Ecuador has been very positive for the two contract growers we work with. Jobs have been created for approximately 75 people at each location, in areas where unemployment is very high. The contracts have given both farms additional financial security at a time when the economies of both countries have been stressed by conversion to US dollar currency in Ecuador, strengthening of the Colombian peso, increased freight rates, inflation and general economic fragility associate with political uncertainty. Suppliers to the growers (fertilizers, chemicals, boxes, sleeves, freight etc.) have also benefited from the increased demand for their services by the two growers.

B3. Conclusions

Key conclusions

- An analysis of the biology of carnation shows no potential for gene dispersal as a result of import of cut-flowers. The flowers are not invasive and there is no opportunity for the cut-flowers to become "weeds". Carnation is not a weed in Europe and 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. No hybrid between carnation and any other *Dianthus* species has ever been recorded in the wild.
- FLORIGENE Moonaqua™ (123.8.12) has a history of safe use outside the EU.
- Field trial results show substantial equivalence between FLORIGENE Moonaqua™ (123.8.12) and the recipient plant, aside from a significantly reduced production of anthers, stamens and styles in the transgenic line.
- There is no evidence that carnation flowers in general, and the transgenic line that is the subject of this application specifically, have any pathogenic, phytotoxic, toxic or allergenic properties.

Specific considerations relevant to GMHPs outlined in Directive 2001/18/EC

1. **Likelihood of the GMHP becoming more persistent than the recipient plant in agricultural settings or more invasive in natural habitats.** The imported carnation flowers will not be used in an agricultural setting, and there is no opportunity for discarded flowers to become established in natural habitats. Survey of areas adjacent to where the GMHP is grown in large numbers in South America has not found any established populations.
2. **Any selective advantage or disadvantage conferred to the GMHP.** The GMHP differs from the recipient in flower colour. Although the introduced delphinidin pigment is not found in any cut flower carnation, it is found in many other plant species such as *Gentiana, Petunia, Centaurea, and Delphinium*. No reports are available that suggest competitive advantages of these plants over non-delphinidin producing plants. This application seeks to import transgenic flowers for which there is a history of use, and no selective advantages have been observed in field trials (measure for example by increased vigour, plant size, or enhanced reproductive ability). Although the GMHP has an herbicide resistance gene, there are no management differences between the GMHP and other carnations, as sulphonylureas herbicides are not used during carnation production.

3. **Potential for gene transfer to the same or other sexually compatible plant species.** There is no potential for gene dispersal from imported cut flowers of the GMHP.
4. **Potential and/or delayed environmental impact resulting from direct and indirect interaction between the GMHP and 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 GMHP and non-target organisms.
5. **Potential and/or delayed effects on human health.** There are no expected short or long term effects on human health. Carnation is not normally a food, and delphinidin is found naturally, and considered to be beneficial, in many ornamental and food species. The genetic modification does not result in production of gene-products whose function is unknown and no potentially toxic compounds have been identified as a result of expression of the genes inserted.
6. **Potential and/or delayed effects on animal health.** The imported products will not be used in animal feed.
7. **Potential effects on geobiochemical processes.** As the products are to be imported as cut flowers, no production related issues relating to fertilizer usage, water usage degradation of waste material and their possible impact on soils, waterways and the atmosphere are relevant
8. **Possible direct and indirect environmental impacts of the specific cultivation, management and harvesting techniques used for the GMHP.** Not applicable. Importation, distribution and use are no different to those used for other carnation flowers.

<u>Possible medium to long term effects</u>

FLORIGENE Moonaqua™ (123.8.12) is genetically stable, measured by flower colour. The variety is vegetatively propagated, and likely to remain genetically stable, based on experience to date. The conclusions on potential impacts on the environment and health are therefore also valid in the medium to long term.

SECTION C
Additional information as required in Annex IV of Directive 2001/18/EC

C1. Proposed commercial names of the products and names of GMOs contained therein, and any specific identification, name or code used by the notifier to identify the GMO

The product is cut flowers of the carnation (*Dianthus caryophyllus*) variety FLORIGENE Moonaqua™ (123.8.12). The product is a standard carnation variety exhibiting modified flower colour. The product also carries a gene encoding resistance to sulphonylurea-type herbicides. This resistance is only important for selecting transgenic lines during the tissue culture process.

FLORIGENE Moonaqua™ (123.8.12) is the trade name for the variety. Plant Breeders Rights have been applied for in Colombia and Ecuador under the name Floricordierite.

Florigene codes and unique identifier numbers for the variety are as shown in the table below.

Trade name	Florigene codes	Unique Identifier number
FLORIGENE Moonaqua™	123.8.12 (also 40689)	FLO-40689-6

The Unique identifier number has been allocated based on the OECD system, and are consistent with Commission regulation (EC)No.65/2004.

C2. Name and full address of the person established in the community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor

Ms. Juliette Gray
Suntory Ltd.
Buchanan House
3 St. James's Square,
London SW1Y4JU,
U.K.
Phone 44-20-7839-9370
Fax 44-20-7839-9379
e-mail juliette.gray@suntory-uk.co.uk

C3. Name and full address of the supplier of control samples

Control samples and samples of transgenic material may be obtained from Florigene, 1 Park Drive, Bundoora, VIC 3083, Australia.

C4. Description of how the product and the GMO as or in the product are intended to be used. Differences in use or management of the GMO compared to similar non-genetically modified products should be highlighted

The product is imported cut flowers. The flowers will be imported in the same way as other carnation cut flowers, by air freight. Flowers are produced in Colombia and Ecuador and will primarily be imported into London, Amsterdam and Frankfurt.

The imported GMO is intended to be use in exactly the same way as other carnation flowers imported from outside the EU. Main use will include;

- Unpacking and repacking for hydration at point of arrival.
- Repacking into bouquets and arrangements at point of arrival.
- Distribution of flowers to wholesale markets, florists and supermarkets
- Consumption by florists in flower arranging
- General purchase by the general public at all outlets where flowers may be sold, such as florists, garages, supermarkets, hypermarkets etc.
- Distribution as gift items between members of the general public

There are no differences in use or management compared to non-genetically modified carnation flowers..

C5. Description of the geographical area(s) and types of environment where the product is intended to be used within the community, including, where possible, estimated scale of use in each area

The imported product is intended to be used anywhere in the community. In practice it is expected flowers will be imported into the Netherlands, United Kingdom, Germany and Scandinavia. Consumption is likely to be in the countries that flowers are imported into and concentrated in the major metropolitan areas. There is likely to be some cross border trade – for example from Germany to Poland and Austria and from the Netherlands to Belgium and France.

The flowers will be used in the same types of environment as other imported cut flowers, including airports, wholesalers, florists, supermarkets, restaurants, weddings, funerals, homes and any other situation where floral decoration is desired.

We estimate annual consumption for the variety will be approximately 25 million flowers eventually. In the first year 1 million flowers are likely to be consumed across the whole EU. Typically, flowers are consumed by many consumers in small numbers. We expect flowers to be sold to 10 – 20 wholesalers, who would then sell smaller numbers (10-100 flowers) to individual florists.

C6. Intended categories of users of the product e.g. industry, agriculture and skilled trades, consumer use by public at large

Flower importers, flower auctioneers, flower wholesalers, distributors, retailers, florists and the general public will use the flowers. FLORIGENE Moonaqua™ (123.8.12) has been consumed in the USA for several years now.

C7. Information on the genetic modification for the purposes of placing on one or several registers modifications in organisms, which can be used for the detection and identification of particular GMO products to facilitate post-marketing control and inspection. *Confidential information that can not be placed on the public register should be identified*

(a) Detail of nucleotide sequences necessary to identify the GMO. A PCR based identification technique is outlined in Attachment C1. This attachment includes the nucleotide sequences necessary to identify the GMO. Whilst the GMHP flowers can be readily identified by their colour, the PCR technique may be used on any tissue.

(b) Methodology for identifying and detecting the GMO product. Refer to Attachment C1.

(c) Experimental data demonstrating the specificity of the methodology. Refer to Attachment C1.

C8.Proposed labelling on a label or in an accompanying document

The labelling strategy is directed to the earlier parts of the supply chain. We do not believe it is feasible and practical to label further along the chain. This is because it is common practice in the industry to use flowers in arrangements, which are often exchanged as gifts. It is unreasonable to guarantee labelling in those types of situations. In the industry it is also usual practice for retailers to remove any packaging used by growers (sleeves for example) before display for retail. This also hampers ability to effectively label.

<u>Written information to distributors</u>

Florigene will carry out the following actions;

In compliance with article 4(1) of EC/1830/EC a letter in English to all importers, listing;

- Commercial name of the product, unique identifier and name of the GMO
- Restrictions on sales of the flowers outside of the EU
- Contact details at Florigene Pty. Ltd. should they want more information
- In compliance with article 4(2) of EC/1830/EC a request to importers that sales by wholesaler and variety are recorded, and are held for five years, and that their customers are required to do the same.

Labels

In compliance with article 6 of EC/1830/EC, products will be labeled. The proposed wording to be used will be;

“These flowers are genetically modified to alter the flower colour and are for ornamental use only”. This wording makes it clear that the carnations are definitely not for feed and food use.

Flowers of a transgenic carnation variety, FLORIGENE Moonshadow™ (C/NL/97/1431363A) are already imported into and sold in the EU. For these flowers, we place a label inside every box or on every sleeve that is shipped to the EU. The wording on this label is:

"These flowers are genetically modified to alter the flower colour and are only produced for use as an ornamental product."

Label formats

Label formats have been chosen that are practical. It is impractical to tag or label individual flowers. Florists would remove them, defeating the objective of labeling.

- **Sleeves.** Sleeves will carry the wording, and will also include the Florigene website address. We accept that sometimes the flowers may not be sold to the general public in the sleeves they were shipped in, and that this is out of our control.
- **The website** will carry unique identifier information and identify the flowers as genetically modified. The Florigene website has been updated, and now provides in English, German and French the commercial name of authorised products, the name of the GMO and the unique identifier. These parts of the website also carry a brief explanation of the genetic modification for colour, contact details at Florigene Pty. Limited (Australia) should they want more information and details of the permit approvals. A links section to the Biosafety protocol and European and another regulatory agencies
- Florigene will provide **accessories** for the use of florists that will carry the Florigene web site address. Examples are florists ribbon and papers.
- **Stickers, tags and gift cards** will be provided to florists for use with the final bouquet arrangements. These will carry the wording, to the effect that some flowers in the arrangement are genetically modified.

Point of purchase materials will be distributed to retail outlets via importers and wholesalers.

Shipping documents

With each shipment of flowers to the EU there will be a manifest (packing list) from the farm, an airway bill and a phytosanitary certificate. Florigene will send an invoice electronically direct to the

importer, from Australia. We have actively been seeking advice from the Australian government, so that if necessary we can modify our shipping documents to be consistent with the requirements of the Biosafety treaty. Our understanding is that there are still no specific documentation requirements, but our intention is to adapt the commercial invoice sent from Australia to include any information required under the treaty.

C9. Measures to take in case of an unintended release or misuse

Through diligent effort, a carnation grower or breeder, or a reasonably well trained horticulturist or amateur gardener could attempt to vegetatively propagate plants from nodes or axillary meristems isolated from the stems of the imported cut flowers. Conceivably, there could therefore be an unintended release or misuse in this context.

Were a professional breeder or grower to attempt illegal propagation Florigene would use the patent protection and PBR protection covering the varieties to prevent production. We would expect the market to alert us to such illegal activities.

In the event of an isolated incident of amateur horticulture, there would be no associated risk, as there is no realistic avenue for gene dispersal in carnation. This is addressed in the environmental risk assessment at Section B, and was already considered at the time Florigene was granted marketing approval for the transgenic carnation varieties FLORIGENE Moondust™, which was approved for commercial production within the EU in 1997 (C/NL/96/14-11) and FLORIGENE Moonshadow™, which was approved for commercial production within the EU in 1998 (C/NL/97/13-1363A)

C10. Specific instructions or recommendations for storage and handling

No specific directions are necessary for the use of the products. The products can be handled and stored according to general practice in handling carnations. FLORIGENE Moonaqua™ (123.8.12) has been consumed in the USA for several years now in exactly the same way as other carnations.

C11. Specific instructions for carrying out monitoring and reporting to the notifier and, if required, the competent authority, so that the competent authorities can be effectively informed of any adverse effect. These instructions should be consistent with AnnexVII part C

The result of the environmental risk assessment, section B of this application, was that there was no significant risk of adverse effects as a result of import of the GMOs. Whilst Florigene have undertaken certain general monitoring steps, described in section D, these are driven by the notifier, and there are no specific instructions required in answer to this question.

C12. Proposed restrictions in the approved use of the GMO, for example where the product may be used and for what purposes.

There are no proposed restrictions.

C13. Proposed packaging

No specific packaging will be used for transport or marketing of flowers. The flowers will be handled according to general practice in handling carnations. Flowers will most likely be imported in cardboard boxes, within which 10 to 25 bunches of flowers will be packed, “sleeved” in plastic flower sleeves for protection. Each sleeve will contain 10 to 15 flowers.

The photographs below show Florigene flowers being packed ready for export to the USA.

Flowers graded for quality and sleeved ready for boxing. The flowers are in hydration solution

Hydrated flowers, still in plastic sleeves, packed dry in cardboard boxes for air freight. Flowers are packed very tightly

Tops of boxes are added, and boxes strapped to secure base to lid for transportation

C14. Estimated production in and/or imports to the community

Consumption will depend on demand and is difficult to estimate as the popularity of flower colour is highly sensitive to changes in consumer's taste. The estimated annual consumption in Europe for FLORIGENE Moonaqua™ (123.8.12) is expected to be between 2.5 and 25 million flower stems.

C15. Proposed additional labeling

There is no proposed additional labelling other than that described in answer to question C8.

C.16 Classification under existing community rules concerning the protection of human health and/or the environment

Carnation is a member of the *Dianthus* genus, and so subject to inspection prior to import into the EU under Directive 2000/29/EC (replaces 77/93/EEC). *Dianthus* is also "subject to contamination" in the same directive as a carrier of bacterial and fungal diseases. Imported cut flower re subject to post entry inspection. Trade in *Dianthus* plant material (not applicable in the present application) requires a "plant passport" (Directive 92/105/EEC) and is subject to Directives 98/56/EC, 93/49/EEC and 1999/68/EC.

SECTION D
Monitoring plan as required in Annex VII of Directive 2001/18/EC

Type of monitoring plan

The monitoring plan described in the submission is based on general surveillance. We believe this is a realistic approach, applicable to the genetically modified carnation lines already approved in Europe, as well as any additional future lines. Once purchased by an end consumer, perhaps in the form of a bouquet, the imported flowers could be used anywhere in the EU, and it is not possible to track the final distribution of all flowers. In reviewing a previous application (C/NL/04/02) in Europe, the GMO panel of EFSA concluded that case-specific monitoring for marketing of colour-modified, genetically-modified carnation was not appropriate (EFSA, 2006). As no potentially adverse affects have been identified for the GM product described in this application, we believe case-specific monitoring is also not appropriate for FLORIGENE Moonaqua™ (123.8.12), as the guideline parameters that are recommended for case specific monitoring do not apply. For example;

- Because there is no avenue for gene dispersal it is not possible to document the spread, persistence and accumulation of transgenes and recombinant proteins.
- There are no apparent organisms, food chains or habitats that are affected by conventional carnation flower imports, and which could therefore be the subject of specific attention for transgenic carnation.
- It is not possible to quantify a baseline environment is the absence of the imported carnation flowers in the floral trade distribution environment and household environment.
- As the flowers will not be grown in the EU, there is no requirement for monitoring of production locations within the EU

Monitoring at production sites

Though Florigene are not required to undertake specific monitoring activity as a condition of approvals to grow and sell FLORIGENE Moonaqua™ (123.8.12) in USA or South America we will continue to monitor the production site areas in South America for the presence of our carnation, particularly in the area where the waste from the carnation production is recycled.. Growers are expected to provide Florigene feedback on;

- Any increase in disease susceptibility
- Any unusual increases in the incidence or type of pests

- Report any adverse reactions to handling the flowers
- Incidence of genetic off types

In addition Florigene staff visit the farms frequently, and personally inspect the areas where flowers are discarded, and the natural vegetation close to the growing areas, for any “escapes”

Traceability

Information collected will be from general surveillance, but the nature of the floricultural business is such that there will be a reasonably accurate record of the flow of flowers to wholesale outlets. This information will be accessible to Florigene, and a database is maintained at Florigene of all individual exports to the EU.

- Florigene will maintain exact records of all exports to Europe – exact number of flowers, customer details, date and arrival airport will be recorded, which can be tallied to independent records of the freight forwarders, importers and airlines.
- Importers will record sales to wholesalers and supermarkets on a variety basis. This is done for them to evaluate the relative commercial success of specific products. The information could be used to identify date and customer name.
- Some wholesalers are very sophisticated and keep bar code systems that will allow the sale of the transgenic carnation to be traced by date, customer and volume. Not all wholesalers have such business systems.

A PCR based test has been developed that will allow the specific identification of the line that is described in this proposal, to support detailed investigation if necessary. This detection method is described in Attachment C1 of this application.

Monitoring methods

Importer feedback

Importers will be asked to monitor their markets for any suppliers selling flowers that resemble the Florigene product. Samples will be collected for analysis if necessary. On a six monthly basis our European importers will be asked for feedback in questionnaire format. The questionnaire is attached at the end of this document.

Consumer feedback

The Florigene web site now has a regulatory link, in German, English and French at which consumers are provided contact details through which to comment on Florigene products.The names and locations of our importer customers are listed on the website.

Expert monitoring group

Florigene has engaged the services of breeders and botanists with interests in *Dianthus* biology. We have asked them to alert us to any unusual hybrids that might find during their routine survey work. We will undertake to carry out molecular analysis and test for resistance to herbicides to eliminate the possibility of transgenic hybrid discovery, if these experts request it. The experts group, as at October 10, 2006 is shown in the table below;

Expert monitoring group; Breeders/botanists

Breeders	
Name	**Address**
Dr. Lin Garland	Floranova Ltd, Norwich Road, Foxley, Dereham, Norfolk, NR20 4SS, United Kingdom.
Dr. Flavio Sapia	Hybrida srl Strada Villetta 19 18038 Sanremo, Italy
Dr. Traude Mundinger	Selecta Klemm GmbH & Co. KG. Hanfäcker 10 70378 Stuttgart, Germany
Botanists	
Name	**Address**
Dr. Domingo Alcaraz Segura	Dpto. Biología Vegetal y Ecología Universidad de Almería La Cañada - E04120 Almería, Spain
Dr. Bengt Oxelman	Department of Systematic Botany, Uppsala University Norbyvägen 18 D SE-752 36, Uppsala, Sweden
Dr. Kit Tan	INSTITUTE OF BIOLOGY Department of Population Biology, Portfløj 1.16 Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark
Dr. Andreas Erhardt	Institut für Natur-, Landschafts- und Umweltschutz Abteilung Biologie Universität Basel St. Johanns-Vorstadt 10 CH-4056 Basel, Switzerland
Prof. Jacqui Shykoff	Laboratoire d'Écologie Systématique et Évolution CNRS UPRESA 8079 Université de Paris-Sud (XI) Bâtiment 360, F-91405 Orsay Cedex, France
Dr. Eleni Maloupa	Balkan Botanic Garden PO Box 458 T.K. 570.01 Thermi Thessaloniki, Greece

Binding agreements/contracts have not been made with the experts as we feel this would discourage

participation in the monitoring activity. Participation of the core group of experts is supported by an annual honorarium of 500 Euro, for which we would expect a brief annual statement on their survey work applicable to the monitoring. The breeders will not be paid. Active participation will be encouraged through contact by phone and email and during visits by Florigene staff to the EU. It is envisaged that the surveys carried out by the contact group would be part of any survey work normally undertaken by the experts, rather than a specific expedition mounted to seek theoretical hybrids. The monitoring parameters will comprise an indication of the locations surveyed, area surveyed and if any *Dianthus* species are identified. Through the contact Florigene will keep with the experts we expect to be advised of the location and timing of surveys and will advise them of known carnation growing areas. Our expectation is that at least one of the primary designated experts would participate in such a survey each year. If not, Florigene will provide support to carry out a survey during one of the summer months in the carnation growing region of southern Spain, in the vicinity of Chipiona, Andalusia. This region has been chosen because of the high density of carnation plants, grown in relatively open conditions, in a region of Europe where several *Dianthus* species do occur. We will undertake to carry out molecular analysis and test for resistance to herbicides to eliminate the possibility of transgenic hybrid discovery, if these experts request it.

Mailing list

Florigene has compiled a mailing list of key herbaria, national botanic survey networks, plant protection services and botanic gardens in Europe, and we will write to these institutions to notify them of the importation of the product into Europe, and alert them to take this into account when reviewing any *Dianthus* collections that may come into their possession.
A copy of the mailing list is provided at Attachment D1.

Annual report

The Florigene office in Australia will collate all information from importers and any comments from consumers. Florigene will provide an annual report to the Dutch CA describing the outcome of the monitoring activity, on the anniversary of the consent date. The report will include;

- Number of flowers imported into Europe in each week of the year, by variety, importer and importer location
- List of surveys carried out by Florigene expert groups with date, location and area surveyed
- All reports of putative *Dianthus* hybrids identified
- All reports of any wild carnation populations
- Response to mail out to herbaria, national botanic survey networks and plant protection services and botanical gradens

- Results of monitoring at production sites in South America

Citation

Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the notification (Reference C/NL/04/02) for the placing on the market of the genetically modified carnation Moonlite 123.2.38 with a modified colour, for import of cut flowers for ornamental use, under Part C of Directive 2001/18/EC from Florigene (Question No EFSA-Q-2005-282). *The EFSA Journal* (2006) 362, 1-19
Opinion adopted on 17 May 2006

Questionnaire

Questionnaire **Number FLORIGENE TO COMPLETE**

As part of the conditions for marketing approval of Florigene varieties in the EU. Florigene are required to monitor for any unexpected effects that may be associated with the import and consumption of our flowers. Your help in completing this questionnaire is very much appreciated. If you tick YES to any question a representative of Florigene will contact you as soon as possible for more details, including variety and circumstances.

Your feedback can be returned to us electronically to Florigene@florigene.com.au or by Fax to +61 3 9416 1761.

Your name **WILL BE COMPLETED BY FLORIGENE**

Your company **WILL BE COMPLETED BY FLORIGENE**

Today's date_____

Are you aware of any reports of illegal growing of Florigene varieties?

YES _____ **NO** _____

Has any of your staff or re-packers reported any adverse or unexpected response to handling Florigene flowers?

YES _____ **NO** _____

Have any of your customers reported to you any adverse or unexpected effects of handling Florigene flowers?

YES _____ **NO** _____

If there any other comments you wish to make, please type here;

SECTION E

SNIF PART 2: Summary information format for products containing genetically modified higher plants (GMHPs)

GENERAL INFORMATION

E.1. Details of notification

(a)	**Member State of notification:** The Netherlands
(b)	**Notification number:** C/NL/06/01
(c)	**Name of the product (commercial and other names):** FLORIGENE Moonaqua™ (123.8.12)
(d)	**Date of acknowledgement of notification:**

E.2. Notifier

(a)	**Name of notifier:** Florigene Pty. Limited (Australia)
(b)	**Address of notifier:** 1 Park Drive, Bundoora, VIC 3083, Australia
(c)	**Is the notifier domestic manufacturer:** No **Importer** Yes
(d)	**In case of an import the name and address of the manufacturer shall be given:** Florigene Pty. Limited, 1, Park Drive, Bundoora, VIC 3083, Australia.
(e)	**Name and full address of the person established in the Community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor;** Ms. Juliette Gray Suntory Ltd. Buchanan House 3 St. James's Square, London SW1Y4JU, U.K. Phone 44-20-7839-9370 Fax 44-20-7839-9379 e-mail juliette.gray@suntory-uk.co.uk

E.3. General Description of the product

(a)	**Name of recipient or parental plant and the intended function of the genetic modification:** Recipient plant is *Dianthus caryophyllus L.* (UK carnation, NL anjer, ESP clavel). The product consists of a carnation variety in which the flowers have a modified flower colour as the result of genes enabling the biosynthesis of delphinidin pigment in the flowers. The flowers also carry an herbicide resistance gene to facilitate selection *in vitro*.
(b)	**Any specific form in which the product must not be placed on the market (seeds, cut-flower, vegetative parts, etc.) as a proposed condition of the authorization applied for:** None
(c)	**Intended use of the product and types of users:** The flower product will be sold in the cut flower market in the same way as other carnation flowers. Users include flower importers, flower auctioneers, flower wholesalers, retailers, and florists. Flowers will ultimately be sold to the general public.
(d)	**Any specific instructions and/or recommendations for use, storage and handling, including mandatory restrictions proposed as a condition of the authorization applied for:** There are no specific requirements.
(e)	**If, applicable, geographical areas within the EU to which the product is intended to be confined under the terms of the authorization applied for:** None
(f)	**Any type of environment to which the product is unsuited:** None
(g)	**Any proposed packaging requirements:** No specific packaging will be used for transport or marketing of the cut-flowers. The flowers will be handled according to general practice in handling carnations.
(h)	**Any proposed labeling requirements in addition to those required by law:** Product information, including written advice and associated labeling and information will be provided with the imported flowers. Proposed wording is; "These flowers are genetically modified to alter the flower colour and are for ornamental use only".
(i)	**Estimated potential demand:** The popularity of flower colour is highly sensitive to changes in consumer's taste. Currently, the estimated annual consumption in Europe is expected to be between 2.5 and 25 million flower stems.

(j) Unique identification codes(s) of the GMO(s):

Trade name	Florigene codes	Unique Identifier number
FLORIGENE Moonaqua™	123.8.12 (also 40689)	FLO-40689-6

E.4. Has the GMHP referred to in this product been notified under Part B of Directive 2001/18/EC and/or Directive 90/220/EEC?

Yes ☐	No ☑

If no, refer to risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC: The transformation experiment resulting in line FLORIGENE Moonaqua™ (123.8.12) was executed by Florigene Europe B.V. in the Netherlands. The plants were propagated and planted in trials in the Netherlands, at the premises of Florigene Europe B.V. under permit number BGGO 95/12-2 (SNIF B/NL/95-012/02).

FLORIGENE Moonshadow™, a carnation variety produced by use of the same transformation vector, has previous marketing approval in the EU (C/NL/97/13-1363A), under directive 90/220/EC.

E.5 (a) Is the product being simultaneously notified to another Member State?

Yes ☐	No ☑

(i) If no, refer to risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC: An environmental risk assessment is provided with the application.

E.5 (b) Has the product been notified in a third country either previously or simultaneously?

Yes ☑	No ☐

(i) If yes, specify: FLORIGENE Moonaqua™ (123.8.12) has previous approval for production in Ecuador, and Colombia and for importation into Canada and the USA.

E.6. Has the same GMHP been previously notified for marketing in the Community?

Yes ☐	No ☑
(i) If yes, give notification number and Member State:	

E.7. Measures suggested by the notifier to take in case of unintended release or misuse as well as measures for disposal and treatment

None.

NATURE OF THE GMHP CONTAINED IN THE PRODUCT INFORMATION RELATING TO THE RECIPIENT OR (WHERE APPROPRIATE) PARENTAL PLANTS

E.8. Complete name

(a)	**Family name:**	Caryophyllaceae
(b)	**Genus:**	*Dianthus*
(c)	**Species:**	*caryophyllus*
(d)	**Subspecies:**	Not applicable
(e)	**Cultivar/breeding line:**	Recipient variety FE123
(f)	**Common name:**	Carnation

E.9 (a) Information concerning reproduction

(i) Mode(s) of reproduction: The cultivated carnation is vegetatively propagated and to produce plants for cut flower production cuttings are taken from vegetative 'mother plants' which are continually pruned to produce a high number of vegetative cuttings from axillary buds. These cuttings are rooted in conditions of high humidity, after treatment with rooting powder.

Rooted plants may be planted in soil or grown hydroponically, and are kept for 1-2 years.

Flowers are produced in flushes, beginning 3-5 months after rooted cuttings are planted.

Picking of <u>all</u> flowers is essential and flowers must be harvested in tight bud (or closed bud for spray types) for distribution and marketing.

Carnation is not reproduced by seed, and seed cannot form during cultivation. Carnation pollen can only be dispersed by lepidopteran insects such as moths. Pollen is not wind dispersed.

(ii) **Specific factors affecting reproduction, if any:** Imported cut-flowers have no capacity for gene dispersal by seed formation or pollen dispersal.

(iii) **Generation time:** Cultivated carnation is grown for 1 to 2 years. The application is for import of cut-flowers only.

E.9 (b) Sexual compatibility with other cultivated or wild plant species

Carnations are double-flowered cultivars and in the general trade and botanical and horticultural literature carnation cultivars are considered to belong to the species *Dianthus caryophyllus.* The common name for *Dianthus caryophyllus* is carnation. However, the exact taxonomic and breeding history of carnation is not known and it is almost certain that carnation is a hybrid involving two or more *Dianthus* species, one of which is *Dianthus caryophyllus*

Whilst there are wild *Dianthus* species in Europe, there is no compatibility between these plants and imported carnation flowers. There is no potential for hybridization. No report exists of spontaneous hybridization between carnation cultivated in Europe and either wild *Dianthus* types or species of other genera.

E.10. Survivability

(a) **Ability to form structures for survival or dormancy:** The survival structures carnation can produce are seeds and pollen, though it is impossible for imported carnation flowers to form seed.

(b) **Specific factors affecting survivability, if any:** Imported carnation flowers will not survive more than 3 weeks in the hands of the consumer. During this time seed set is impossible.

Discarded carnation flowers have no vegetative propagation ability.

E.11. Dissemination

(a) **Ways and extent of dissemination:** Genetic material from cultivated carnation plants could theoretically be disseminated through seed or insect pollination or vegetative propagation. None of these avenues are realistic avenues for gene dispersal in the case of the carnation flowers imported into Europe.

(b) **Specific factors affecting dissemination, if any:** Not applicable.

E.12. Geographical distribution of the plant

The carnation is a cultivated plant and is not found in the wild, but is grown worldwide. In Europe, main production countries are Italy, Spain and the Netherlands. Carnation flowers are imported into the EU from Africa, South America and the middle East. Wild. *Dianthus caryophyllus* is very rare and can only be found in specific coastal regions of Corsica, Sardinia, France and Italy.

E.13. In the case of plant species not normally grown in the Member States(s), description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts

Carnation is cultivated and has no natural habiitat. Carnation flowers are routinely imported into the EU from Africa, South America and the middle East, and are also widely grown in Europe. Several billion carnation flowers are distributed within the EU per annum.

E.14. Potentially significant interactions of the plant with other organisms in the ecosystem where it is usually grown, including information on toxic effects on humans, animals and other organisms

The product is imported cut flowers, and the receiving environment is the commercial environment of airports, warehouses, trucks and shops, and the home. The product will not be grown in Europe. Discarded flowers will be dead, or soon die, have no ability to survive after use and will not enter human or animal food chains.

Carnation has been used safely by humans for ornamental purposes for centuries. The modification in the GMHP (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.

Direct tests of potential toxicity indicate no potential for harm to plant, animal or human health.

There is now a long history of safe use of the products. Carnation is not reported to be a poisonous plant, and there is no evidence that the transgenic line has, or could, cause an adverse reaction.

E.15. Phenotypic and genetic traits

The product consists of imported flowers from carnation plants that have a modified flower colour and are herbicide resistant.

Phenotype

Flower colour is generally the result of the relative concentration and type of two pigment types - carotenoids and flavonoids. Carotenoids are responsible for yellow through orange colours however most plants do not contain carotenoid pigments. Anthocyanins are flavonoid based coloured pigments. There are three groups of anthocyanins, the delphinidins that generally produce blue flower colour, cyanidins that produce red or pink flower colour, and pelargonidins that produce orange or brick red flower colour. Non-genetically modified carnations lack the part of the anthocyanin biosynthetic pathway that is responsible for the production of delphinidin, as they lack a gene encoding the enzyme flavonoid 3’5’ hydroxylase that converts dihydrokaempferol (DHK) to dihydroquercetin (DHQ) and then to dihydromyricetin (DHM). In the genetically modified carnations commercialized by Florigene a gene encoding DFR has also been introduced as the particular non-genetically modified starting material used lacked both F3’H and DFR activity. The enzyme DFR can use either DHK, DHQ or DHM as substrate. Delphinidin is thus produced as a result of the combined expression of the introduced genes DFR and F3’5’H together with endogenous genes in the anthocyanin biosynthetic pathway. The production of delphinidin results in a change in flower colour.

Genotype

Three genes have been transferred;

- The **petunia DFR gene**, coding for dihydroflavonol 4-reductase (DFR), derived from *Petunia* X *hybrida*. The petunia DFR enzyme is only capable of using dihydroquercetin and dihydromyricetin as substrate, not dihydrokaempferol. This ensures that most or all of the anthocyanidin produced is delphinidin. A constitutive promoter drives the petunia *DFR-A* cDNA derived gene.
- the **pansy F3’5’H gene,** coding for flavonoid 3' 5' hydroxylase (F3’5’H), derived from *Viola sp.* F3’5’H acts by converting the dihydroflavonols dihydrokaempferol and/or dihydroquercetin into the dihydroflavonol dihydromyricetin. The cDNA for F3’5’H encodes the enzyme F3’5’H allowing transgenic plants normally lacking this enzyme to produce violet or blue delphinidin derived pigments.
- ***ALS gene (SuRB),*** coding for a mutant acetolactate synthase protein (ALS), derived from *Nicotiana tabacum.* Expression of the mutation confers resistance to sulfonylurea herbicides.

INFORMATION RELATING TO THE GENETIC MODIFICATION

E.16. Description of the methods used for the genetic modification

Genetic material was inserted into carnation by *Agrobacterium*-mediated transformation using the disarmed *Agrobacterium tumefaciens* strain AGL0 carrying the transformation vector pCGP1991, developed by Florigene Pty. Limited, Bundoora, Australia.

E.17. Nature and source of the vector used

The transformation vector pCGP1991, developed by Florigene Pty. Limited, Bundoora, Australia.

E.18. Size, source of the vector used

Position (nt)	Genetic element	Origin	Function
27119-571 (27444)	LB	*Ti* plasmid *A. tumefaciens* Octopine strain	Defines junction between T-DNA and plant genomic DNA. Utilized in transfer of insert to the plant.
572-579	polylinker	pBluescript/pUC, E. coli	Residual sequences from vectors used in assembling transformation vector.
580-770	35S promoter	Cauliflower Mosaic Virus	Constitutive promoter in plants*.
771-831	*Cab 5'utr*	*Petunia* X *hybrida*	Chlorophyll a/b binding protein cDNA 5' untranslated region (utr)
832-4596	*SuRB* (ALS)	Tobacco, *Nicotiana tabacum*	Encodes Acetolactate Synthase. Chlorsulfuron-resistance gene with terminator. Chlorsulfuron is only used during the tissue culture process8.
4597-4861	polylinker	*pBluescript/pUC series E. coli vectors*	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
4862-9819	DFR genomic clone	*Petunia* X *hybrida*	Encodes the dihydroflavonol reductase protein with its own promoter and terminator; a key enzyme in the anthocyanin biosysnthesis pathway. The gene is comprised of 6 exons and 5 introns (see map)*.
9820-9847	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
9848-11019	CHS promoter	*Antirrhinum majus*	Flavonoid pathway promoter from a gene encoding chalcone synthase.
11020-11063	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
11064-12841	F3'5'H cDNA	*Viola* sp.	Encodes the flavonoid 3'5'hydroxylase protein. A key enzyme in the anthocyanin biosynthesis pathway leading to the biosynthesis of delphinidin*.
12842-12854	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
12855-13673	'D8' terminator	*Petunia* X *hybrida*	Terminator sequence from petunia 'D8', a gene encoding a phospolipid transfer homologue.
13674-13860	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
13861-15693	RB	*Ti* plasmid *A.tumefaciens* Octopine strain	Defines junction between T-DNA and plant genomic DNA. Utilized in transfer of insert to the plant*.

Position (nt)	Genetic element	Origin	Function
15694-15703	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.
15704-23618	pVS1 replicon	*Pseudomonas aeruginos*	For replication in *A. tumefaciens*. This is a broad spectrum replicon, which allows plasmid replication in a wide range of bacteria. Includes flanking sequences either side of origin of replication*.
23619-25591	Tetracycline resistance gene complex	*Escherichia coli*	Used for the selection of bacteria carrying the transformation vector. This DNA has a known function and encodes a membrane associated protein that prevents tetracycline from entering bacterial cells.
25592-27118	Modified pACYC184 replicon	*Escherichia coli*	This low copy replicon allows replication in *E. coli* only. Includes flanking sequences either side of origin of replication*.

INFORMATION RELATING TO THE GMHP

E.19 Description of the trait(s) and characteristics, which have been introduced or modified

Flowers of the genetically modified carnation product of this application produce delphinidin whilst carnations which are not modified do not. The production of delphinidins results in a change in flower colour. The flower products of this application are a shade of light mauve, compared to the cream-white flowers of the line from which the transgenic line was derived.

E.20 Information on the sequences actually inserted/deleted/modified

(a) Size and structure of the insert and methods used for its characterization, including information on any parts of the vector introduced in the GMHP or any carrier or foreign DNA remaining in the GMHP:

The T-DNA is 27,444 base pairs. The size and structure of the inserts have been analyzed by Southern blot analysis and T-DNA between the left and right borders of pCGP1991 remains in the GMHP. Through Southern Blot analysis it has been shown that no DNA from outside the T-DNA borders is present in the GMHP and that the introduced DNA is present as three loci.

No carrier (*Agrobacterium*) remains in the GMHP.

(b) In the case of deletion, size and function of the deleted region(s):

Not applicable.

(c) Location of the insert in the plant cells (integrated in the chromosome, chloroplast, mitochondrion, or maintained in a non-intergrated form) and method for its determination:

The insert is integrated into the plant chromosome.

(d) Copy number and genetic stability of the insert: The T-DNA is present at three integration loci, as summarized in the table below.

Estimated copy numbers of probes that span the region within the T-DNA in transgenic tissue of the line FLORIGENE Moonaqua™(123.8.12)

Probe	Estimated Copy Number FLORIGENE Moonaqua™(123.8.12)		
	Locus 1	**Locus 2**	**Locus 3**
LB	1	0	0
SuRB	1	0	0
F3'5'H	1	1	1
gDFR	1	0	0
RB	1	2	1

FLORIGENE Moonaqua™ (123.8.12) has been vegetatively propagated since 1999. Since 2000 plants have been in continuous commercial production in Ecuador and Colombia. Measured by flower colour, the line is genetically stable.

(e) In case of modification other than insertion or deletion, describe function of the modified genetic material before and after modification as well as direct changes in expression of genes as a result of the modification: Not applicable.

E.21 Information on the expression of the insert

(a) Information on the expression of the insert and methods used for its characterization:

Expression of the insert has been determined by the presence of delphinidin-type pigments using TLC and HPLC techniques. Flowers of the product contain approximately 0.1 mg delphinidin per gram fresh weight, determined by HPLC.

Northern analysis of expression of three introduced genes was carried out in FLORIGENE Moonaqua™ (123.8.12) and the parental line it was derived from (FE 123). The *SuR*B(ALS) gene is under the direction of a CaMV 35S promoter which generates transcript in numerous plant tissues including the petal. A strong hybridization signal indicates the introduced ALS mRNA is present in petal tissue.The petunia DFR gene used is under the direction of its own promoter which is relatively weak and typically strongest early in flower development in petunia. The pansy F3'5'H gene is under the control of an *Antirrhinum* CHS promoter which typically directs expression through most stages of flower development.

The parental line FE123 has no closely homologous ALS gene, is a DFR mutant and has no F3'5'H gene. Hence controls show no detectable transcript for all the probes used.

(b) **Parts of the plant where the insert is expressed (e.g. roots, stem, pollen, etc):** Flowers of the genetically modified carnation product produce delphinidin whilst carnations which are not modified do not. The production of delphinidin results in a change in flower colour. Transgenic flowers are light mauve, compared to the cream-white flowers of the control line. Delphinidin production has not been observed in other tissues of the transgenic flowers and plants, such as stems, nodes, leaves and roots. Production of delphinidin is confined to the petals as result of the use of floral specific promoters for some genes and because the biochemical pathway leading to anthocyanin biosynthesis is induced to coincide with flower development.

E.22 Information on how the GMHP differs from the recipient plant in:

(a) **Mode(s) and/or rate of reproduction:** There no avenue of reproduction from imported cut flowers of either recipient or GMHP.

(b) **Dissemination:** There are three theoretical avenues of gene dispersal from an imported carnation flower;

1. Vegetative spread of the imported cut flowers, leading to the formation of wild clonal populations.
2. Formation and dispersal of seed from the imported cut flower as a result of self fertilization or fertilization with pollen from an external source.
3. Formation of seed by a recipient plant, fertilized by pollen dispersed from the imported cut flower.

The probability of gene dispersal from a carnation flower, of recipient or GM origin, is negligible to nil.

(c) **Survivability:** Imported flowers of the GMHP have no greater ability to survive than flowers from any other carnation variety, including the recipient.

(d) **Other differences:** The primary difference between FLORIGENE Moonaqua™ (123.8.12) and the recipient plant is in the colour of the flowers, because of the production of delphinidin in the GMHP. The transgenic line FLORIGENE Moonaqua™ (123.8.12) produces smaller flowers than the parental line it is derived from, and these flowers have a much reduced number of anthers, styles and stamens. The styles and stamens are also significantly shorter in the transgenic line.

E.23 Potential for transfer of genetic material from the GMHP to other organisms

There is an extremely low risk of gene dispersal for imported carnation flowers. The imported flowers from the GMHP have no enhanced ability to transfer genetic material.

E.24 Information on any harmful effects on human health and the environment, arising from the genetic modification

Carnation has been used safely by humans for ornamental purposes for centuries. The modification in the GMHP (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. Carnation is not reported to be a poisonous plant, or to cause allergic reactions, and there is no evidence that the transgenic line has, or could, cause an adverse reaction. There is now an extensive history of safe use of the product overseas. Carnation is not used as a food but there is a slight possibility that some home consumers may decide to eat flower petals, or garnish foods with flower petals. In the event that this did occur we do not believe the transgenic carnation poses any health risk because the novel products in the GMHP are found naturally in many foods. Open reading frame analysis of introduced regions of DNA reveals that the deduced amino acid sequences of the transgenic carnation lines in this application appear not to be homologous to any known toxic or allergenic proteins. Direct tests of potential toxicity indicate no potential for harm to plant, animal or human health.

There is no potential for gene dispersal. The settings 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 effect on the environment.

E.25 Information on the safety of the GMHP to animal health, where the GMHP is intended to be used in animal feedstuffs, if different from that of the recipient/parental organism(s)

Not applicable. The product is not intended to be used as animal feed.

E.26 Mechanism of interaction between the GMHP and target organisms (if applicable), if different from that of the recipient/parental organism(s)

Not applicable. There are no target organisms.

E.27 Potentially significant interactions with non-target organisms, if different from the recipient or parental organism(s)

The flowers from the GMPH are intended to be used for human consumption as an ornamental product, in the same way as other carnation flowers. There are no changes in this interaction as a result of the genetic modification.

E.28 Description of detection and identification techniques for the GMHP, to distinguish it from the recipient or parental organism(s)

The GMHP can be distinguished using DNA based identification methods, such as Southern analysis and PCR. A PCR based identification technique has also been developed that will allow the product to be distinguished from other transgenic carnation lines.

Flower colour can be used to distinguish the product from the recipient plant, and biochemical tests such as thin layer chromatography may be used to determine that delphinidin is produced. The product, but no non GM carnation variety, is able to produce delphinidin.

INFORMATION ON THE POTENTIAL ENVIRONMENTAL IMPACT FROM THE RELEASE OF THE GMHP

E.29 Potential environmental impact from the release or the placing on the market of GMOs (Annex II, D2 of Directive 2001/18/EC), if different from a similar release or placing on the market of the recipient or parental organism(s)

There is no environmental impact from the placing on the market of the GMHP which would be different to that of placing flowers from the recipient plant on the market. Carnation flowers from many varieties, including the recipient, are a commodity in the EU, and several billion non-GM carnation flowers are consumed per annum in the community. There is no evidence the products would have any adverse

effects;

- An analysis of the biology of carnation shows no potential for gene dispersal as a result of import of cut-flowers. The flowers are not invasive and there is no opportunity for the cut-flowers to become "weeds". Carnation is not a weed in Europe and 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. No hybrid between carnation and any other *Dianthus* species has ever been recorded in the wild.
- FLORIGENE Moonaqua™ (123.8.12) has a history of safe use outside the EU.
- Field trial results show substantial equivalence between FLORIGENE Moonaqua™ (123.8.12) and the recipient plant, aside from a significantly reduced production of anthers, stamens and styles in the transgenic line.
- There is no evidence that carnation flowers in general, and the transgenic line that is the subject of this application specifically, have any pathogenic, phyto-toxic, toxic or allergenic properties.

The transgenic carnation variety FLORIGENE Moonshadow™ has already been approved for commercial production within the EU (C/NL/97/13-1363A). This variety was developed with the same transformation vector as FLORIGENE Moonaqua™ (123.8.12).

E.30 Potential environmental impact of the interaction between the GMHP and target organisms (if applicable), if different from that of the recipient or parental organism(s)

Not applicable. There are no target organisms.

E.31 Possible environmental impact resulting from potential interactions with non-target organisms, if different from that of the recipient or parental organism(s)

(a) **Effects on biodiversity in the area of cultivation:** Not applicable. The products are cut flowers and will not be cultivated.

(b) **Effects on biodiversity in other habitats:** Not applicable. The imported cut flowers have no means to become established in any habitat.

(c) **Effects on pollinators:** Not applicable. Imported cut flowers are very unlikely to come into contact with pollinators in the environment in which they will be used.

(d) Effects on endangered species: Not applicable. The imported cut flowers have no means to become established, and will be consumed in the human household environment in the same way as other carnation flowers.

INFORMATION RELATING TO PREVIOUS RELEASES

E.32 History of previous releases notified under Part B of the Directive 2001/18/EC and under Part B of Directive 90/220/EEC by the same notifier

(a) Notification number: The variety FLORIGENE Moonaqua™ (123.8.12) has not been notified. However, similar products have been approved under Part B of directive 90/220/EEC and two varieties of these approved GM carnation have been imported into the EU;

Trade name	Plasmid	OECD ID No. (Unique Identifier)	EU approval & registration No.
FLORIGENE Moondust™	pCGP1470	FLO-07442-4	C/NL/96/14-11
FLORIGENE Moonshadow™	pCGP1991	FLO-11363-1	C/NL/97/13-1363A

(b) Conclusions of post-release monitoring: Production sites overseas have been monitored for escapes from cultivation of the GMHP and none have been found. There have been no reports from growers and consumers of the product relating to harmful effects on human health.

(c) Results of the release in respect to any risk to human health and the environment (submitted to the competent authority according to Article of Directive 2001/18/EC):

The product has been released in Colombia, Canada, USA and Australia. There have been no reports from growers and consumers of the product relating to harmful effects on human health.

E.33 History of previous releases carried out inside or outside the Community by the same notifier

(a) Inside the community:

The transformation experiments resulting in lines FLORIGENE Moonaqua™ (123.8.12) were executed by Florigene Europe B.V. in the Netherlands. In 1998 the first plants flowered under a contained use permit. Trials were carried out in the summers of 1999 and 2000 under permit number BGGO 95/12-02.

(b) Outside the community:

USA

FLORIGENE Moonaqua™ (123.8.12) has been sold in the USA since July 2001.

Canada

Flowers from FLORIGENE Moonaqua™ (123.8.12) have been imported into Canada in small numbers since August 2002.

Colombia

A resolution permitting commercial release was issued in May 2000. Production of FLORIGENE Moonaqua™ (123.8.12) began in mid-2000 and first flowers were exported from June 2001.

INFORMATION RELATING TO THE MONITORING PLAN- IDENTIFIED TRAITS, CHARACTERISTICS AND UNCERTAINTIES RELATED TO THE GMO OR ITS INTERACTION WITH THE ENVIRONMENTAL THAT SHOULD BE ADDRESSED IN THE POST –COMMERCIALIZATION MONITORING PLAN

E.34 Information relating to the monitoring plan- identified traits, characteristics and uncertainties related to the GMO or its interaction with the environmental that should be addressed in the post – commercialization monitoring plan

Transgenic carnation, and specifically the carnation line that is the subject of this proposal, now has sufficient history of safe use to support the fact that the biology of the crop precludes gene dispersal and dissemination from transgenic carnation at either production locations or after import of flowers.

- In trials and a period of commercial production in Europe, no observations were made to suggest that GMHP behaved any differently to non-genetically modified carnation.
- Transgenic carnation flowers have been imported into the EU on a virtually weekly basis for the past 18 months. We have received no reports from the users of these flowers to suggest any features or characteristics that would require further monitoring.
- Several hundred thousand plants of FLORIGENE Moonaqua™ (123.8.12) have been grown in South America since 2000, and several million flowers produced. Surveys of the production

sites have found no evidence of dissemination from outside of the cultivation area and there have been no adverse effect reports from any of the workers handling the plants or flowers.

- Several million flowers of FLORIGENE Moonaqua™ (123.8.12) have been exported to the USA and Japan with no reports of adverse effects on distributors or end users.
- There is experience of growing and selling two similar transgenic carnation varieties within the EU, without any reports of adverse effects.

A general monitoring plan has therefore been proposed for this product. The environmental risk assessment indicates no risks associated with the import of the GMHP and that issues associated with imports are the same as non GM carnation flowers. As the flowers will not be grown in the EU, there is no requirement for monitoring of production locations within the EU.
Information collected will be from general surveillance, rather than collection of specific data, or tracing products to end use.

SECTION F
Information about previous releases of the genetically modified plant

F.1 Details of release of similar GMOs inside of the community

Two similar GMO varieties have been approved for marketing approval in the EU under directive 90/220/EEC, and are licensed until October 2006. Flowers of these two varieties are currently imported into the EU, but plants are not grown in the EU. Details of the two varieties are shown in the table below.

Trade name	Florigene code	Plasmid	OECD ID No. (Unique Identifier)	EU approval & registration No.
FLORIGENE Moondust™	Vio-lin 11, or 7442	pCGP1470	FLO-07442-4	C/NL/96/14-11
FLORIGENE Moonshadow™	Vio-11363, or 1363A	pCGP1991	FLO-11363-1	C/NL/97/13-1363A

C/NL/96/14 and C/NL/97/13 included several other lines, but these have not been commercialized. There have been no identified concerns associated with the commercial release of these two varieties in terms of potential harm to the environment, plant health, human health or animal health.

- **C/NL/96/14. Issued December 1997.** This approval covered four transgenic carnation lines modified by co-cultivation with *A. tumefaciens* carrying pCGP1470. Commercial production of two varieties was carried out in Spain and in the Netherlands. Flowers were sold through wholesalers in the Netherlands and retailed in France, Netherlands and Germany. Flowers are currently exported from Ecuador primarily to the UK and Netherlands, with small numbers to Sweden and Germany.
- **C/NL/97/13. Issued October 1998.** This approval covered six transgenic carnation lines modified by co-cultivation with *A. tumefaciens* carrying with pCGP1991. pCGP1991 is a binary transformation vector very similar to pCGP1470, except for the source of the flavonoid 3'5' hydroxylase gene, and some regulatory elements. Commercial production of a single line was carried out in Spain. Flowers were sold through wholesalers in the Netherlands and retailed in France, Netherlands and Germany. Flowers are currently exported from Ecuador primarily to the UK and Netherlands, with small numbers to Sweden and Germany.

The carnations of the present application differ from FLORIGENE Moonshadow™ in the shade of

flower colour and flower type (standard-type vs. midi-type).

Florigene Pty. Ltd. has also applied for import into Europe of flowers from the genetically modified variety FLORIGENE Moonlite™. This application (C/NL/04/02) was made under Directive 18/2001 and is currently under consideration in the EU.

F.2 Details of contained release of FLORIGENE Moonaqua™ (123.8.12) in the EU

The transformation experiments resulting in line FLORIGENE Moonaqua™ (123.8.12) were executed by Florigene Europe B.V. in the Netherlands. A timetable of activity is presented in the table below.

Timeline of development of 123.8.12	Date
Transformation	October 1997
Transfer to soil	January 1998
First flower collected	August 1998
Field trial 1 (Florigene Europe and commercial grower close to Florigene Europe)	Planted February 1999 Completed Sep 1999
Field trial 2 (Florigene Europe)	Planted December 1999 Completed Sep 2000

In 1998 the first plants flowered under a contained use permit. The plants were propagated and planted in trials in the Netherlands, at the premises of Florigene Europe B.V. Trials were carried out in the summers of 1999 and 2000. Cuttings were transferred from Holland to Australia and South America in 2000, following which the facility in the Netherlands was closed.

Field trial work was carried out under permit number BGGO 95/12-2 (SNIF B/NL/95-012/02). This permit has now been cancelled.

F.3 Details of release of FLORIGENE Moonaqua™ (123.8.12) outside the Community

FLORIGENE Moonaqua™ (123.8.12) has been released commercially in Canada, Colombia, Ecuador and the USA. Plants have been grown and flowers traded and sold, in the same ways as non-genetically modified carnation.

Neither the products themselves, nor similar products, have been withdrawn anywhere in the world due to safety concerns.

The GMOs that are the subject of this application have been vegetatively propagated since 1999 and as of early September 2006, approximately 8.6 million FLORIGENE Moonaqua™ (123.8.12) flowers

have been produced in South America. Most flowers have been consumed in North America. There have been no environmentally negative effects from the release of the variety. Florigene's decision to grow most of our crop in Ecuador and Colombia has been economically beneficial to the economy of those two countries.

USA

In February 1997, APHIS-USDA de-regulated any carnation variety transformed using the transformation vector pCGP1991. Flowers transformed with pCGP1991may be imported in to the USA under normal market conditions with no special packaging or labelling requirements.

The USA government was provided with a detailed description of FLORIGENE Moonaqua™ (123.8.12) as supplementary information in late 2000, and was provided additional information in February 2001. In line with the expected requirements of the Biosafety Treaty USDA-APHIS were advised in March 2001 of the unique identifier for 123.8.12, and the variety that this designated.

FLORIGENE Moonaqua™ (123.8.12) has been sold across the USA since December 2000.

Canada

The Canadian Government has determined that carnation flowers from carnations transformed using *A. tumefaciens* carrying pCGP1470 or pCGP1991 are not a plant health risk. Accordingly, as is the case in the USA, flowers are imported into Canada under the same conditions as non-modified carnations. Flowers from line FLORIGENE Moonaqua™ (123.8.12) have been imported into Canada in small numbers since August 2002.

Colombia

Florigene has worked with the regulatory agency in the Ministry of Agriculture in Colombia since October 1998. As the regulations were developed in Colombia, Florigene lodged an application for general release of colour-modified carnation (transformed using *A. tumefaciens* carrying pCGP1470 or pCGP1991). A resolution permitting commercial release was issued in May 2000. Commercial production of FLORIGENE Moonaqua™ (123.8.12) began in mid 2000. Flowers have been exported from Colombia since June 2001, and there is no special labeling or packaging conditions.

Ecuador

After review of data submitted in April 1997 the government of Ecuador approved, for growing and flower export under commercial conditions, the release of any carnation genetically transformed using *A. tumefaciens* carrying pCGP1470 or pCGP1991. Permission was granted in August 1997.

Production of FLORIGENE Moonaqua™ (123.8.12) began in early 2000 and first flowers were exported from November 2000. There are no special labeling or packaging requirements.

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C/NL/06/01

ATTACHMENTS

ATTACHMENT A1

PCR analysis for presence of residual *Agrobacterium*

Introduction

We present evidence here for the absence of residual *Agrobacterium* (herein after means "*A. tumefaciens* AGL0") in the transgenic carnation line FLORIGENE Moonaqua™ (123.8.12). The method used is highly sensitive (PCR-based) and specific to the strain used in the original transformation experiments. As controls we have included non-transgenic carnation material, *Agrobacterium* and a 10^3-fold dilution of *Agrobacterium* in the presence of non-transgenic carnation material. Our results show a complete absence of detectable *Agrobacterium* in the transgenic line 123.8.12 (FLORIGENE Moonaqua™).

Methods

The aim of this experiment was to use a PCR assay to look for the presence or absence of the *Agrobacterium* strain AGL0 (Lazo *et al.*, 1991) in glasshouse grown transgenic material by specific amplification of a *Vir*G fragment. The glasshouse material is many cutting generations from the primary transgenic. The *Vir*G gene (Jin *et al.*, 1987) is located on the modified *Ti* plasmid (tumour inducing genes deleted) resident in *A. tumefaciens* strain AGL0 used to transform carnations to generate line FLORIGENE Moonaqua™ (123.8.12). It thus represents an *A. tumefaciens* AGL0 marker.

A 1 mm stem section was taken from each of 15 glasshouse-grown cuttings. The stem sections were pooled for each line and DNA extracted as described by Edwards *et al* (1991). Plant tissue was ground using a disposable grinder in a 1.5mL Eppendorf tube with 400 µL extraction buffer [200 mM Tris-Cl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS]. The sample was mixed using a vortex mixer prior to centrifugation for 5 min at 14,000 rpm in an Eppendorf bench top microfuge. 300 µL of the resultant supernatant was transferred to a fresh 1.5 mL tube and the DNA precipitated by addition of an equal volume of isopropanol and the precipitate pelleted by centrifugation for 10 min at 14,000 rpm. The DNA pellets were dried and resuspended in 100 µL TE (10mM Tris-Cl, pH 8.0, 0.1 mM EDTA).

Total DNA will include any contents of the *Agrobacterium* cell and is comprised of chromosomal and extra-chromosomal (Ti and cryptic plasmids) DNA. *Agrobacterium* (AGL0) DNA used as a positive control was sourced directly from a 30^{o}C overnight culture grown in LB medium (1% (w/v) Tryptone, 1% (w/v) NaCl, 0.5% (w/v) Yeast Extract, 0.01M Tris, pH 8.0). The cell density of the culture was determined by an absorbance reading at 620 nm and using the cell concentration of 1 OD unit being equivalent to $5x10^8$ cells/mL as described by Lin *et al* (1994).

The sequences of the amplification primers used herein are as follows:
*Vir*G-F1 5'-TGG ACA CTT AAT CTC AGG CAA CG-3'
*Vir*G-R1 5'-TGA AGA GGG ACC TAT CGG AAC C-3'
The expected size of the amplification product was ~400bp.
The amplification reaction contained the following components – 1x Qiagen PCR buffer, 200 µM of each dNTP, 125 ng of the *Vir*G-F1 primer, 125 ng of the *Vir*G-R1 primer, 2.5 Units HotStarTaq DNA polymerase (Qiagen) and 1µL of DNA template.

The amplification reactions begin with an initial DNA template denaturation and enzyme activation of 15 min at 95^{o}C followed by 35 cycles of 1 min at 94^{o}C, 1 min at 55^{o}C, 1.5 min at 72^{o}C and then a final extension of 10 min at 72^{o}C. Fragments were resolved through a 1% (w/v) agarose gel using a running buffer containing 1 x TAE (Sambrook et al., 1989).

Results

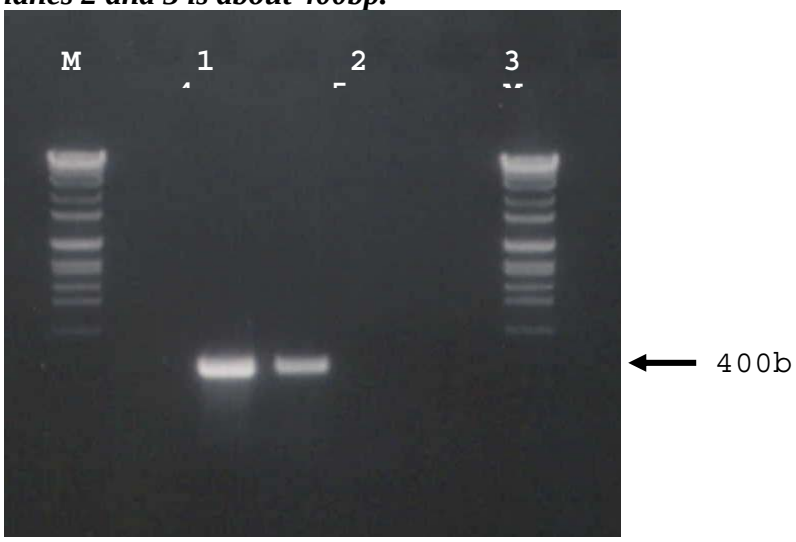
Results are shown in Table 1 and Figure 1.

Table 1. Results of PCR using DNA template in extracts of carnation and Agrobacterium.

Reaction #	DNA Sample	Product	Interpretation
1	negative control, no DNA	-	No contamination
2	1.2x10^{5} cells AGL0	+	*Vir*G detectable in cultured bacteria
3	~100 cells AGL0 + NTG control DNA	+	*Vir*G detectable in DNA sample spiked with ~100 AGL0 cells
4	NTG control DNA	-	*Vir*G/AGL0 absent
5	123.8.12	-	*Vir*G/AGL0 absent

NTG = non-transgenic; + = product detected; - = no product detected.

Figure 1. Scanned photograph of UV illuminated EtBr stained PCR products resolved in 1% (w/v) agarose. Products are from reactions detailed in Table 1. Outside lanes (M) contain EcoRI/SPP-1 DNA size markers. EtBr = ethidium bromide. The large band in lanes 2 and 3 is about 400bp.



Conclusions

The transgenic carnation line FLORIGENE Moonaqua™ (123.8.12) was generated using *Agrobacterium*-mediated transformation (Lu et al, 1991). To address the question of whether any residual *Agrobacterium* remained in plants a sensitive PCR-based assay was developed for the detection of *Agrobacterium*-specific DNA sequences. The carnation plant material sourced was a representative cutting of the transgenic line FLORIGENE Moonaqua™ (123.8.12) and, as a control, non-transgenic parental material.

Agrobacterium tumefaciens AGL0, in common with other strains used in plant transformation harbours a Ti plasmid which carries the virulence genes the products of which facilitate processing,

transfer and integration of T-DNA. The *A. tumefaciens* strain AGL0 is derived from strain A281 for which Ti plasmid sequence data is available (Jin et al, 1987). The *Vir*G gene encodes a transcription factor which is responsible for activation of the 'Vir operon' and we selected this gene to design primers for the ready detection of *A. tumefaciens* AGL0.

The strategy employed was to investigate whether the *Vir*G gene was present or absent in a range of samples. We have confirmed that the *Vir*G primers generate product of the expected size when template derived from *A. tumefaciens* AGL0 was used. Template derived from a sample of the transgenic line and the parental line failed to generate any PCR product using *Vir*G primers. A near 10^3 fold dilution of the *Agrobacterium* culture used as a positive control (estimated to represent ~100 bacterial cells) illustrates (i) the sensitivity of the assay, and (ii) that the presence of carnation DNA template did not inhibit generation of the expected PCR product.

Based on the above results we have concluded that no residual *A. tumefaciens* AGL0 is present in the carnation material used in this study. As the sample is representative of plant material currently grown for flower production worldwide it is reasonable to conclude that carnations in production are free of residual *A. tumefaciens* AGL0.

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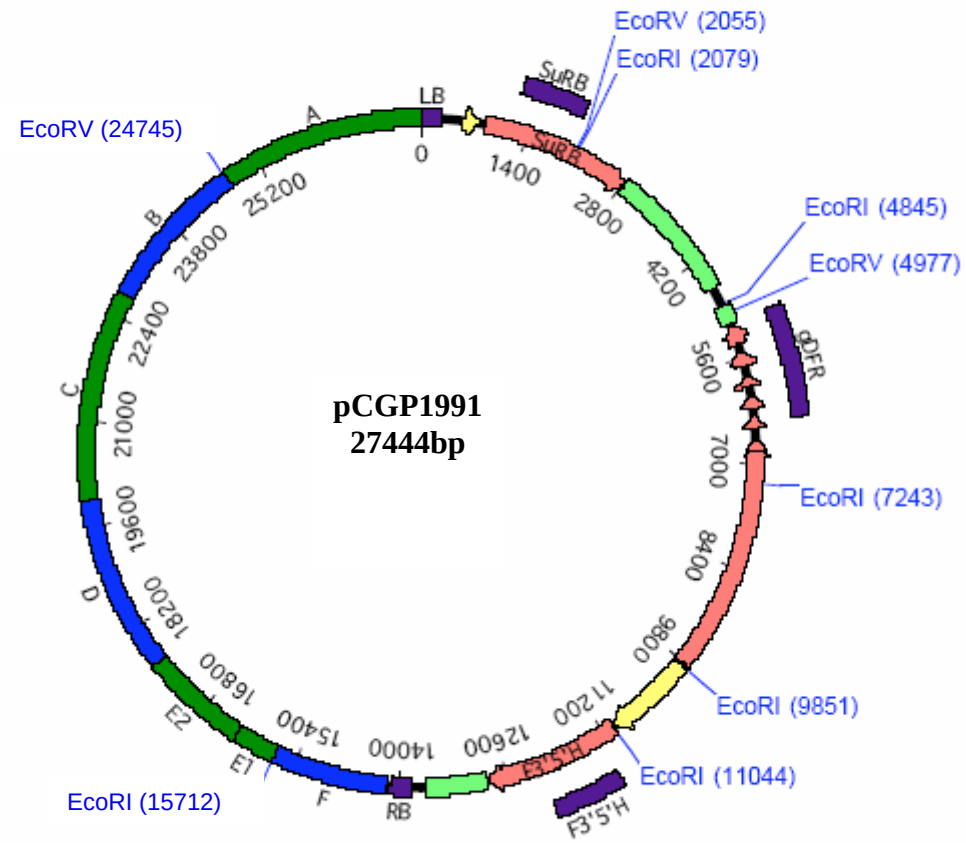
ATTACHMENT A2

Map of transformation vector pCGP1991 and details of its construction

A map of transformation vector pCGP1991, showing key restriction enzyme sites is shown in Figure 1. Figures 1a and 1b show details of the construction of the transformation vector.

Figure 1
A restriction endonuclease map of the pCGP1991 transformation vector, the three genes transferred in the T-DNA and the probes used in Southern analysis



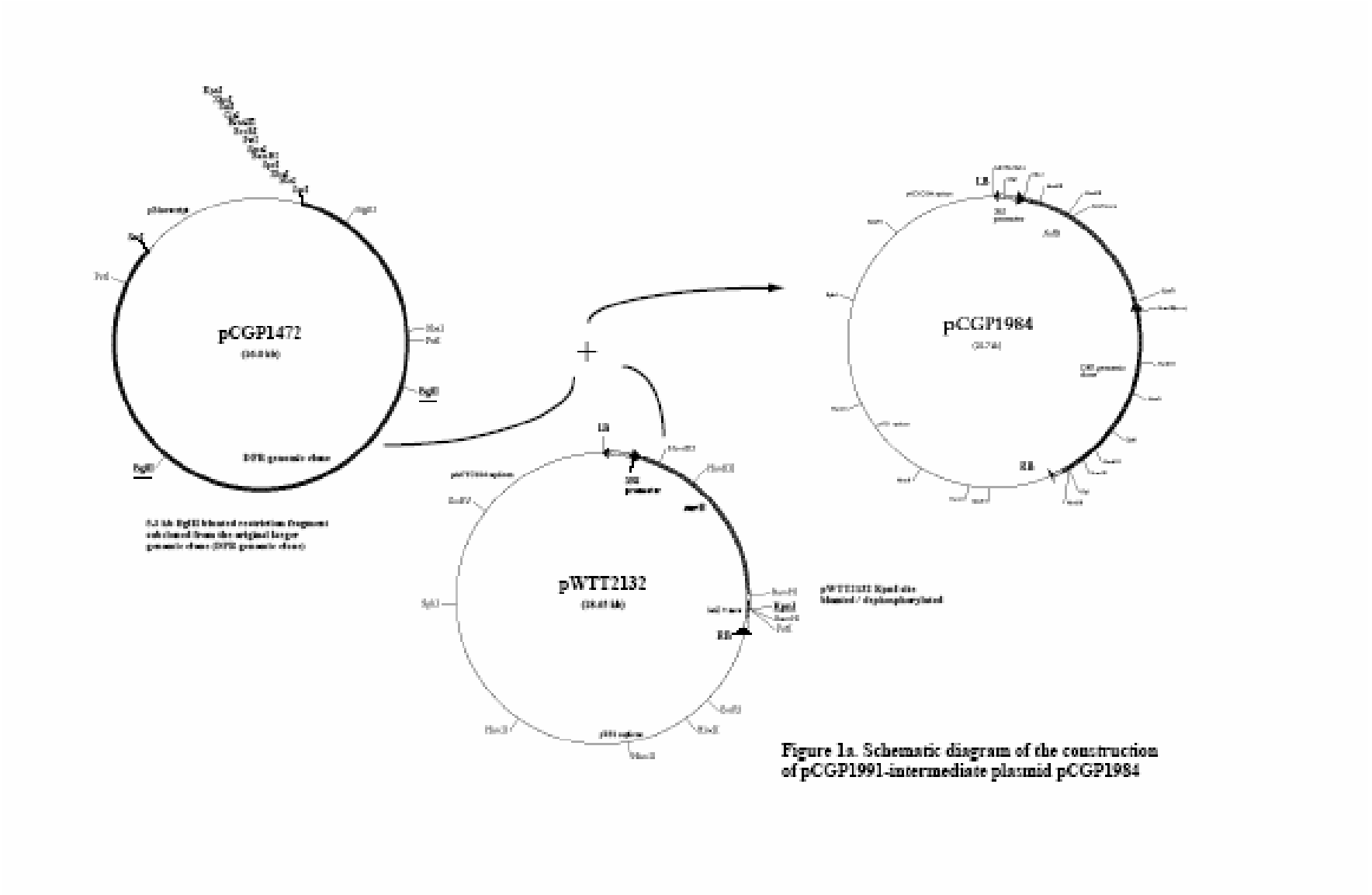


Figure 1a. Schematic diagram of the construction of pCGP1991-intermediate plasmid pCGP1984

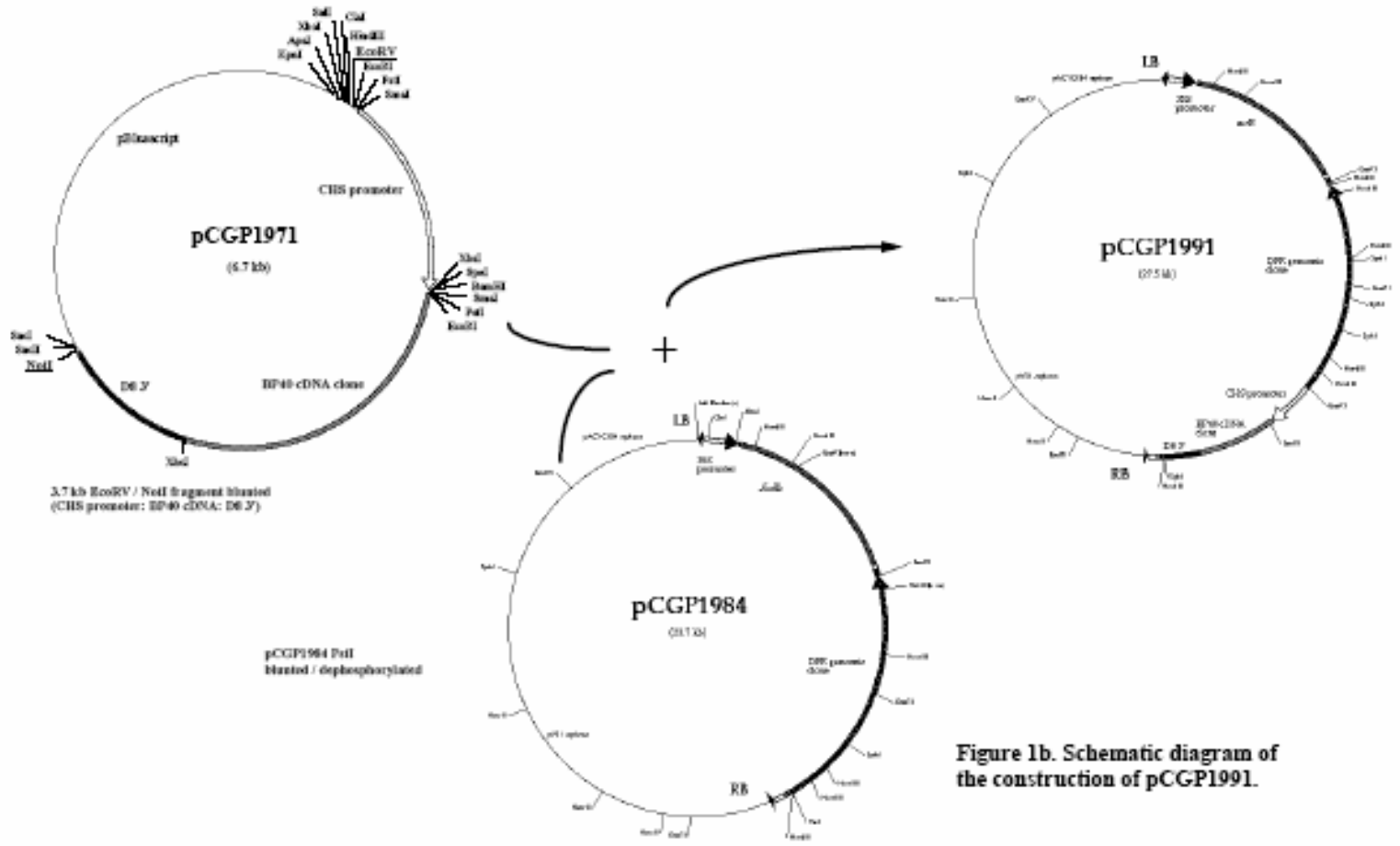


Figure 1b. Schematic diagram of the construction of pCGP1991.

ATTACHMENT A3

Sequence of transformation vector and origin and function of genetic elements

Table 1 lists the genetic elements of pCGP1991. The annotated sequence in Figure 1 identifies the position of the nucleotides numbered in the table below.

Table 1. pCGP1991; Genetic elements, origin and function

Position (nt)	Genetic element	Origin	Function	Encompasses open reading frame	Citations
27119-571 (27444)	LB	*Ti* plasmid *A. tumefaciens* Octopine strain	Defines junction between T-DNA and plant genomic DNA. Utilized in transfer of insert to the plant.	No	Ref (16, 18)
572-579	polylinker	pBluescript/pUC series *E. coli* vectors	Residual sequences from vectors used in assembling transformation vector.	No	Ref (14, 17)
580-770	35S promoter	Cauliflower mosaic virus	Constitutive promoter in plants.	No	Ref (4)
771-831	Cab 5'utr	*Petunia* X *hybrida*	5' untranslated region (utr) of the Chlorophyll a/b binding protein gene	No	Ref (5)
832-4596	*SuRB* (ALS)	Tobacco, *Nicotiana tabacum*	Encodes Acetolactate Synthase. Chlorsulfuron-resistance gene with terminator. Chlorsulfuron is only used during the tissue culture process.	Yes	Ref (12)
4597-4861	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
4862-9819	*DFR* genomic clone	*Petunia* X *hybrida*	Encodes the dihydroflavonol reductase protein with its own promoter and terminator; a key enzyme in the anthocyanin biosysnthesis pathway. The gene is comprised of 6 exons and 5 introns .	Yes	Ref (2)
9820-9847	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
9848-11019	*CHS* promoter	*Antirrhinum majus*	Flavonoid pathway promoter from a gene encoding chalcone synthase.	No	Ref (15)
11020-11063	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
11064-12841	*F3'5'H* cDNA	*Viola* sp.	Encodes the flavonoid 3' 5'hydroxylase protein. A key enzyme in the anthocyanin biosynthesis pathway leading to the biosynthesis of delphinidin.	Yes	Ref (7, 8, 19)
12842-12854	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
12855-13673	'D8' terminator	*Petunia* X *hybrida*	Terminator sequence from petunia 'D8', a gene encoding a phospholipid transfer protein.	No	Ref (6)
13674-13860	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
13861-	RB	*Ti* plasmid	Defines junction between T-DNA and plant	No	Ref (16, 18)

15693		*A.tumefaciens* Octopine strain	genomic DNA. Utilized in transfer of insert to the plant.		
15694-15703	polylinker	pBluescript/pUC series *E. coli* vectors	Residual pBluescript and/or pUC series vector DNA used for making the construct. Cloning & ligation sites.	No	Ref (14, 17)
15704-23618	pVS1 replicon	*Pseudomonas aeruginos*	For replication in *A. tumefaciens*. This is a broad spectrum replicon, which allows plasmid replication in a wide range of bacteria. Includes flanking sequences either side of origin of replication.	No	Ref (9, 10, 11)
23619-25591	Tetracycline resistance gene complex	*Escherichia coli*	Used for the selection of bacteria carrying the transformation vector. This DNA has a known function and encodes a membrane associated protein that prevents tetracycline from entering bacterial cells.	Yes	Ref (1)
25592-27118	Modified pACYC184 replicon	*Escherichia coli*	This low copy replicon allows replication in *E. coli* only. Includes flanking sequences either side of origin of replication.	No	Ref (3, 13)

Information on donor organisms

Scientific & other names

Scientific name	Other Name
Agrobacterium tumefaciens	crown gall bacterium
Escherichia coli	"E.coli"
Petunia X hybrida	petunia
Viola sp.	pansy
Pseudomonas aeruginosa	pseudomonas
CaMV	Cauliflower mosaic virus
Nicotiana tabacum	tobacco
Antirrhinum majus	snapdragon

Pathogenic characteristics of the donor organism

Organism	Characteristics
Agrobacterium tumefaciens	The donor organism causes crown gall disease in a number of plant species. Short sequences from each border sequence in pCGP1991 are transferred from the donor organism to the plant.
Escherichia coli	Specific strains of the donor organism can cause serious infection in human beings. The donated sequences in pCGP1991 are not involved in the pathogenic characteristics of the bacteria.
Viola sp.	None
Pseudomonas aeruginosa	Some *Pseudomonas* species are associated with treatable infection in humans (e.g. urinary tract infection).
Petunia	None
CaMV	The Cauliflower mosaic viruses (CaMV) are responsible for a number of diseases in several important crops. Cauliflower Mosaic Virus infects a number of cruciferous crops, reducing productivity, yield and quality. A promoter sequence from CaMV has been used to drive the expression of one of the introduced genes.
Tobacco	None
Antirrhinum majus	None

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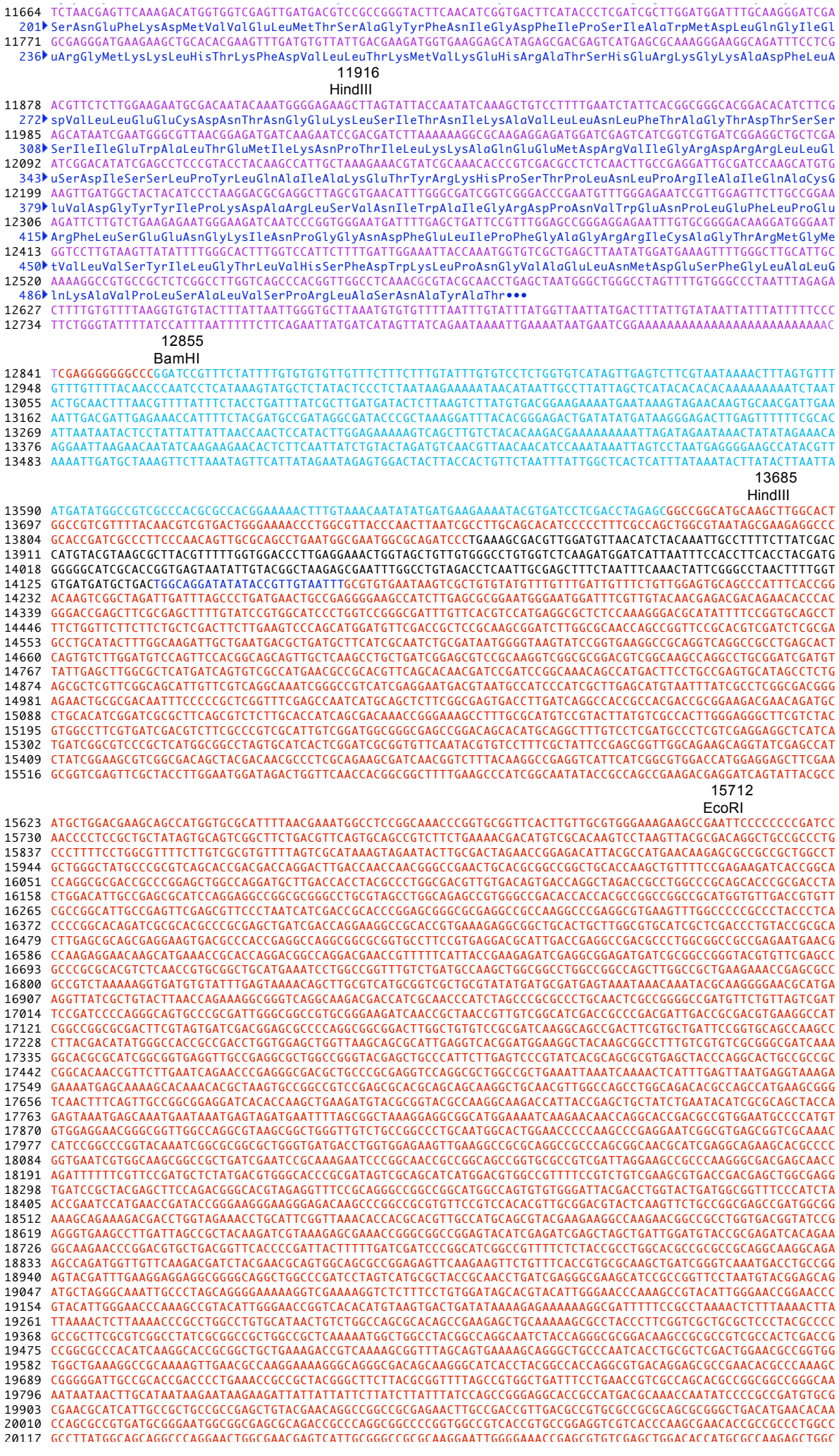
Figure 1 (following pages). Annotated sequence of transformation vector pCGP1991. Sequence is coloured as an aid to reading.


1196
HindIII
1961
HindIII
2055
EcoRV
2079
EcoRI
2223
XbaI
3987
XbaI
4597
XbaI
4603
BamHI
4845
EcoRI
4877
HindIII
4977
EcoRV
5109
HindIII
5138
XbaI


6446
HindIII
7243
EcoRI
8984
HindIII
9387
HindIII
9829 XbaI
BamHI 9835
9851
EcoRI
11014
XbaI
11026
BamHI
11044
EcoRI


11916
HindIII
12855
BamHI
13685
HindIII
15712
EcoRI

```
20224 CGACGCCTACGAAACCCAGGCAACCGAGCTTGACCAGGTGCGGGCCGCCGCCGAAGCGGCCGACCAGGCGCACCAGGTACAAAGTCAGCAGGTCGCCGGCGAGCTGG
20331 CCAGCGTGCGGACAGAATTGGCCCAGGCTGTCACCAGGGCCGAACGTGCGGAAGCGAAGGCCGACGAGATCGAGCACCGTGCCCGCGAGCTGCGGGCCGAGCTGGAC
20438 AGGGCACACCAGGACGCCGACCAGGCGCGGGCTGCCGTGACCGATGCGCAGCAGGCTACCCAGGCAGTGACCGCGCAACTCGACCAGGTACGCGCCGAACTGGCGAC
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20652 AACGCGACCAGGCCAGAGAGGAAGCCGGCAGCGCCCGCGAGCGAGCCGCCGAGCTGGCCGGCCAGCTCACCGCACACCAGGAACAGGCGGCCGCGTTGCTGGCACGC
20759 CTGGCACCGGCCGAAACCAAAACACCCAGGGCGAAGAAGTAGGGCGGTGGATTGGTCTGGCGGTAGTGATTGAAGTGTCAACAAAGAGCCGCTCCGCCCCTCAAGTG
20866 TCAGTAGGCCGCCGCTATGGCGAGGGGCTGATAGCGACCGGCGTTTTTAGGTGTTCATTGACACTTGAGGGGCGGGGTGTACCGCCTCTTTCCTTGGCGTCCAACCT
20973 TGTTTTTTTGGGCGGTCATGTGGCCGTGGAAAGCATGGGGAAAGTGGTGTTTCCTCGTTGTGGAAACAGCAAGGAAATCGTTGCTTTCTTGTGCAAAGCAGAGTTTT
21080 GGAAAATTCGATTAAACCGCGATTTTGGCACGGTTTAATGCCGATTTAAGCCCGTTTTTCCCGCTGTAGAGCCCCTATTAGGCCGATTACAGGCCGCTTTAACCCGA
21187 TTGCATGGCAATCGGTATGCGATAGCGCATGAATTGCACAAGAATCCAACTGCTGTACTATGAGTATTCGAGGCTGAATAGCGGGCAGGATGTGTGAAGTAGGCCCA
21294 CCCGTAAACGGGTTGTCCTTCTTCACTTCCCCTTATTCGCACCTGCGGTGCTCAACGGGAATCCTGCCCTGCGGGGCCAGCCGGCAGCGCCGGCCAAAGAGACGATA
21401 GGAGGGCTTGCCGATGGCGGCAGAGAACGACAAACCAACGCGCAAGGGCAGCCCGCCGATCAAGGTGTATTGCCTGCCCGAAGAGCGGGCCGAGATCGAGGCGCTGG
21508 CGAAGGCCAGCGGCCACAGCGCATCGACTTATTTGCGCTTGGTTGGGCAGGGCTACCAGGTCACCGGCGTGACCGATTACGAGAACGTGCGCGAGCTGGTGCGCGTG
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22471 GACCGTCAAGCCAAGCACGCTTGGGCGGGATCTATCCAAGCCAGCTTTGGAAAAACGGCTGGGCACTTTTGAGCCGTCGCCCGAGCGGCAGGCAGAGCCGAAACGGC
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23434 CGAGGGCGCGAGCATCCCAACGCCGAAGCCTGCACCGATTCCGGCAATCAATGACCAGGAACGGCGAAAAGCGAAGCTGCAAGCCGAGTTGAACGCCAAGAAAGAGA
23541 AGGCGAAACAGCGGAAAGGGGGAGGGGATGTTGTCTACATGGCTCTGCTGTAGTGAGTGGGTTGCGCTCCGGCAGCGGTCCTGATCAATCGTCACCCTTTCTCGGTC
23648 CTTCAACGTTCCTGACAACGAGCCTCCTTTTCGCCAATCCATCGACAATCACCGCGAGTCCCTGCTCGAACGCTGCGTCCGGACCGGCTTCGTCGAAGGCGTCTATC
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24611 GTCTCGCTGGCCGGCGCCACTGTGGACTACGCCATCATGGCGACAGCGCCTTTCCTTTGGGTTCTCTATATCGGGCGGATCGTGGCCGGCATCACCGGGGCGACTGG
                                  24745
                                EcoRV
24718 GGCGGTAGCCGGCGCTTATATTGCCGATATCACTGATGGCGATGAGCGCGCGCGGCACTTCGGCTTCATGAGCGCCTGTTTCGGGTTCGGGATGGTCGCGGGACCTG
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25467 TAACAACGTGGAACGGGTGGGCATGGATTGCAGGCGCTGCCCTCTACTTGCTCTGCCTGCCGGCGCTGCGTCGCGGGCTTTGGAGCGGCGCAGGGCAACGAGCCGAT
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                        25698
                       XbaI
25681 GATAAATTGCACTGAAATCTAGAAATATTTTATCTGATTAATAAGATGATCTTCTTGAGATCGTTTTGGTCTGCGCGTAATCTCTTGCTCTGAAAACGAAAAAACCG
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27179 TCAAATACTTTGCGATCGTGTTGCGCGCCTGCCCGGTGCGTCGGCTGATCTCACGGATCGACTGCTTCTCTCGCAACGCCATCCGACGGATGATGTTTAAAAGTCCC
27286 ATGTGGATCACTCCGTTGCCCCGTCGCTCACCGTGTTGGGGGGAAGGTGCACATGGCTCAGTTCTCAATGGAAATTATCTGCCTAACCGGCTCAGTTCTGCGTAGAA
                                                  27444
27393 ACCAACATGCAAGCTCCACCGGGTGCAAAGCGGCAGCGGCGGCAGGATATAT
```

ATTACHMENT A4

Southern blot analysis – *Eco*RI

An electronic file is included with this Attachment; **MAEcoRISURBhighres.TIF**

Introduction

Genomic DNA isolated from transformed and non-transformed lines was probed using a number of fragments spanning the entire transformation vector (pCGP1991) to identify integrated sequences and indicate the complexity of the integrated DNA and copy number of the introduced genes. Our aim was to compare transgenic lines with the recipient line using Southern blot analysis based on probes covering the entire transformation vector used to engineer FLORIGENE Moonaqua™ (123.8.12). Genomic DNA was analysed in two sets of experiments, one in which DNA was digested with the restriction endonuclease *Eco*RI, and one in which DNA was digested with the restriction endonuclease *Bgl*II (see Attachment A5).

Experimental rationale

Petal material analyzed was collected from plants of each line grown in Melbourne, Australia. Petal material was frozen and stored at –70°C. Petal samples of the non-transgenic control were taken from plants also grown in Melbourne. Genomic DNA isolated from carnation line FLORIGENE Moonaqua™ (123.8.12) was hybridized with probes from inside, on and outside the T-DNA borders, comprising the entire transformation vector pCGP1991 (see Figure 1). Twelve different probes were used. By hybridizing Southern blots with each of the probes our purpose was to:

- Estimate transgene copy number,
- Determine whether extra-border sequences from the pCGP1991 transformation vector have been integrated into the carnation genome, and
- Confirm that genomic DNA isolated from the transgenic carnations does not interfere with hybridization of the probes with any homologous sequences.

Methods

In general, the methods followed were as described in:

- Molecular Cloning: A Laboratory Manual, Sambrook *et al.* (1989),
- Plant Molecular Biology Manual (2nd edition), Gelvin and Schilperoot (eds), Kluwer Academic Publisher, The Netherlands, 1994
- Plant Molecular Biology Labfax, Croy (ed), Bios scientific Publishers, Oxford, UK, 1993.

The cloning vectors pBluescript and pCR script were obtained from Stratagene, USA. The cloning vector pCR2.1 was obtained from Invitrogen, USA.

***(i) Isolation and purification of DNA fragments* -** Fragments were generally isolated on a 1% (w/v) agarose gel and purified using the QIAEX II Gel Extraction kit (Qiagen) or Bresaclean Kit (Bresatec, Australia) following procedures recommended by the manufacturer.

(ii) Polymerase Chain Reaction (PCR) - Unless otherwise specified, PCR conditions using plasmid DNA as template iused 2 ng of plasmid DNA, 100 ng of each primer, 2 µL 10 mM dNTP mix, 5 µL 10 x Taq DNA polymerase buffer, 0.5 µL Taq DNA polymerase in a total volume of 50 µL. Cycling conditions comprised an initial denaturation step of 5 min at 94ºC, followed by 35 cycles of 94ºC for 20 sec, 50ºC for 30 sec and 72ºC for 1 min with a final treatment at 72ºC for 10 min before storage at 4ºC. PCRs were performed in a Perkin Elmer GeneAmp PCR System 9600.

(iii) DNA Sequence Analysis - DNA sequencing was performed using the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by

the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). Sequencing runs were generally performed by the Australian Genome Research Facility at The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) or in-house on an automated 373A DNA sequencer (Applied Biosystems).

(iv) Preparation of genomic DNA and Southern blots - Total plant genomic DNA was isolated from leaf or petal tissue essentially as described by Dellaporta et al. (1983). The DNA preparations were further purified by CsCl buoyant density centrifugation (Sambrook et al., 1989).

The genomic DNA (5 µg) was digested for 16 to 48 hours with 120-200 units of the restriction endonuclease *Eco*RI and electrophoresed through a 0.8% (w/v) agarose gel in a running buffer of TAE (40 mM Tris, pH 7.6, 50 mM Acetic acid, 50 mM EDTA). The DNA was transferred to Hybond NX (Amersham) membranes as described by the manufacturer. Samples on all gels were:

1. *Hin*dIII-digested lambda DNA standard markers equivalent to 23.13, 9.42, 6.56, 4.36, 2.32 and 2.03 kb respectively.
2. 5 µg of *Eco*RI-digested genomic DNA isolated from petals of the transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™).
3. 5 µg of *Eco*RI-digested genomic DNA from petals of the non-transgenic carnation parental line FE123 (negative control).
4. 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA was added to the transgenic and non-transgenic genomic DNA samples described in 2 & 3.
5. 11.5, 57.5, 115, 230, 575 and 1150 pg of plasmid DNA (pCGP1991) digested with *Eco*RI. Based on an estimate of the size of the carnation genome , the 115 pg DNA digest was expected to provide hybridization signals approximate to a single copy T-DNA insert. These lanes also provide a positive hybridization control.

Following electrophoresis of the digested DNA in agarose, the DNA was: depurinated in 0.25 M HCl for 15 minutes, denatured in 1.5 M NaCl/0.5 M NaOH for 1 to 1.5 hours, then neutralised in 0.5 M Tris-HCl (pH 7.5), 0.48 M HCl and 1.5 M NaCl for 1 to 1.5 hours. DNA was then transferred to a Hybond-NX nylon transfer membrane (Amersham Life Science) using 20 x SSC (3 M sodium chloride, 0.3 M Tris-sodium citrate, pH 7.0).

(**v) Preparation of probes -** Twelve probes were used for Southern blot analysis (Attachment A3, Figure 1 of this attachment and Table 1 below). Probes were generated by restriction digests at convenient sites.

Table 1: Summary of fragments used as probes on Southern blots

Probe	Plasmid	Digest	Fragment size (kb)	Region spanned by probe on pCGP1991 vector
*SuR*B	pCGP1651	*Hin*dIII	0.77	1196-1965
F3'5'H	pCGP1961	*Xho*I/*Sal*I	0.84	11321- 12159
gDFR	pCGP2786	*Hin*dIII	1.34	5110- 6446
LB	pCGP2003	*Eco*RI/*Hin*dIII	0.25	7-261
RB	pCGP3203	*Eco*RI	0.29	13845-14133
A	pCGP1667	*Eco*RI	2.71	24734-27432
B	pCGP1673	*Eco*RV/*Bam*HI	2.01	22725-24733
C	pCGP1679	*Cla*I/*Kpn*I	2.78	19955-22730
D	pCGP1669	*Hin*cII (from *Sph*I/*Hin*cII digest)	2.30	17655-19957
E1	pCGP1677	*Hin*cII/*Eco*RI	0.54	15701-16243
E2	pCGP1676	*Hin*cII	1.40	16244-17654
F	pCGP1674	*Eco*RI	1.55	14151-15700

The LB, RB, A and F probes were originally generated by PCR using pWTT2132 as template. pWTT2132 is the parental transformation vector from which pCGP1991 is derived (see Attachment 1, Figure 2). The PCR products were subcloned into pCR2.1 (Invitrogen) and their identity confirmed by sequence determination along with restriction endonuclease mapping and Southern blot hybridisation with digested plasmid DNA. The plasmids were designated pCGP2003, pCGP3203, pCGP1667 and pCGP1674 respectively (see Table 1). Restriction endonuclease digests of the respective plasmids (see Table 1) were used to generate fragments for ^{32}P-labeling. The fragments were purified after agarose gel electrophoresis. In some cases the probe fragment length exceeds the vector coordinate-derived region due to carry over of restriction sites during cloning.

The *SuR*B, B, C, D, E1 and E2 probes were originally generated by restriction endonuclease digestion of pWTT2132. The isolated fragments were then cloned into pBluescript II (Stratagene) and their identity confirmed by sequencing, restriction endonuclease mapping and Southern blot hybridisation with digested plasmid DNA. The plasmids were designated pCGP1651, pCGP1673, pCGP1679, pCGP1669, pCGP1677 and pCGP1676 respectively (see Table 1). Restriction endonuclease digests of the respective plasmids (see Table 1) were used to generate fragments for ^{32}P-labeling reactions. The fragments were purified after agarose gel electrophoresis.

The *F3'5'H* probe fragment was generated by restriction endonuclease digestion of the plasmid containing the original cDNA, pCGP1961 and its identity confirmed by restriction endonuclease mapping, Southern blot hybridisation with digested plasmid DNA and DNA sequencing.

The *gDFR* probe fragment was generated by restriction endonuclease digestion of the plasmid pCGP1472 containing the original petunia genomic *DFR* clone. The isolated 1.3kb *Hin*dIII fragment containing exons 2 to 6 of the *DFR* genomic clone was cloned into pBluescript KSII (Stratagene) and its identity was confirmed by restriction endonuclease mapping and Southern blot hybridisation with digested plasmid DNA. The resultant plasmid was designated pCGP2786.

(**vi) ^{32}P-Labelling of DNA Probes -** DNA fragments (50 to 100 ng) were radioactively labeled with 50 µCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks) or a Decaprime kit (Ambion). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on Sephadex G-50 (Fine) columns.

(vii) Hybridization - Hybridization conditions included a prehybridization step in 50% (v/v) deionised formamide, 1 M NaCl, 1% (w/v) SDS and 10% (w/v) dextran sulphate at 42°C for at least 1 hr. Denatured ^{32}P-labelled fragments of the probes (LB, *SuR*B, *F3'5'H*, *gDFR*, RB, A, B, C, D, E (1+2) and F) were then added to the hybridization solution and hybridization was continued at 42ºC for a further 16 hours. Membranes were typically washed in 2 x SSC, 1% (w/v) SDS at 65°C for 1 to 2 hours and then in 0.2 x SSC, 1% (w/v) SDS at 65°C for 0.5 to 1.0 hour. Membranes hybridized with the RB probe were washed in 2 X SSC, 1% (w/v) SDS at 65^{o}C for 1-2hrs. Membranes were exposed to AR (Kodak X-ray X-OMAT AR or Fuji Medical X-ray RX) film with an intensifying screen at –70°C for 16 hours to 7 nights. X-ray AR films were developed using an X-ray developing machine (AGFA). Hybridized ^{32}P-labelled fragments of probes were removed from Southern blots by pouring a boiling solution of 0.1% (w/v) SDS over the membrane and then incubating the membranes in 0.1% (w/v) SDS in a 65°C incubator for 30 minutes. This process was repeated. The 0.1% (w/v) SDS solution was then allowed to cool down to room temperature and the membrane was then rinsed in 2 X SSC prior to being exposed to Kodak X-ray X-OMAT AR film with an intensifying screen at –70°C for 16 hours to check for efficiency of removal of the probe.

Results

Results obtained following hybridization of Southern blots with each of the twelve probes are shown in Figures 2 to 12. The results are summarised below in Tables 2, 3 and 5.

Each probe hybridised with expected bands in the *Eco*RI-digested pCGP1991 plasmid DNA alone (Panel C in Figures 2-12) and with those in the lanes containing the *Eco*RI-digested pCGP1991 plasmid DNA together with *Eco*RI digested carnation genomic DNA (Panel B in Figures 2-12). These results confirm that the samples of carnation genomic DNA did not interfere with hybridisation of the probes with homologous sequences.

Table 2. Sizes of expected hybridizing bands*

Probes	*Eco*RI-digested pCGP1991	*Eco*RI-digested genomic DNA
LB, *SuR*B, E1, E2, D, C, B, A	13.8kb (A to E2, LB, 35S, *SuR*B 5' coding)	>2kb (LB, 35S, *SuR*B 5' coding and plant genome)
gDFR	2.4kb (*DFR* genomic)	2.4kb (*DFR* genomic)
RB, *F3'5'H*, F	4.7kb (RB, *F3'5'H*, F)	>3kb (RB, *F3'5'H*, F and plant genome)

**Sizes of expected bands with the various probes used on EcoRI-digested pCGP1991 plasmid DNA and EcoRI-digested plant genomic DNA isolated from transgenic carnation line FLORIGENE Moonaqua™ (123.8.12). The expectation is based on simple integration of an intact T-DNA. The sequences contained within the fragments are also indicated in brackets.*

Table 3 summarises the estimated size (in kb) and relative intensities of the hybridising bands detected with each probe in the *Eco*RI-digested transgenic genomic DNA isolated from FLORIGENE Moonaqua™ (123.8.12) (see Figures 2-12).

***Table 3. Summary of hybridizing bands detected in* FLORIGENE Moonaqua™ *(123.8.12)*[#]**

Probe	Estimate of sizes (kb) and relative intensities of hybridizing bands
LB	8 ($^1/_{10}$+)
*SuR*B	8 (+)[*]
F3'5'H	4.2 (+), 3.4 (++)
gDFR	2.4 (+)
RB	4 ($^1/_2$+), 3.8 ($^1/_2$+), 3.5 ($^1/_2$+), 3.4 ($^1/_2$+)

[#] Summary of hybridising bands detected in line FLORIGENE Moonaqua™ (123.8.12) using probes that span the region within the T-DNA. +: Indicates relative intensity of the hybridising band with "+" indicating a relative signal equivalent to a single copy (1) as indicated by the plasmid DNA control.

[*]: There is hybridization to an endogenous sequence (1.0 kb) in both the transgenic and control. This can be seen more clearly in the TIF image of the gel that is provided with this attachment (**MAEcoRISURBhighres.TIF)**

In the case of the F3'5'H probe signals (Figure 4) there are faint signals in the transgenic DNA lane which are not present in the non-transgenic parent lane. We believe there are two possible explanations for this;

(i) The probe sequence is derived from a clone of the entire F3'5'H sequence. On occasion, gel purified probe preparations contain unintended carryover of DNA from other regions of the clone. These may label and give faint signal. Such signals may be based on low abundance homology or homology to polylinker sequences (common to other regions of the T-DNA) which though they have very short stretches of homology will nevertheless generate detectable signal. As a result signal could be obtained to the partial F3'5'H integration observed in locus 3 (1.5kb+) or unrelated T-DNA sequences (carrying polylinker for example).

(ii) Though considerable care is taken to ensure endonuclease digestions continue to completion, incomplete digestion of genomic DNA may occur. This may be differential such that some sites are digested to completion and others are not completely digested, giving faint background signal (eg, the 6kb signal).

Because of the large difference in signal intensity between the 3.2 kb and 4.2 kb F3'5'H bands and background, we believe the 1 and 6 kb signals obtained using the F3'5'H probe do not reflect integrated sequences which have not been cloned and subsequently sequenced. No additional bands were observed on the *Bgl*II Southerns (attachment A5) and the location of the *Eco*RI sites in loci 1 and 2 (Figure 13) indicates that 1 or 6kb fragments would not be expected. Locus 3 has no *Eco*RI sites.

Copy number

Table 4 summarises the estimated copy number of each probe based on the number and the relative intensities of hybridizing bands (see Figures 2 to 6).

Table 4. Estimated copy numbers of probes*

Probe	Estimated Copy Number	Probe	Estimated Copy Number
LB	1	*gDFR*	1
*SuR*B	1	RB	4
F3'5'H	3		

**Estimated copy numbers of probes (or fragments thereof) that span the region within the T-DNA in the transgenic tissue of the line* FLORIGENE Moonaqua™ *(123.8.12).*

Several different methods are reported in the literature to estimate gene copy number and gene copy equivalents of T-DNA integrations in transgenic plants (Chee et al., 1991; Croy, 1993). Here we have assumed our T-DNA integrations are similar to those in published studies (De Neve et al., 1997) and are frequently rearranged, ligated and integrated as repeats at single or several independent loci.

The genome size of a *Dianthus caryophyllus* has been reported as 613 Mbp (Bennett and Leitch, 1995; Figueira et al., 1992; http:/www.rbgkew.org.uk/cval/homepage.html). We have assumed that the parental variety is diploid. This assumption is consistent with the opinion of the breeder.

The calculation we have used for copy number is based on:
(see http://www.med.umich.edu/tamc/spike.html)

ASSUMPTION 1: The haploid content of the carnation genome is 6.13×10^8 bp:
ASSUMPTION 2: We have 5 µg of genomic DNA in each gel electrophoresis lane, however we use 2.5 µg in the calculation since the transgenic lines are heterozygous (propagation of carnation is vegetative).

(Mass of transgene/2.5 µg genomic DNA) = (N bp transgene DNA/6.13×10^8 bp genomic DNA)

Therefore for pCGP1991;

(Mass of transgene/2.5 µg genomic DNA) = (27432 bp transgene DNA/6.13×10^8 bp genomic DNA)
And mass of transgene = 0.000112 µg = 112 pg

Therefore to prepare 1 standard copy we would add 112 pg of pCGP1991 to the 5 µg of genomic DNA.

Integration of DNA external to the T-DNA region

Under the conditions used with 5 µg petal genomic DNA, no hybridizing bands were detected in line *FLORIGENE Moonaqua*™ (123.8.12) when a selection of probes spanning the region outside the T-DNA were used (see Table 5).

Table 5. Summary of hybridising bands detected in line* FLORIGENE Moonaqua™ *(123.8.12).

Probe(s)	Bands
A	none
B	none
C	none
D	none
E1 and E2	none
F	none

Under the hybridisation conditions used and described here the probe *SuR*B (acetolactate synthase gene) generated some signal (~1.0 kb) in the non-transgenic control DNA in addition to the transgenic line 123.8.12. This suggests homologous sequences occur in carnation genomic DNA. All Carnations must have one or more genes encoding acetolactate synthase (ALS) as ALS catalyses an essential step in the biosynthesis of branched-chain amino acids.
Under the hybridisation conditions used there were no uniquely hybridizing bands detected with probes A, B, C, D, E1, E2 and F in line 123.8.12 (FLORIGENE Moonaqua™). These results are consistent with there being no integration of sequences outside the transformation vector T-DNA borders in line 123.8.12.

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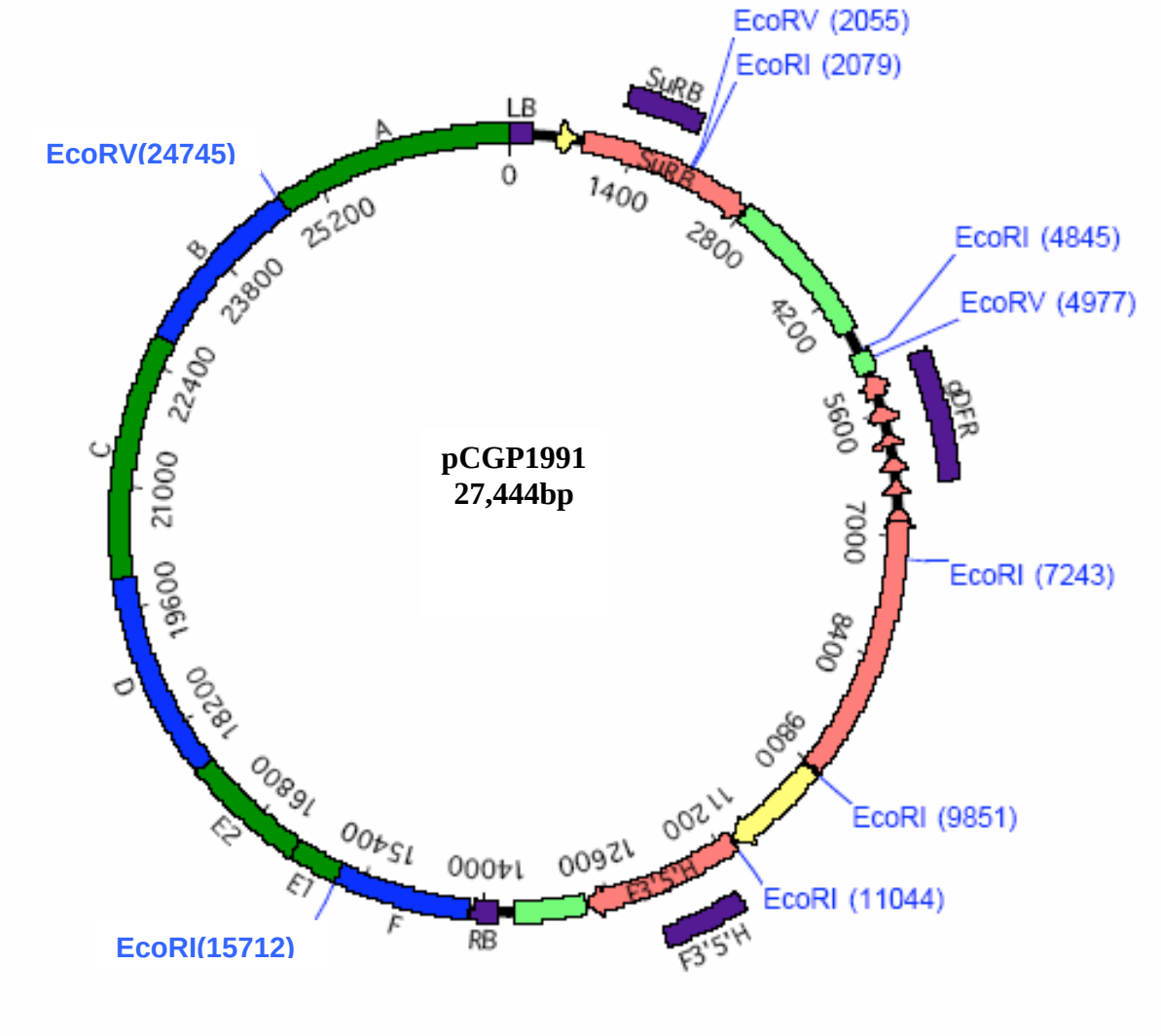
Figure 1

A restriction endonuclease map of the pCGP1991 transformation vector, the three genes transferred in the T-DNA and the probes used in Southern analysis



Southern Blot Analysis of Carnation Genomic DNA Using Probe LB

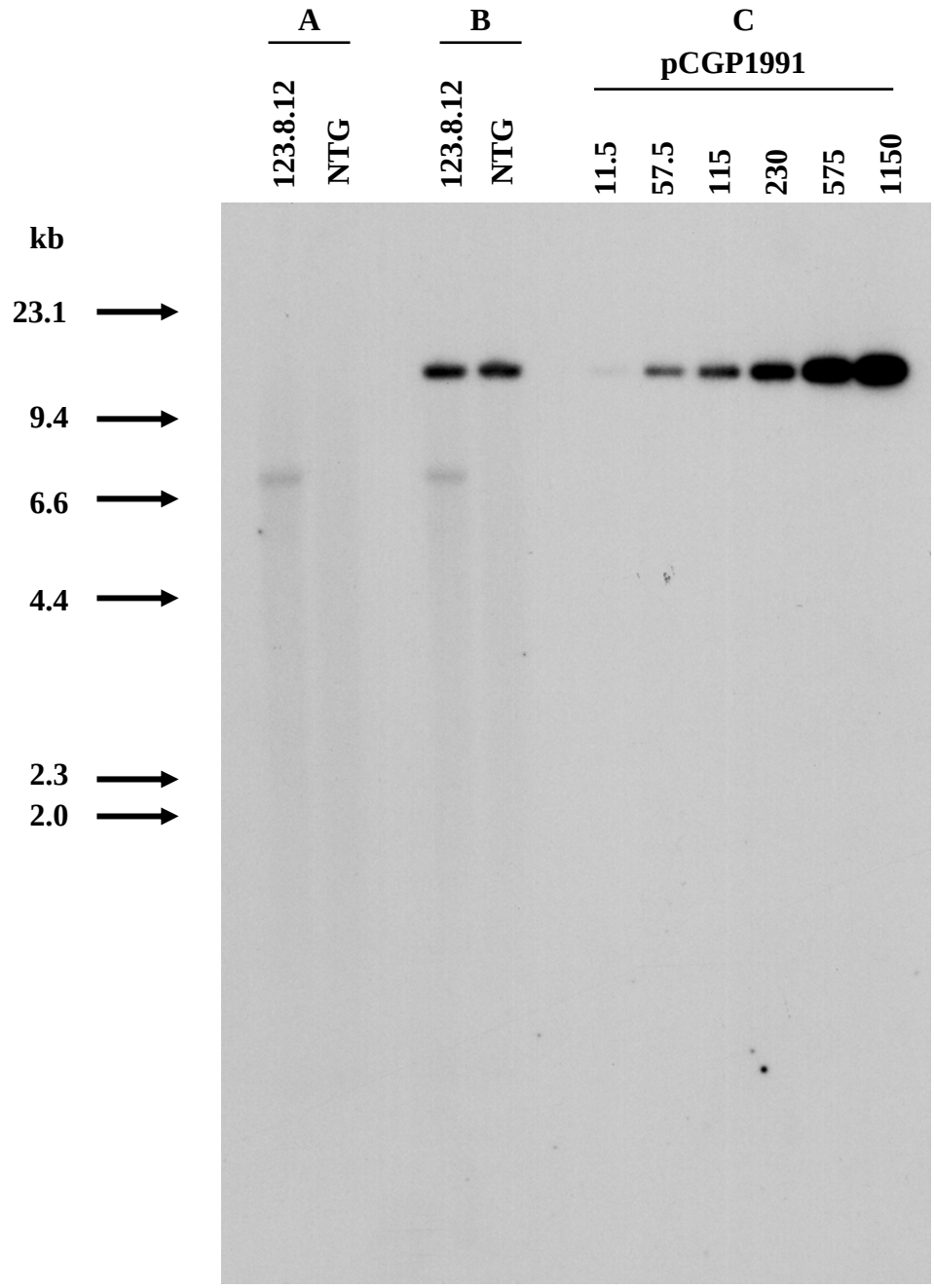


Figure 2 – Probe LB

High-resolution scan of an autoradiograph of a Southern blot hybridised with Probe LB (refer to Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123(NTG) or as indicated above each gel lane., with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. In addition to the hybridising band detected in Panel A, a further hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

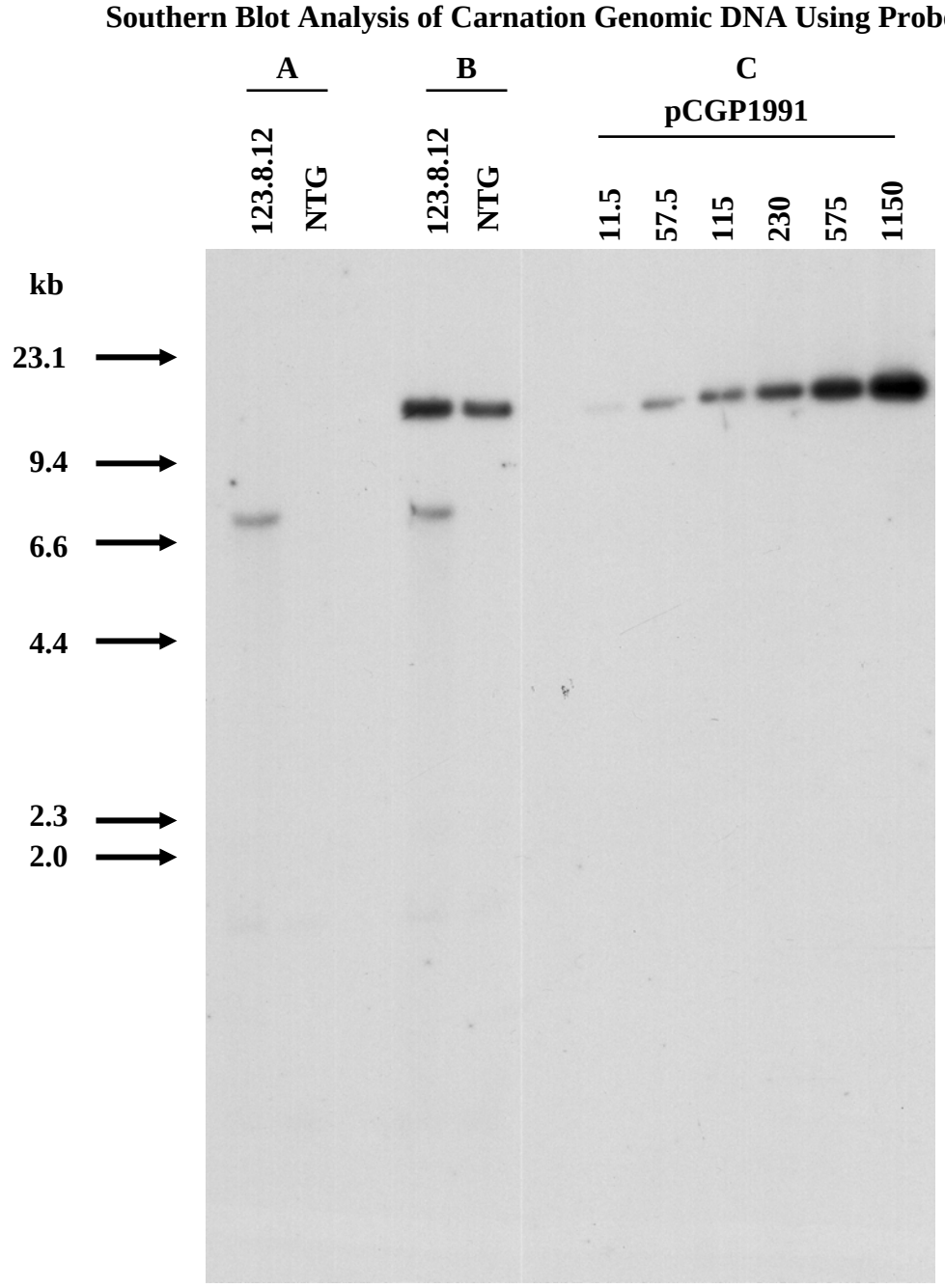


Figure 3 – Probe *SuR*B

High-resolution scan of an autoradiograph of a Southern blot probed with Probe *SuR*B (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane. Results are summarized in Table 3.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane., with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. In addition to the hybridising band detected in Panel A, a further hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

Southern Blot Analysis of Carnation Genomic DNA Using Probe *F3'5'H*

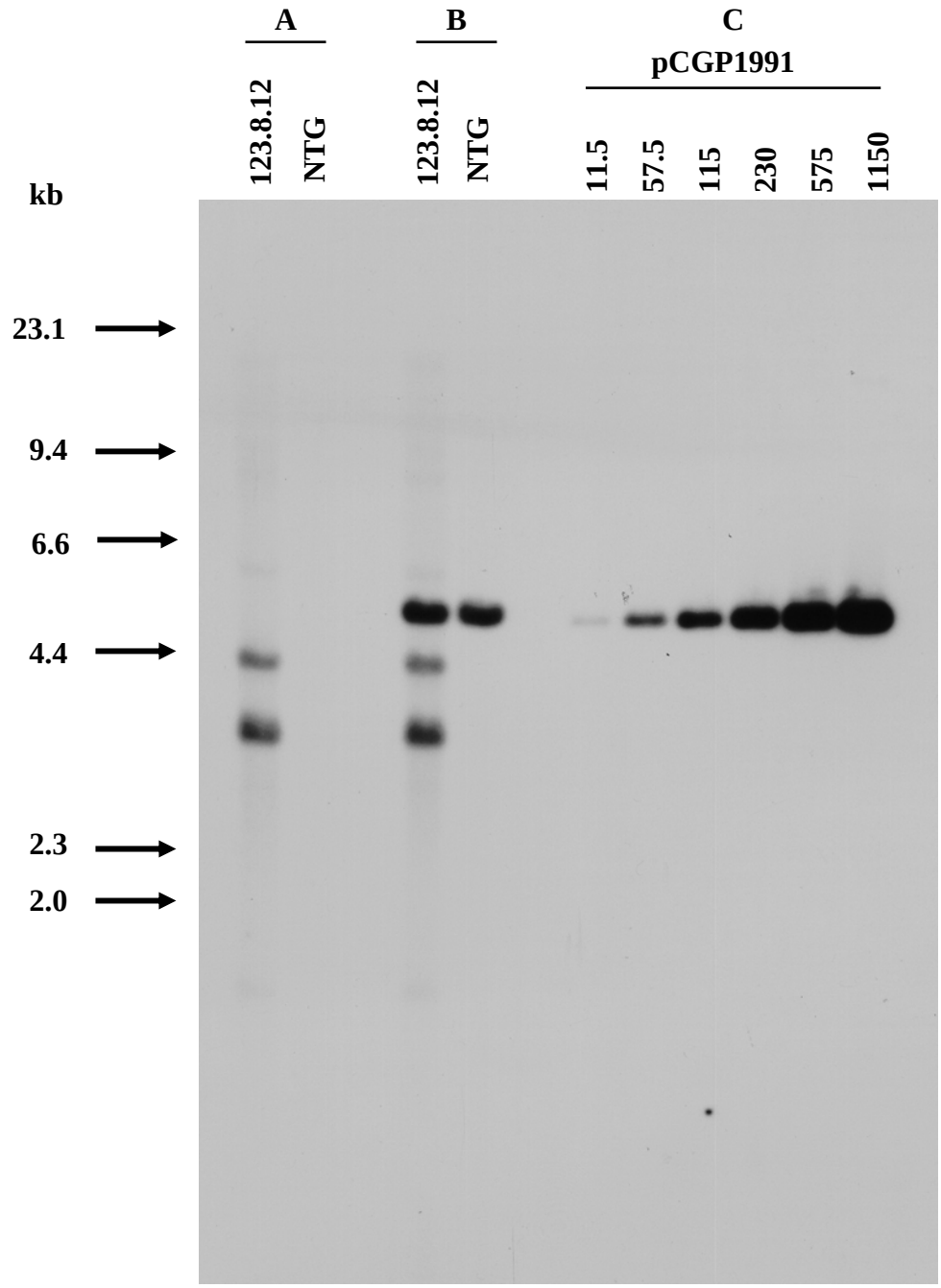


Figure 4 – Probe *F3'5'H*
High-resolution scan of an autoradiograph of a Southern blot probed with Probe *F3'5'H* (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. Results are summarized in Table 3.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. In addition to the hybridising bands detected in Panel A, a further hybridising band of approximately 4.9 kb was detected in both lines. The signal intensity of the 4.9 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

Southern Blot Analysis of Carnation Genomic DNA Using Probe *gDFR*

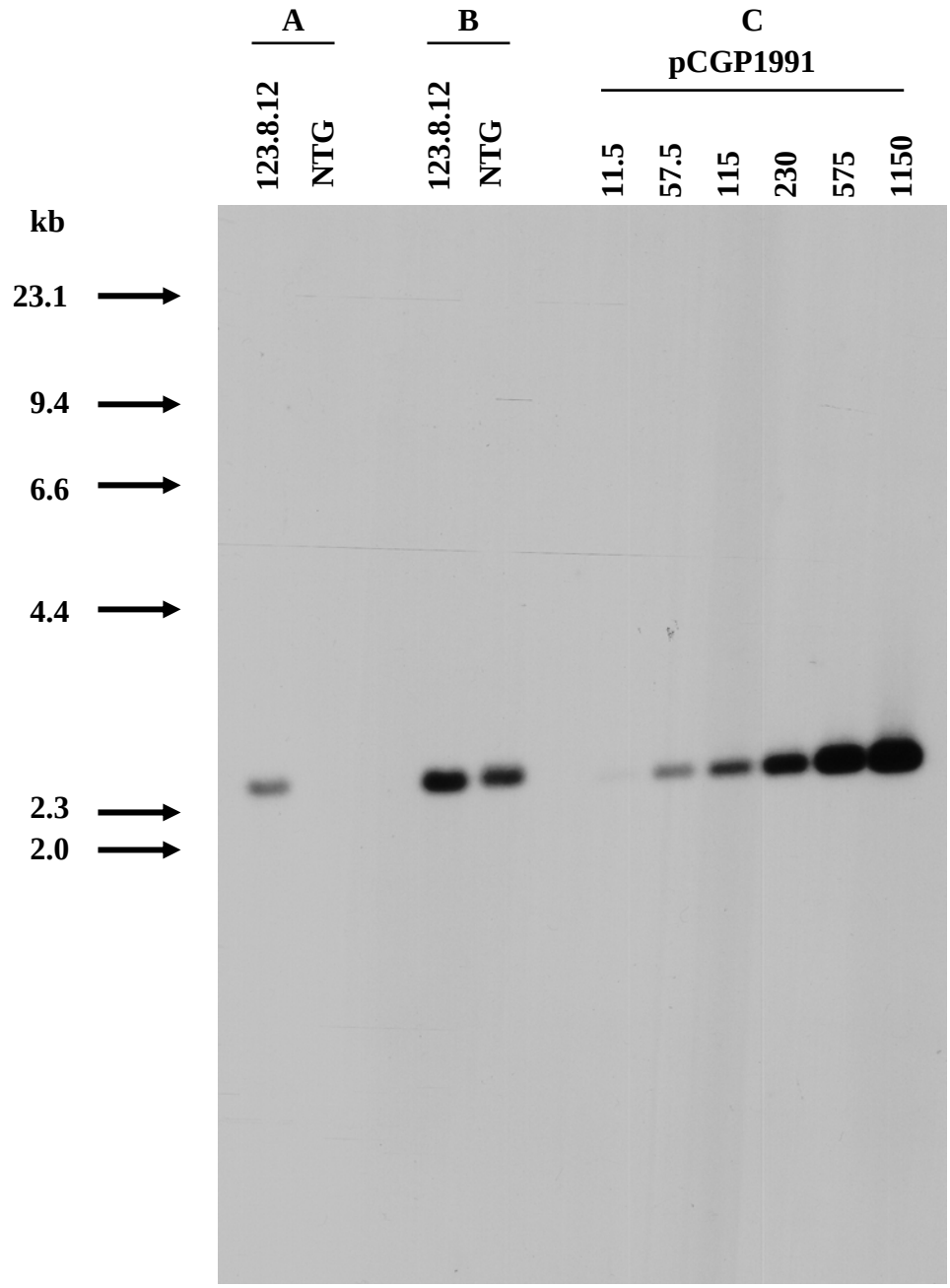


Figure 5 – Probe *qDFR*

High-resolution scan of an autoradiograph of a Southern blot probed with Probe *gDFR* (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. Results are summarized in Table 3.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. In addition to the hybridising bands detected in Panel A, a further hybridising band of approximately 2.4 kb was detected in the NTG line. The signal intensity of the 2.4 kb band in the NTG line is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

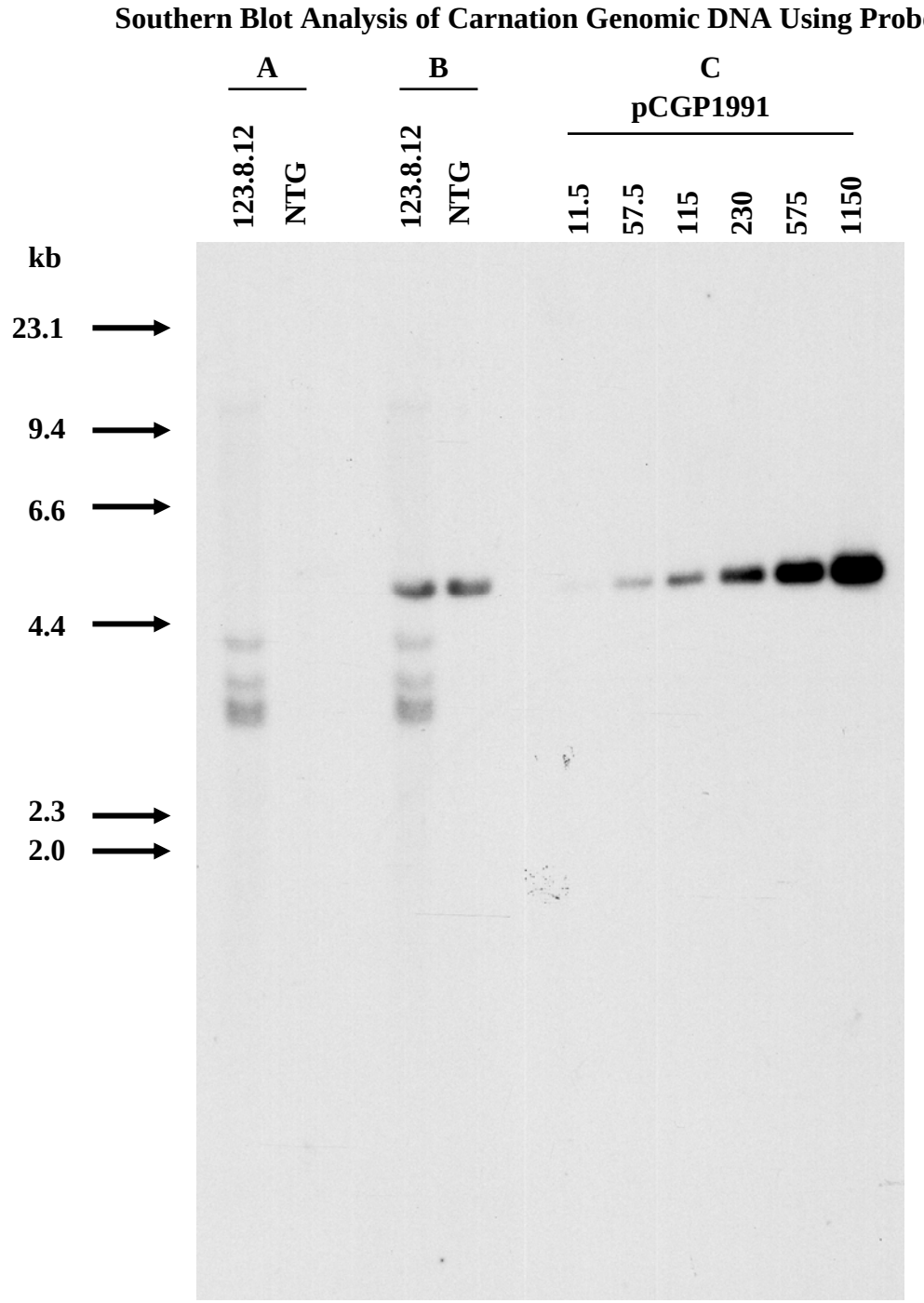


Figure 6 – Probe RB

High-resolution scan of an autoradiograph of a Southern blot probed with Probe RB (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123(NTG) or as indicated above each gel lane. Results are summarized in Table 3.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. In addition to the hybridising bands detected in Panel A, a further hybridising band of approximately 4.7 kb was detected in both lines. The signal intensity of the 4.7 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

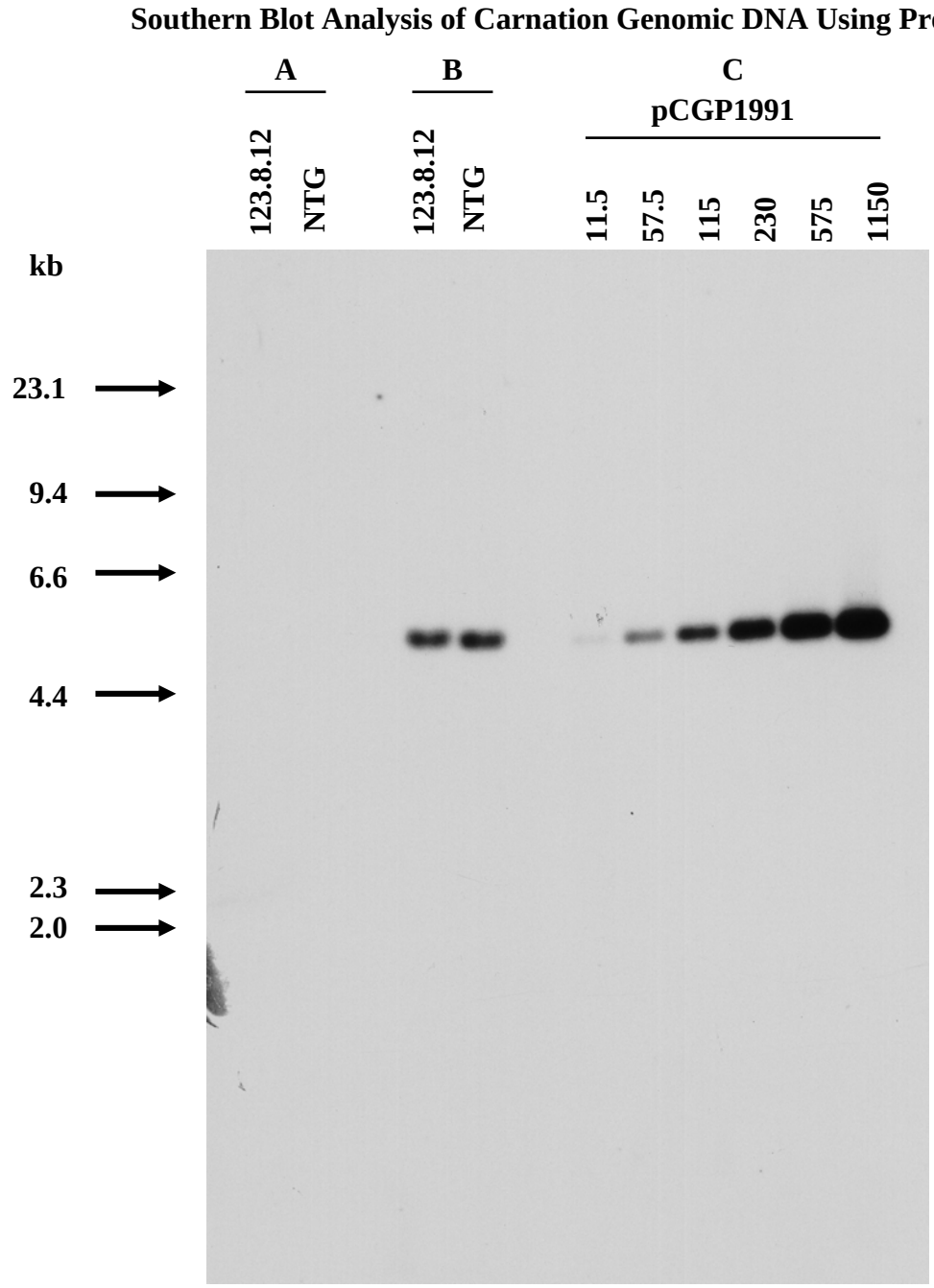


Figure 7 – Probe F
High-resolution scan of an autoradiograph of a Southern blot probed with Probe F (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probe F (see Section 4.2). Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising bands of approximately 4.7 kb was detected in both lines. The signal intensity of the 4.7 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

Southern Blot Analysis of Carnation Genomic DNA Using Probe E1 and 2

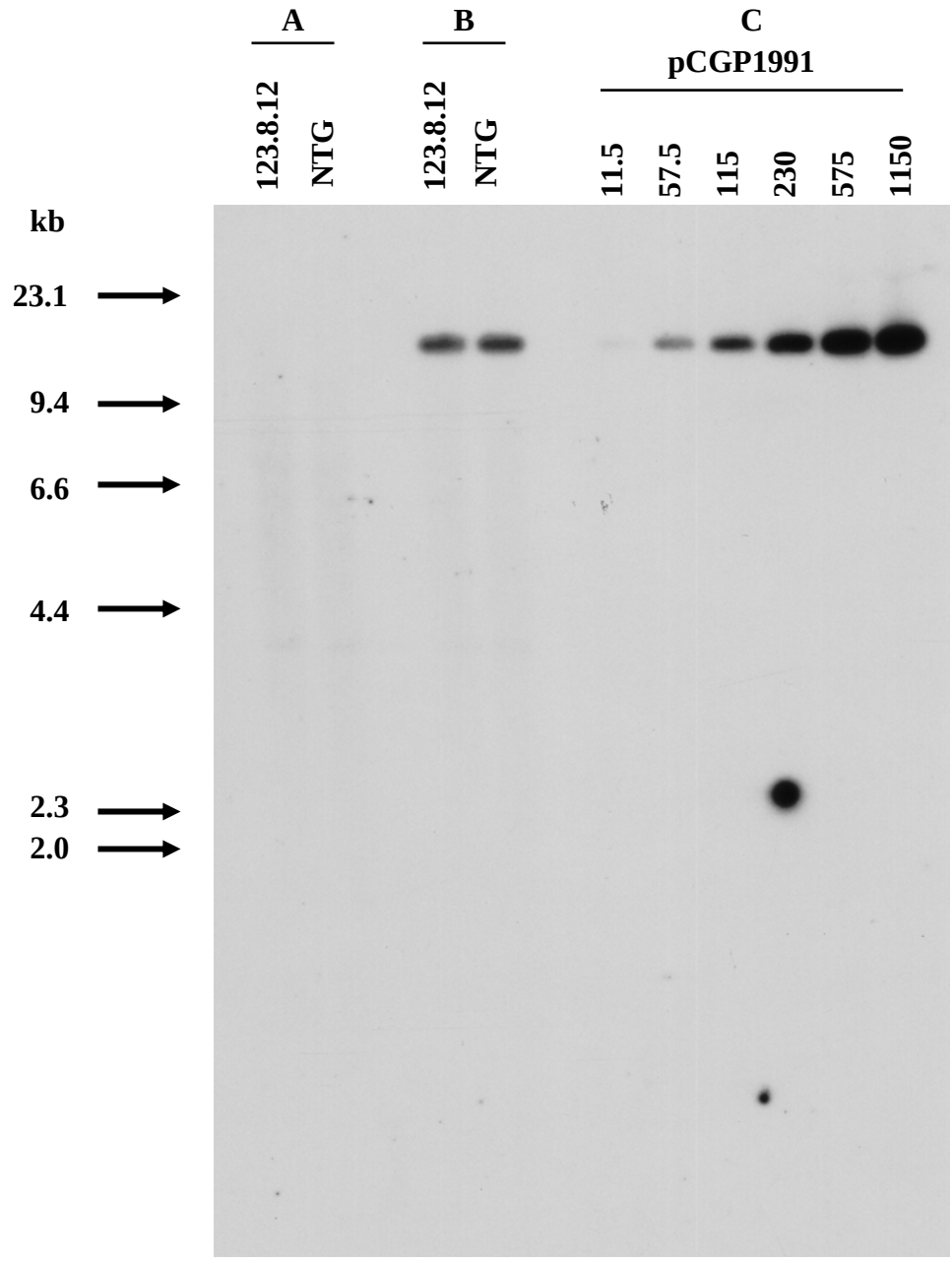


Figure 8 – Probes E1 & E2

High-resolution scan of an autoradiograph of a Southern blot probed with Probes E1 and E2 (see Figure 1). The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probes E1 and E2. Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE 123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

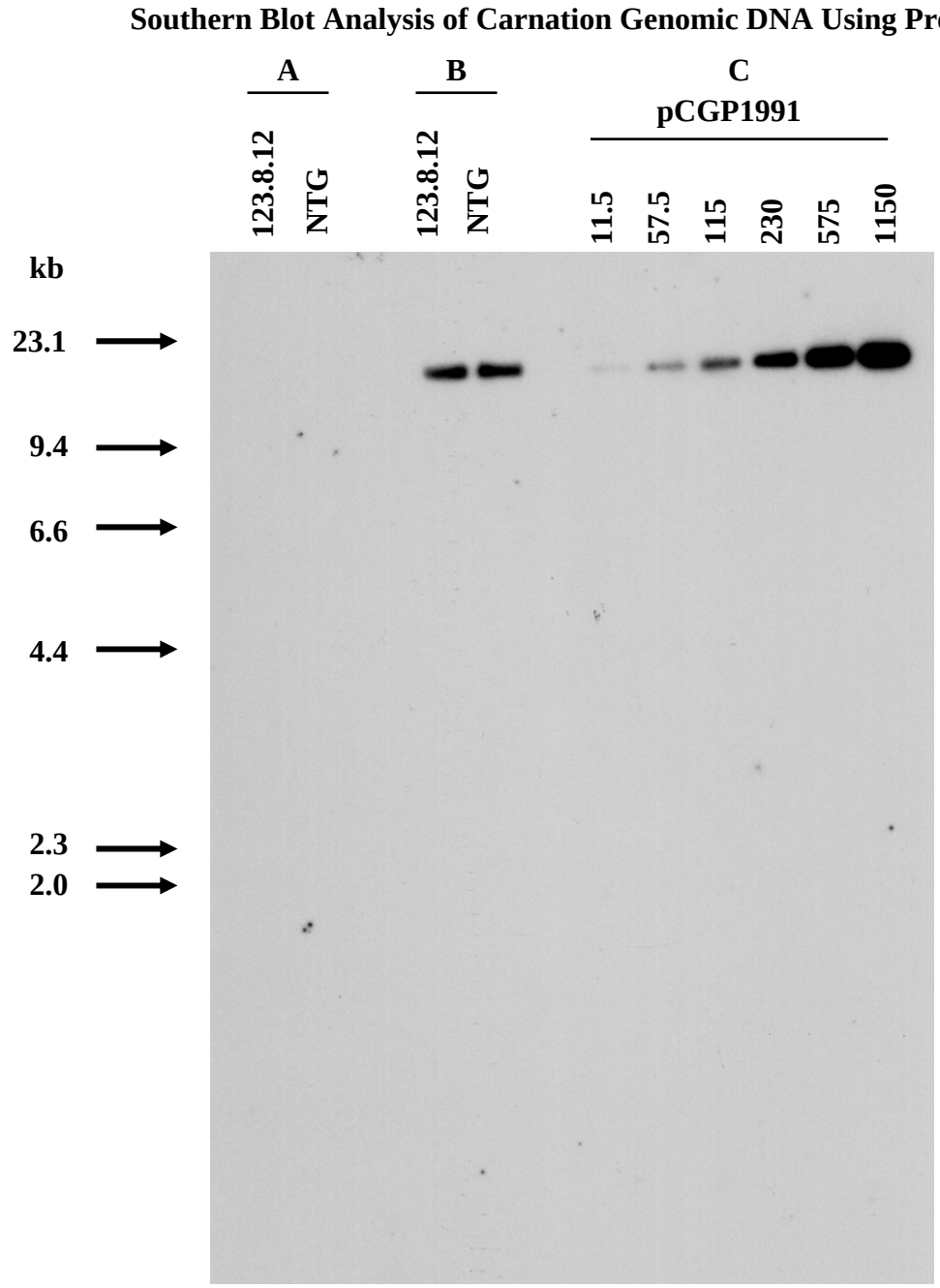


Figure 9 – Probe D

High-resolution scan of an autoradiograph of a Southern blot probed with Probe D (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probe D. Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

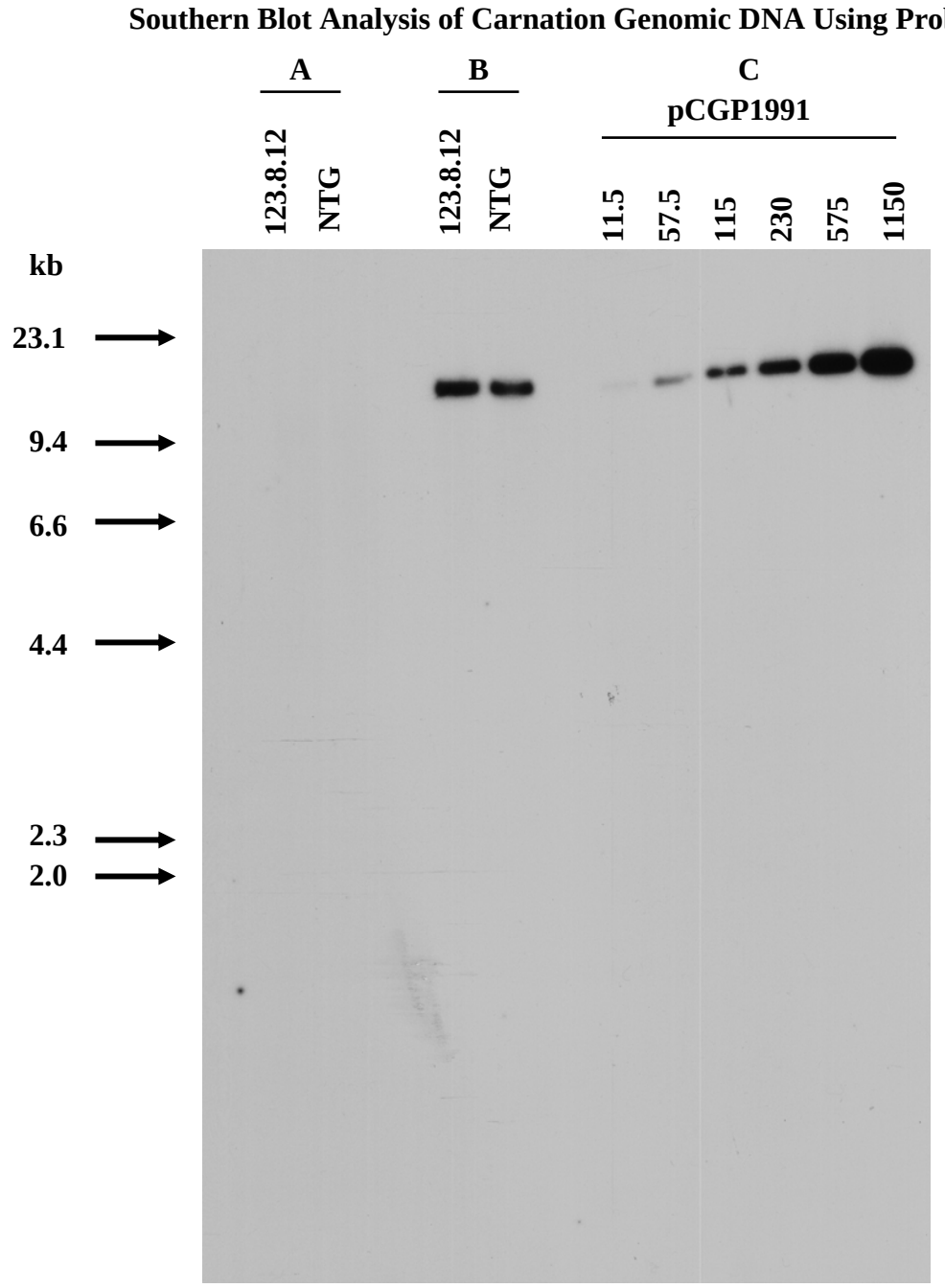


Figure 10 – Probe C

High-resolution scan of an autoradiograph of a Southern blot probed with Probe C (see Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probe C. Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

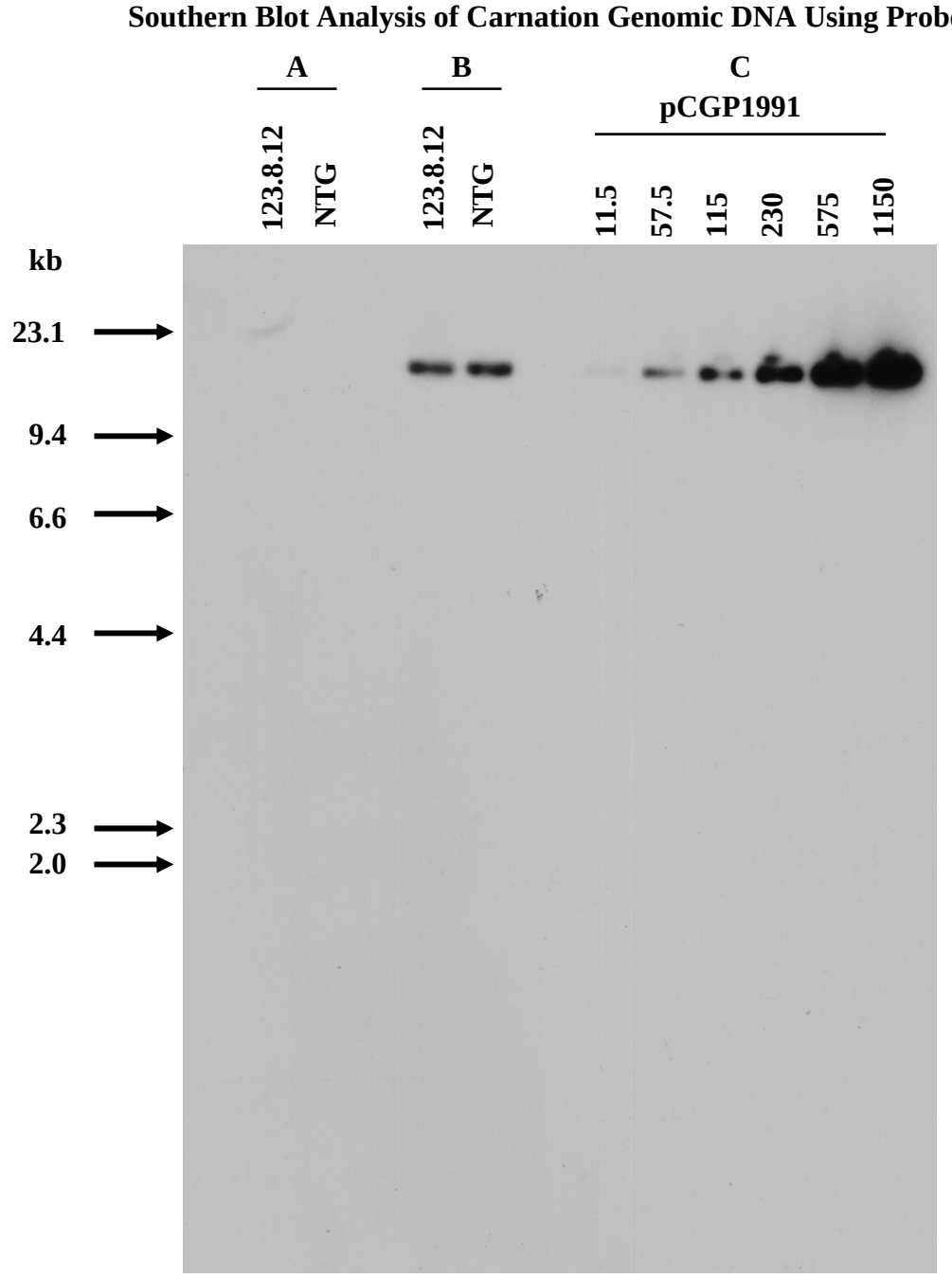


Figure 11 – Probe B
High-resolution scan of an autoradiograph of a Southern blot probed with Probe B (Figure 1).
The Southern was generated via gel electrophoresis of:

Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probe B. Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

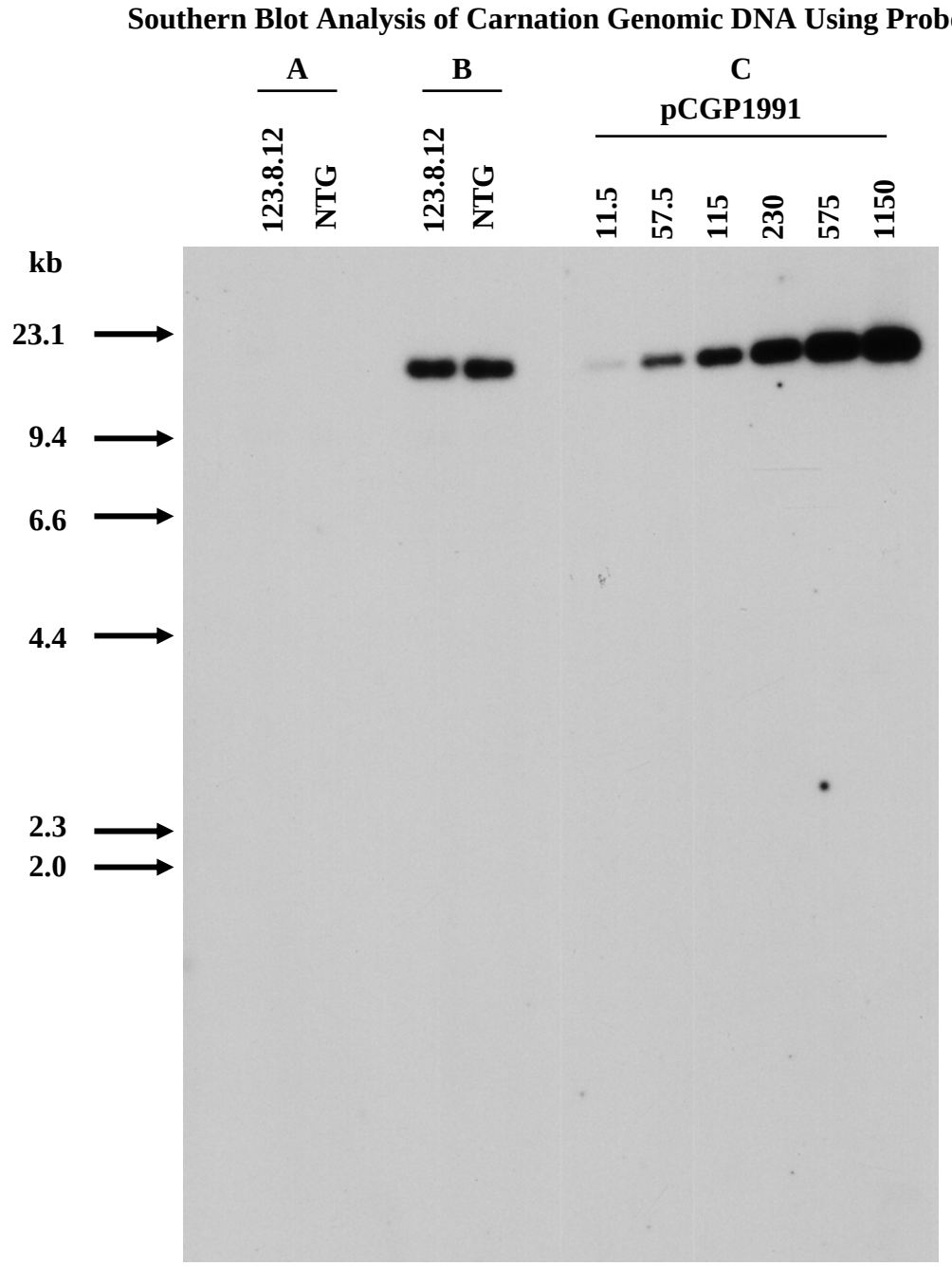


Figure 12 – Probe A
High-resolution scan of an autoradiograph of a Southern blot hybridised with Probe A (Figure 1).
The Southern was generated via gel electrophoresis of:

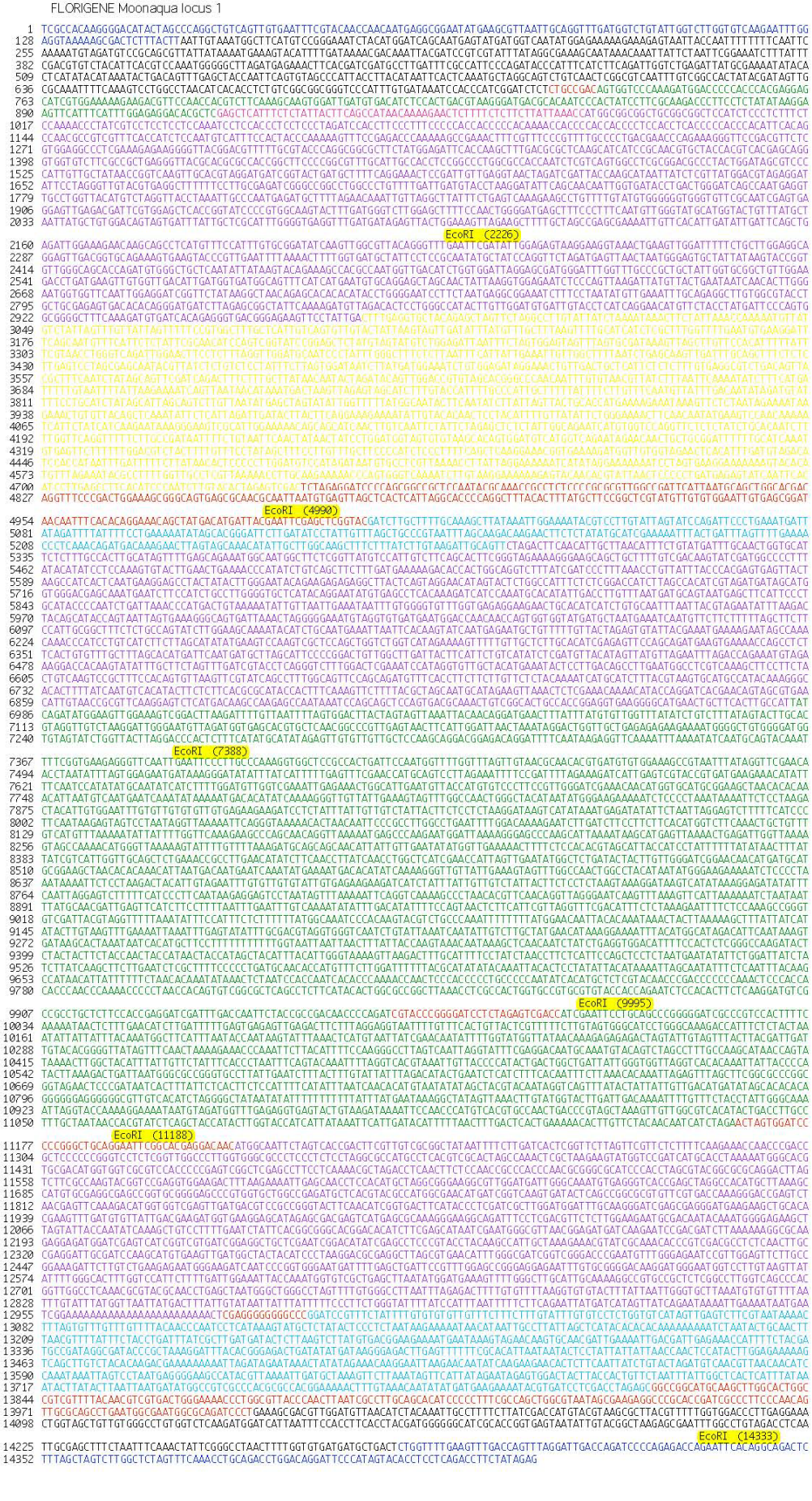
Panel A: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane. No hybridisation signal can be detected in the NTG or transgenic line demonstrating there is no integration of DNA outside the T-DNA in these lines corresponding to the region of the transformation vector spanned by the Probe A. Results are summarized in Table 5.

Panel B: 5 µg *Eco*RI-digested total petal DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or from non-transgenic carnation line FE123 (NTG) or as indicated above each gel lane, with 115 pg of *Eco*RI-digested pCGP1991 transformation vector DNA added to each. A hybridising band of approximately 14 kb was detected in both lines. The signal intensity of the 14 kb band in both lines is consistent with that for 115 pg of *Eco*RI-digested pCGP1991 plasmid DNA that was added to the samples (see Panel C).

Panel C: 11.5, 57.5, 115, 230, 575 and 1150 pg of *Eco*RI-digested pCGP1991 plasmid DNA. These lanes approximate 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 gene copy equivalents respectively for the line 123.8.12 (FLORIGENE Moonaqua™).

Figure 13

Sequences of Locus 1 and Locus 2, with *Eco*RI sites indicated.




FLORIGENE Moonaqua locus 2
EcoRI (1894)

ATTACHMENT A5

Southern blot analysis – *Bgl*II

Introduction

Table 1 below lists the probes covering the entire transformation vector and the hybridization results previously obtained by Southern blot analysis for *Eco*RI-digested DNA isolated from FLORIGENE Moonaqua™ (123.8.12) (refer Attachment A4)

***Table 1. Summary of fragments used as probes and corresponding hybridization detected in* FLORIGENE Moonaqua™ *(123.8.12) genomic DNA digested with Eco*RI**

Probes	Hybridization
LB	+
*SuR*B	+
F3'5'H	+
gDFR	+
RB	+
A	-
B	-
C	-
D	-
E1	-
E2	-
F	-

"+": Indicates detection of uniquely hybridizing bands with these probes. "-": Indicates that uniquely hybridizing bands were not detected using these probes, i.e., these sequences were not integrated into Florigene Moonaqua™ (123.8.12) during plant transformation.

To better understand the organization of the integrated sequences shown in Table 1, and to determine the number of loci at which these sequences are integrated, further Southern blot experiments were carried out. In these experiments genomic DNA was digested with the restriction endonuclease, *Bgl*II, for which no recognition sequences occur within the plant transformation vector pCGP1991 (See Attachment A3). The use of such a restriction endonuclease ensures that any vector-derived sequences (within the T-DNA and outside it) remain intact at each point of integration within the transgenic plant DNA and are detectable as independent integration loci.

Experimental rationale

Petal and leaf material analyzed was collected from plants grown in Melbourne, Australia. Material was frozen and stored at –70°C. Genomic DNA isolated from carnation line FLORIGENE Moonaqua™ (123.8.12) was hybridized with the five probes from the transformation vector pCGP1991 which had previously been shown to produce uniquely hybridizing bands in this line (LB, *SuR*B, *F3'5'H*, *gDFR*, and RB) (Table 1). By hybridizing Southern blots with each of these probes our purpose was to:

- Estimate transgene copy number,
- Determine the number of loci at which vector-derived sequences have been integrated into the carnation genome,
- Better understand the organization of integrated sequences at each locus, and

- Confirm that genomic DNA isolated from the transgenic carnations does not interfere with hybridization of the probes with any homologous sequences.

Methods

In general, the methods followed were as described in:

- Molecular Cloning: A Laboratory Manual, Sambrook et al. (1989),
- Plant Molecular Biology Manual (2nd edition), Gelvin and Schilperoot (eds), Kluwer Academic Publisher, The Netherlands, 1994
- Plant Molecular Biology Labfax, Croy (ed), Bios scientific Publishers, Oxford, UK, 1993.

The cloning vectors pBluescript and pPCR script were obtained from Stratagene, USA. The cloning vector pCR2.1 was obtained from Invitrogen, USA.

(i) Isolation and purification of DNA fragments - Fragments were generally isolated on a 1% (w/v) agarose gel and purified using the QIAEX II Gel Extraction kit (Qiagen) or Bresaclean Kit (Bresatec, Australia) following procedures recommended by the manufacturer.

(ii) Polymerase Chain Reaction (PCR) - Unless otherwise specified, PCR conditions using plasmid DNA as template included using 2 ng of plasmid DNA, 100 ng of each primer, 2 µL 10 mM dNTP mix, 5 µL 10 x Taq DNA polymerase buffer, 0.5 µL Taq DNA Polymerase in a total volume of 50 µL. Cycling conditions comprised an initial denaturation step of 5 min at 94ºC, followed by 35 cycles of 94ºC for 20 sec, 50ºC for 30 sec and 72ºC for 1 min with a final treatment at 72ºC for 10 min before storage at 4ºC. PCRs were performed in a Perkin Elmer GeneAmp PCR System 9600.

(iii) DNA Sequence Analysis - DNA sequencing was performed using the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). Sequencing runs were generally performed by the Australian Genome Research Facility at The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) or in-house on an automated 373A DNA sequencer (Applied Biosystems).

(iv) Preparation of genomic DNA and Southern blots - Total plant genomic DNA was isolated from petal or leaf tissue essentially as described by Dellaporta et al. (1983). The DNA preparations were further purified by CsCl buoyant density centrifugation (Sambrook et al., 1989). The genomic DNA (10 µg) was digested for 16 to 48 hours with 120-200 units of the restriction endonuclease *Bgl*II and then electrophoresed through a 0.8% (w/v) agarose gel in a running buffer of TAE (40mM Tris, pH 7.6, 50mM Acetic acid, 50 mM EDTA). The DNA was transferred to Hybond NX (Amersham) membranes as described by the manufacturer.

Samples run on all gels included:

1. *Hin*dIII-digested lambda DNA standard markers equivalent to 23.13, 9.42, 6.56, 4.36, 2.32 and 2.03 kb respectively.
2. 10 µg of *Bgl*II-digested genomic DNA isolated from the leaves of transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™).
3. 10 µg of *Bgl*II-digested genomic DNA from petals of the non-transgenic carnation line 123 (negative control).
4. 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA was added to the transgenic and non-transgenic genomic DNA samples described in 2 & 3. The 230 pg DNA digest was expected to provide hybridization signals approximate to a single copy T-DNA insert. These lanes also provide a positive hybridization control.

Following electrophoresis of the digested DNA in agarose, the DNA was: depurinated in 0.25 M HCl for 15 minutes, denatured in 1.5 M NaCl/0.5 M NaOH for 1 to 1.5 hours, then neutralized in 0.5 M Tris-HCl (pH 7.5), 0.48 M HCl and 1.5 M NaCl for 1 to 1.5 hours. DNA was then transferred

to a Hybond-NX nylon transfer membrane (Amersham Life Science) using 20 x SSC (3 M sodium chloride, 0.3 M Tris-sodium citrate, pH7.0).

(v) Preparation of probes - Five probes was used for Southern blot analysis (see Figure 1 and Table 2 below). These probes were generated by restriction digests at convenient sites.

Table 2. Summary of fragments used as probes on Southern blots

Probe	Plasmid	Digest	Fragment size (kb)	Region spanned by probe on pCGP1991 vector (Attachment A3)
*SuR*B	pCGP1651	*Hin*dIII	0.77	1196-1965
F3'5'H	pCGP1961	*Xho*I/*Sal*I	0.84	11321-12159
gDFR	pCGP2786	*Hin*dIII	1.34	5110- 6446
LB	pCGP2003	*Eco*RI/*Hin*dIII	0.25	7-261
RB	pCGP3203	*Eco*RI	0.29	13845-14130

The LB and RB probes were originally generated by PCR using pWTT2132 as template. pWTT2132 is the parental transformation vector from which pCGP1991 is derived (see Attachment A2). The PCR products were subcloned into pCR2.1 (Invitrogen) and their identity confirmed by sequencing along with restriction endonuclease mapping and Southern blot hybridization with digested plasmid DNA. The plasmids were designated pCGP2003, pCGP3203 and pCGP1667 respectively (see Table 2). Restriction endonuclease digests of the respective plasmids (see Table 2) were used to generate fragments for ^{32}P-based radiolabeling. The fragments were purified after agarose gel electrophoresis. In some cases the probe fragment length exceeds the vector coordinate-derived region due to carry over of restriction sites during cloning. The *SuR*B probe was originally generated by restriction endonuclease digestion of the pWTT2132 transformation vector. The isolated fragment was then cloned into pBluescriptII (Stratagene) and the identity confirmed by sequencing, restriction endonuclease mapping and Southern blot hybridization with digested plasmid DNA. The plasmid was designated pCGP1651 (see Table 2). Restriction endonuclease digests of the plasmid (Table 2) was used to generate fragments for ^{32}P-dependent radiolabeling reactions. The fragments were purified after agarose gel electrophoresis.

The *F3'5'H* probe fragment was generated by restriction endonuclease digestion of the plasmid containing the original cDNA, pCGP1961 and its identity confirmed by restriction endonuclease mapping, Southern blot hybridisation with digested plasmid DNA and DNA sequencing.

The *gDFR* probe fragment was generated by restriction endonuclease digestion of the plasmid pCGP1472 containing the original petunia genomic *DFR* clone. The isolated 1.3 kb *Hin*dIII fragment containing exons 2 to 6 of the *DFR* genomic clone was cloned into pBluescript KSII (Stratagene) and its identity was confirmed by restriction endonuclease mapping and Southern blot hybridisation with digested plasmid DNA. The resultant plasmid was designated pCGP2786.

(vi) ^{32}P-Labelling of DNA Probes - DNA fragments (50 to 100 ng) were radioactively labeled with 50 µCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks) or a Decaprime kit (Ambion). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on Sephadex G-50 (Fine) columns.

(vii) Hybridization - Hybridization conditions included a prehybridization step in 50% (v/v) deionised formamide, 1 M NaCl, 1% (w/v) SDS and 10% (w/v) dextran sulphate at 42°C for at least 1 hr. Denatured ^{32}P-labelled fragments of the probes (LB, *SuR*B, *F3'5'H*, *gDFR* or RB) were then added to the hybridization solution and hybridization was continued at 42ºC for a further 16 hours. Membranes were typically washed in 2 x SSC, 1% (w/v) SDS at 65°C for 1 to 2 hours and then in 0.2 x SSC, 1% (w/v) SDS at 65°C for 0.5 to 1.0 hour. Membranes hybridized with RB or LB probes were washed in 2 X SSC, 1% (w/v) SDS at 65^{o}C for 1-2hrs. Membranes were exposed to AR (Kodak BioMax MS) film with an intensifying screen at –70°C for 16 hours to 7 nights. X-ray AR

films were developed using an X-ray developing machine (AGFA). Hybridized ^{32}P-labelled fragments of probes were removed from Southern blots by pouring a boiling solution of 0.1% (w/v) SDS over the membrane and then incubating the membranes in 0.1% (w/v) SDS in a 65°C incubator for 30 minutes. The process was then repeated. The 0.1% (w/v) SDS solution was then allowed to cool down to room temperature and the membrane was then rinsed in 2 X SSC prior to being exposed to Kodak BioMax MS film with an intensifying screen at –70°C for 16 hours to check for efficiency of removal of the probe.

Results

Each probe is expected to hybridize to *Bgl*II-digested pCGP1991 plasmid DNA. As there are no restriction sites for *Bgl*II within the pCGP1991 plasmid DNA sequence, the probes are expected to detect both open circle and supercoiled forms of uncut plasmid DNA. Typically, uncut plasmid DNA will migrate differentially with respect to linearized DNA of the same plasmid under identical electrophoretic conditions.

In the case of each probe it is impossible to predict the size of hybridizing fragments in *Bgl*II-digested genomic DNA samples due to the absence of *Bgl*II restriction sites within the originating transformation vector.

Results obtained following hybridization of five Southern blots with each of five probes are shown in Figures 2 to 6.The results are summarized below in Table 3.

Each probe hybridized with both open circle and supercoiled forms of pCGP1991 in the lanes containing the *Bgl*II-digested pCGP1991 plasmid DNA combined with *Bgl*II digested carnation genomic DNA (Panel B in Figures 2 to 6). These results confirm that the samples of carnation genomic DNA did not interfere with hybridization of the probes with homologous sequences.

Table 3 summarises the estimated size (in kb) and relative intensities of the hybridising bands detected with each probe in the *Bgl*II-digested transgenic genomic DNA isolated from FLORIGENE Moonaqua™ (123.8.12) (see Figures 2 to 6).

Table 3. Summary of hybridizing bands detected in line Florigene Moonaqua™ (123.8.12 (See also Figures 2 to 6) [#]

Probe	Estimate of sizes (kb) and relative intensities of hybridizing bands
LB	20 (+)
*SuR*B	20 (+)
F3'5'H	20 (+), 13(++), 8.8 ($^1/_2$+)
gDFR	20 (+)
RB	20 (+), 13 (++), 8.8 (+)

[#] *Summary of hybridizing bands detected in line* FLORIGENE Moonaqua™ *(123.8.12) using probes for plasmid vector sequences that are known to be integrated. +: Indicates relative intensity of the hybridizing band with "+" indicating a relative signal equivalent to a single copy (1) as indicated by the plasmid DNA control present in panel B of Figures 2 to 6.*

Copy number

Table 4 summarises the estimated copy number of each probe based on the number and the relative intensities of hybridizing bands (see Figures 2 to 6).

Table 4. Estimated copy number of probes*

Probe	Estimated Copy Number
LB	1
*SuR*B	1
F3'5'H	3
gDFR	1
RB	3

**Estimated copy numbers of probes (or fragments thereof) that span the region within the T-DNA in the transgenic tissue of the line FLORIGENE Moonaqua™ (123.8.12)*

Several different methods are reported in the literature to estimate gene copy number and gene copy equivalents of T-DNA integrations in transgenic plants (Chee et al., 1991; Croy, 1993). Here we have assumed our vector-derived DNA integrations are similar to those in published studies (De Neve et al., 1997) and are frequently rearranged, ligated and integrated as repeats at single or several independent loci.

The results shown in Table 3 above suggest that there are three independent *Bgl*II loci at which pCGP1991 plasmid DNA sequences have been integrated into FLORIGENE Moonaqua™ (123.8.12). This is indicated by the presence of three independent fragments which hybridized to all the pCGP1991-derived fragments used as probes. A 20 kb *Bgl*II fragment (Locus 1) appears to contain single copies of each of LB, *SuR*B, *F3'5'H*, *gDFR* and RB. A second locus (Locus 2) is detected as a 13 kb *Bgl*II fragment and appears to contain at least one copy of *F3'5'H* and RB. Locus 3 (8.8 kb) also appears to contain a copy of *F3'5'H* and RB. Loci 2 and 3 therefore appear to contain partially integrated copies of some of the T-DNA components found in plasmid pCGP1991.

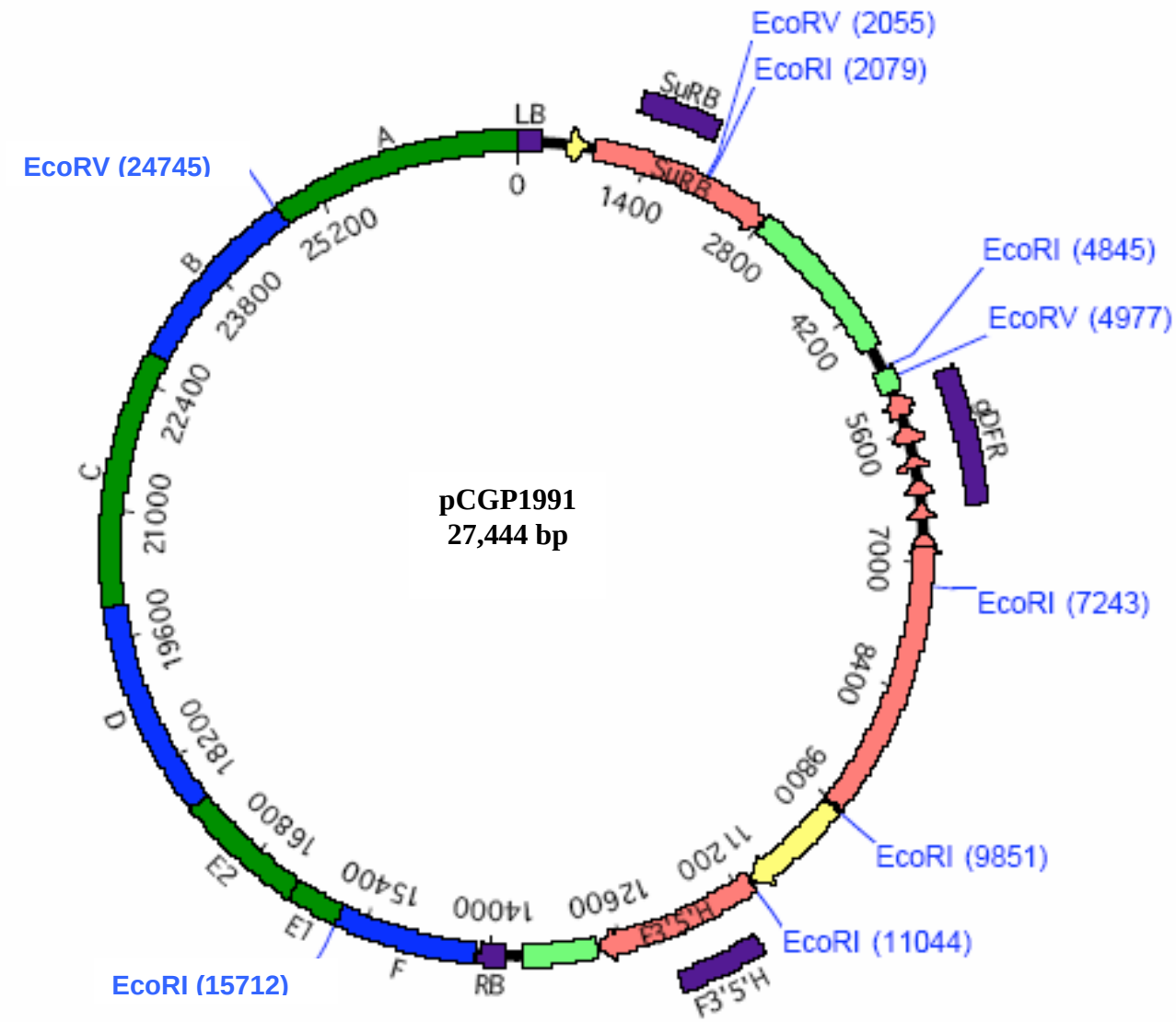
Attachment A6 provides a schematic of the three loci and Attachment A7 their sequence.

Citations

1. Chee, P.P., Drong, R.F. and J.L. Slightom. Using polymerase chain reaction to identify transgenic plants. Plant Molecular Biology Manual C3:1-28. Kluwer Academic Publishers, The Netherlands, 1991
2. Croy, R.R.D. (ed). Plant Molecular Biology Labfax 30-32. BIOSIS Scientific Publishers Limited, 1993
3. Dellaporta, S. J., Wood, J. and Hick, J.B. A plant DNA mini-preparation, version two. Plant Mol. Biol. Rep. 1, 19-21, 1983
4. De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M. and A. Depicker. T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. The Plant Journal 11:15-29, 1997
5. Sambrook, J., Fritsch, E.F. and T. Maniatis. Molecular Cloning: A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory Press, USA, 1989

Figure 1

A restriction endonuclease map of the pCGP1991 transformation vector, the three genes transferred in the T-DNA and the probes used in Southern analysis



Southern Blot Analysis of Carnation Genomic DNA Using Probe LB

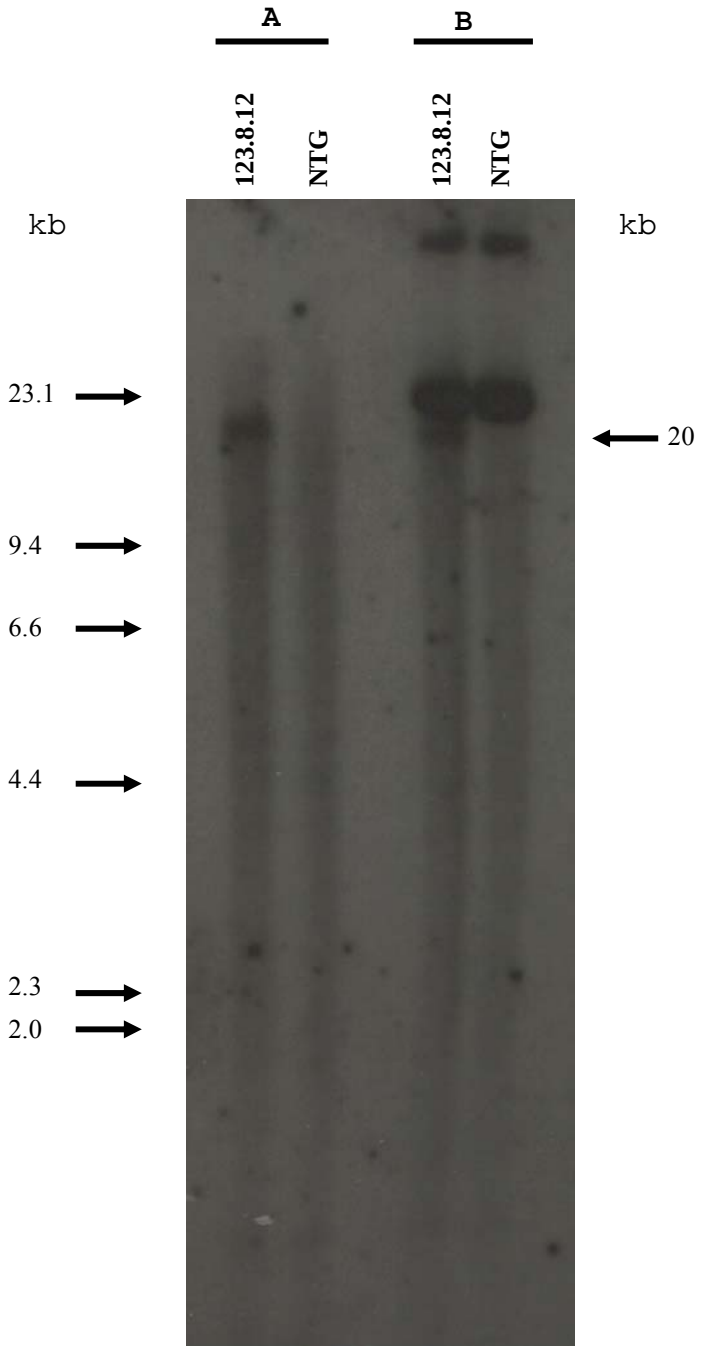


Figure 2 – Probe LB

High resolution scan of an autoradiograph of a Southern blot hybridized with Probe LB (refer to Figure 1). The Southern was generated via electrophoresis of:

Panel A: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane. No hybridization signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane, with 230 pg of *Bgl*II-digested pCGP1991 transformation vector DNA added to each. In addition to hybridizing bands detected in Panel A, further hybridizing bands of approximately 28 kb and >50 kb were detected in all lines corresponding to both supercoiled and open circle forms of uncut plasmid, respectively. The signal intensity of the 28 kb band in the NTG line is consistent with that for 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA that was added to the sample.

Southern Blot Analysis of Carnation Genomic DNA Using Probe *SuR*B

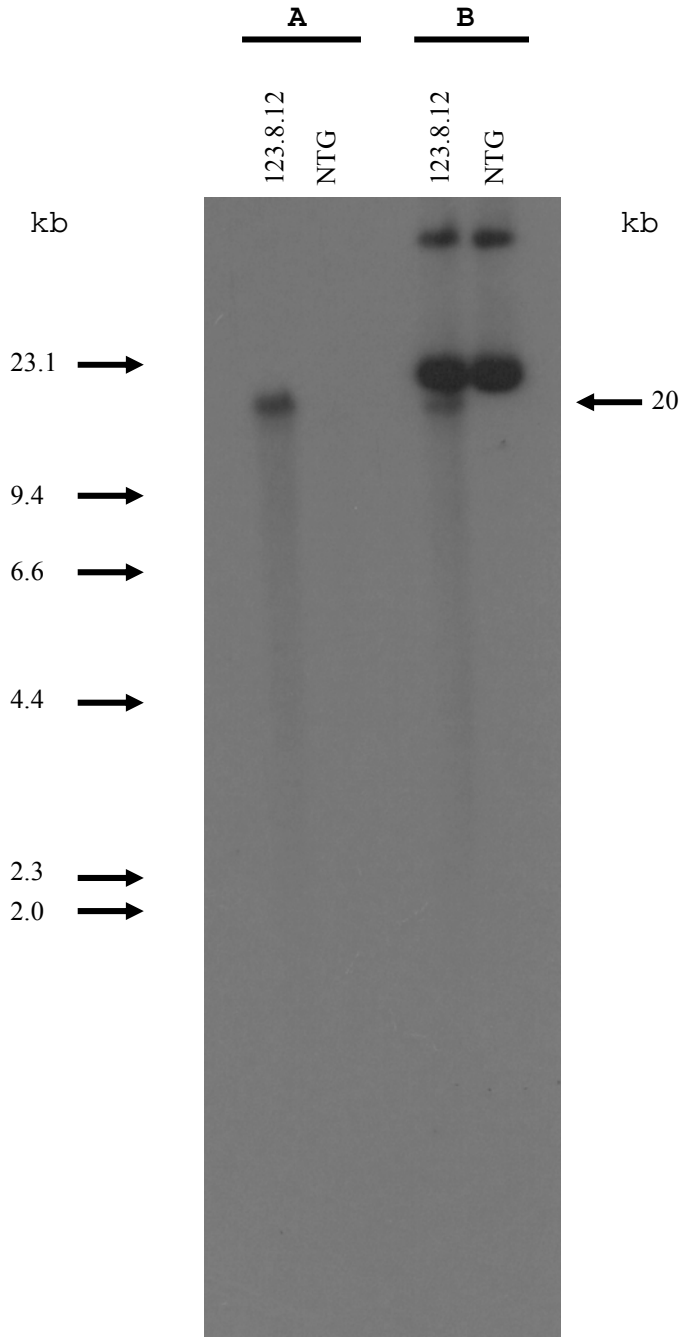


Figure 3 – Probe *SuR*B

High resolution scan of an autoradiograph of a Southern blot hybridized with Probe *SuR*B (refer to Figure 1). The Southern was generated via electrophoresis of:

Panel A: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane. No hybridization signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane, with 230 pg of *Bgl*II-digested pCGP1991 transformation vector DNA added to each. In addition to hybridizing bands detected in Panel A, further hybridizing bands of approximately 28 kb and >50 kb were detected in all lines corresponding to both supercoiled and open circle forms of uncut plasmid, respectively. The signal intensity of the 28 kb band in the NTG line is consistent with that for 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA that was added to the sample.

Southern Blot Analysis of Carnation Genomic DNA Using Probe *F3'5'H*

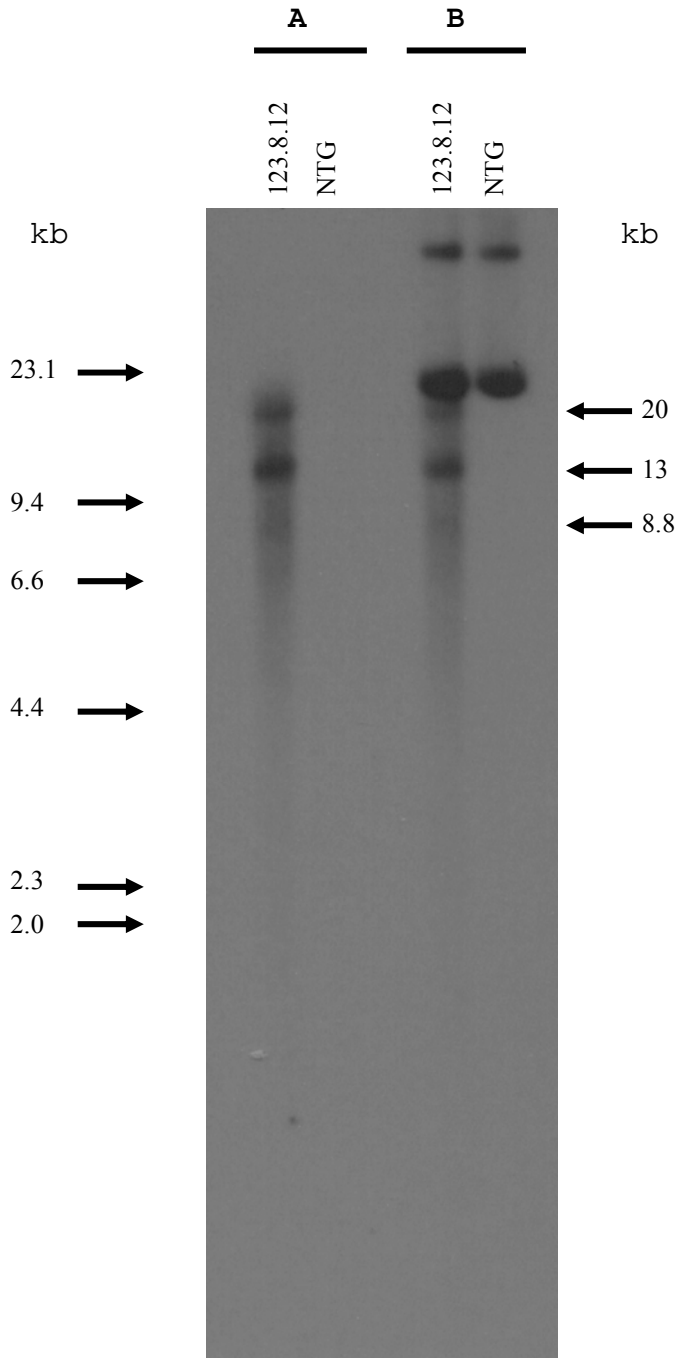


Figure 4 – Probe *F3'5'H*

High resolution scan of an autoradiograph of a Southern blot hybridized with Probe *F3'5'H* (refer to Figure 1). The Southern was generated via electrophoresis of:

Panel A: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane. No hybridization signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua ™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane, with 230 pg of *Bgl*II-digested pCGP1991 transformation vector DNA added to each. In addition to hybridizing bands detected in Panel A, further hybridizing bands of approximately 28 kb and >50 kb were detected in all lines corresponding to both supercoiled and open circle forms of uncut plasmid, respectively. The signal intensity of the 28 kb band in the NTG line is consistent with that for 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA that was added to the sample.

Southern Blot Analysis of Carnation Genomic DNA Using Probe *gDFR*

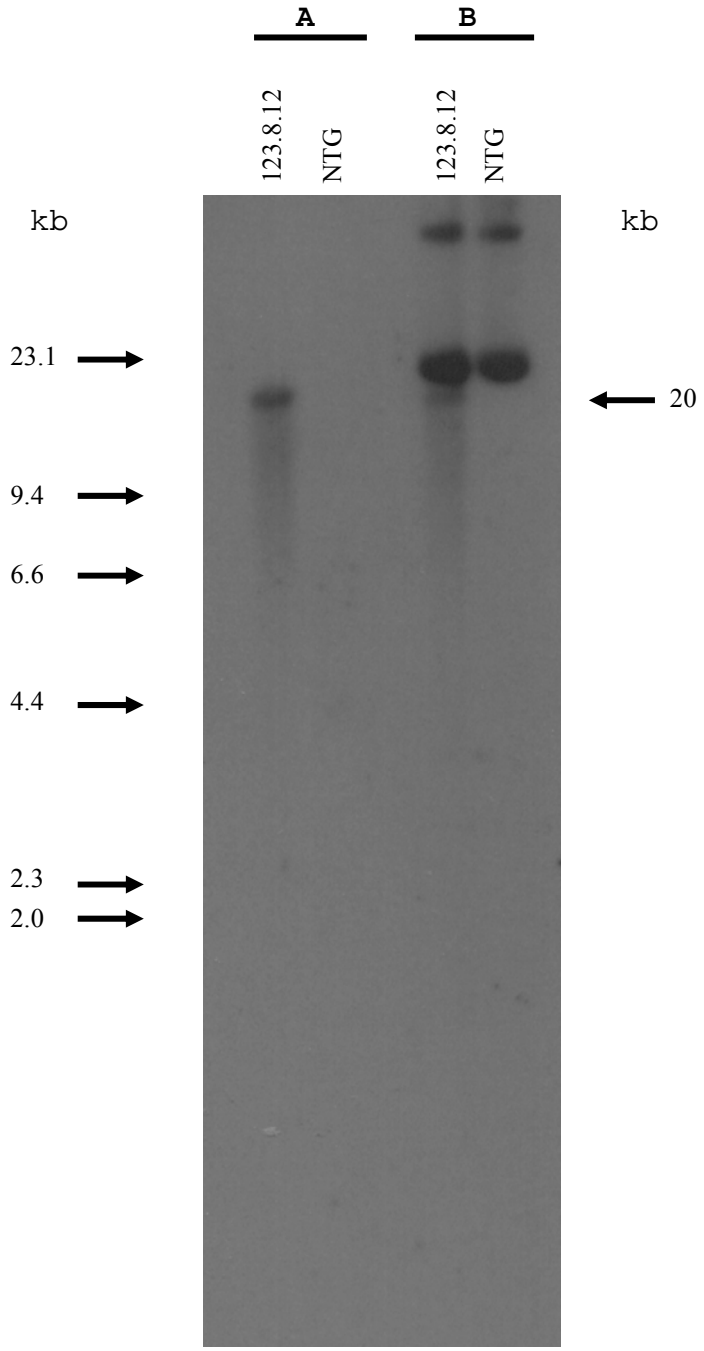


Figure 5 – Probe *gDFR*

High resolution scan of an autoradiograph of a Southern blot hybridized with Probe *gDFR* (refer to Figure 1). The Southern was generated via electrophoresis of:

Panel A: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane. No hybridization signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 10µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (FLORIGENE Moonaqua™) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane, with 230 pg of *Bgl*II-digested pCGP1991 transformation vector DNA added to each. In addition to hybridizing bands detected in Panel A, further hybridizing bands of approximately 28 kb and >50 kb were detected in all lines corresponding to both supercoiled and open circle forms of uncut plasmid, respectively. The signal intensity of the 28 kb band in the NTG line is consistent with that for 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA that was added to the sample.

Southern Blot Analysis of Carnation Genomic DNA Using Probe RB

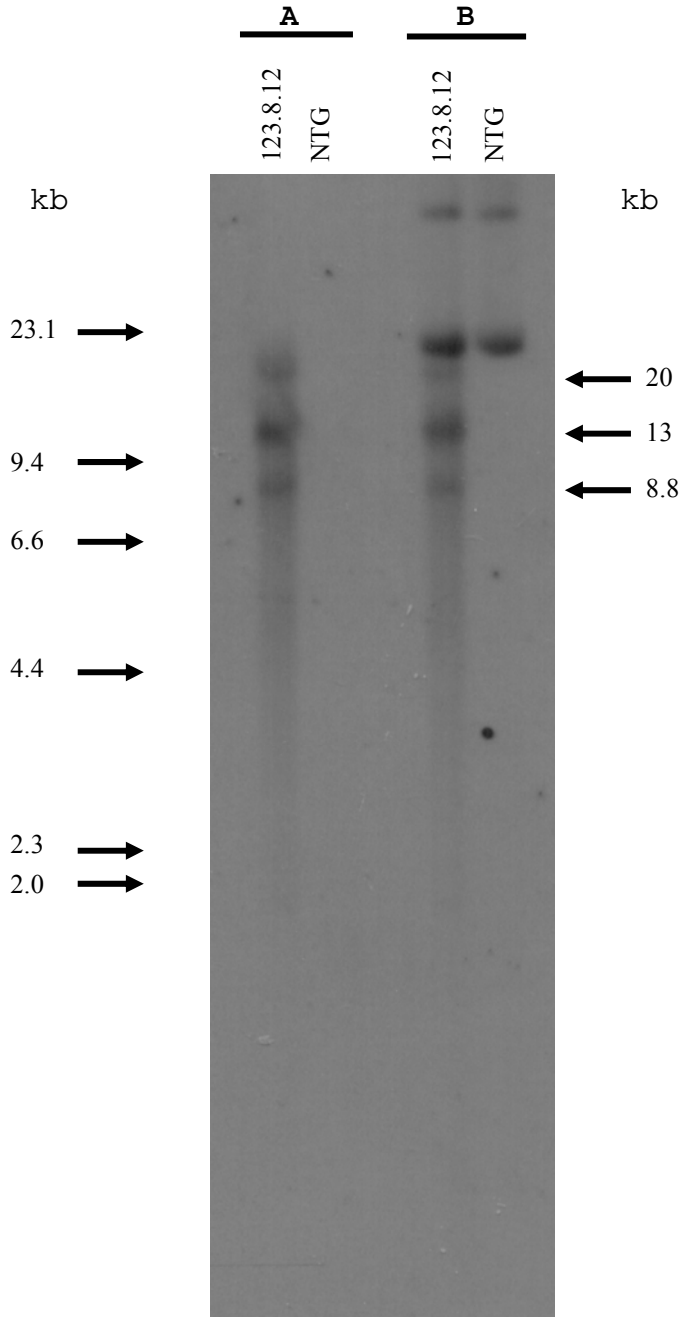


Figure 6 – Probe RB

High resolution scan of an autoradiograph of a Southern blot hybridized with Probe RB (refer to Figure 1). The Southern was generated via electrophoresis of:

Panel A: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (*Florigene Moonaqua ™*) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane. No hybridization signal can be detected in the NTG lane. Results are summarized in Table 3.

Panel B: 10 µg *Bgl*II-digested total leaf DNA isolated from transgenic carnation line 123.8.12 (*Florigene Moonaqua ™*) or 10 µg *Bgl*II-digested total petal DNA isolated from non-transgenic carnation line 123 (NTG) as indicated above each gel lane, with 230 pg of *Bgl*II-digested pCGP1991 transformation vector DNA added to each. In addition to hybridizing bands detected in Panel A, further hybridizing bands of approximately 28 kb and >50 kb were detected in all lines corresponding to both supercoiled and open circle forms of uncut plasmid, respectively. The signal intensity of the 28 kb band in the NTG line is consistent with that for 230 pg of *Bgl*II-digested pCGP1991 plasmid DNA that was added to the sample.

ATTACHMENT A6

Schematic of inserts

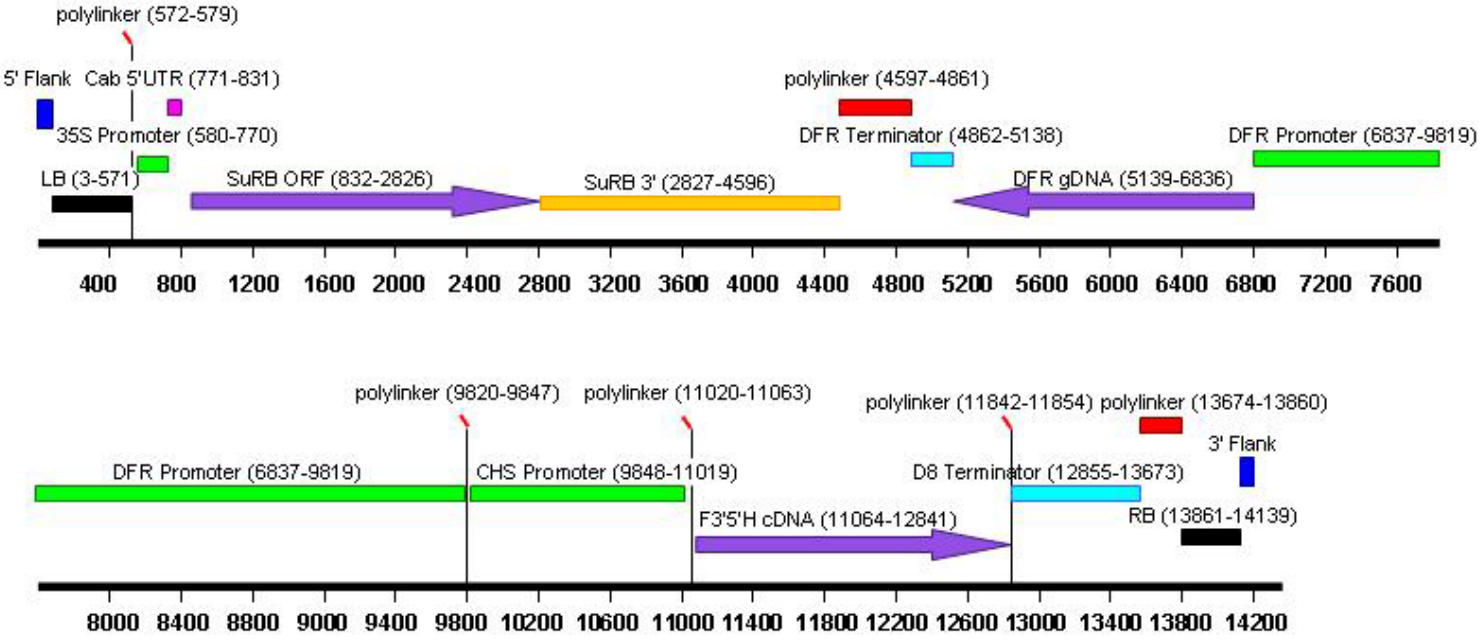
FLORIGENE Moonaqua™ contains three integration loci. The structure of each locus is presented below with reference to the sequence and the table of genetic elements shown in Attachment 3..

The schematic diagrams are colour coded for direct reference to the sequence of the inserts, provided at Attachment A7.

The co-ordinates shown in brackets in the schematic diagrams for Locus 1, Locus 2 and Locus 3 refer to table 1 of attachment A3.

Transformation vector pCGP1991 and pCGP1470 (used in C/NL/04/02) are both based on the vector pWTT2132 and have in common the Tet resistance gene complex and the Modified pACYC184 replicon. In the table below corrections have been made to the sequence numbering of the Tet resistance gene complex and the Modified pACYC184 replicon.

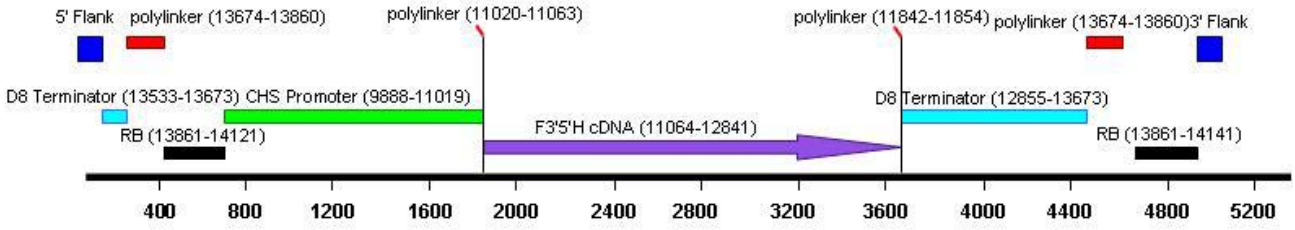
Moonaqua Integration Locus 1 (14433 bp)



Co-ordinates in brackets refer to the corresponding nucleotide position on the transformation vector pCGP1991 (see Attachment A3, Table 1).

5' flank – refers to the 5' flanking genomic DNA sequence.
3' flank – refers to the 3' flanking genomic DNA sequence.
All other components are transformation vector derived and colour coded with reference to the sequence shown in Attachment A7.

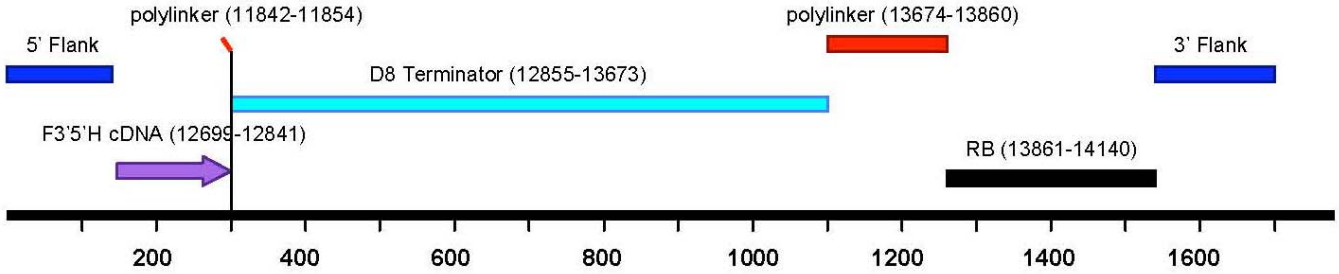
Moonaqua Integration Locus 2 (5140 bp)



Co-ordinates in brackets refer to the corresponding nucleotide position on the transformation vector pCGP1991 (see Attachment A3, Table 1).

5, flank – refers to the 5, flanking genomic DNA sequence.
3, flank – refers to the 3, flanking genomic DNA sequence.
All other components are transformation vector derived and colour coded with reference to the sequence shown in Attachment A7.

Moonaqua Integration Locus 3 (1741 bp)



Co-ordinates in brackets refer to the corresponding nucleotide position on the transformation vector pCGP1991 (see Attachment A3, Table 1).).

5' flank – refers to the 5' flanking genomic DNA sequence.
3' flank – refers to the 3' flanking genomic DNA sequence.
All other components are transformation vector derived and colour coded with reference to the sequence shown in Attachment A7.

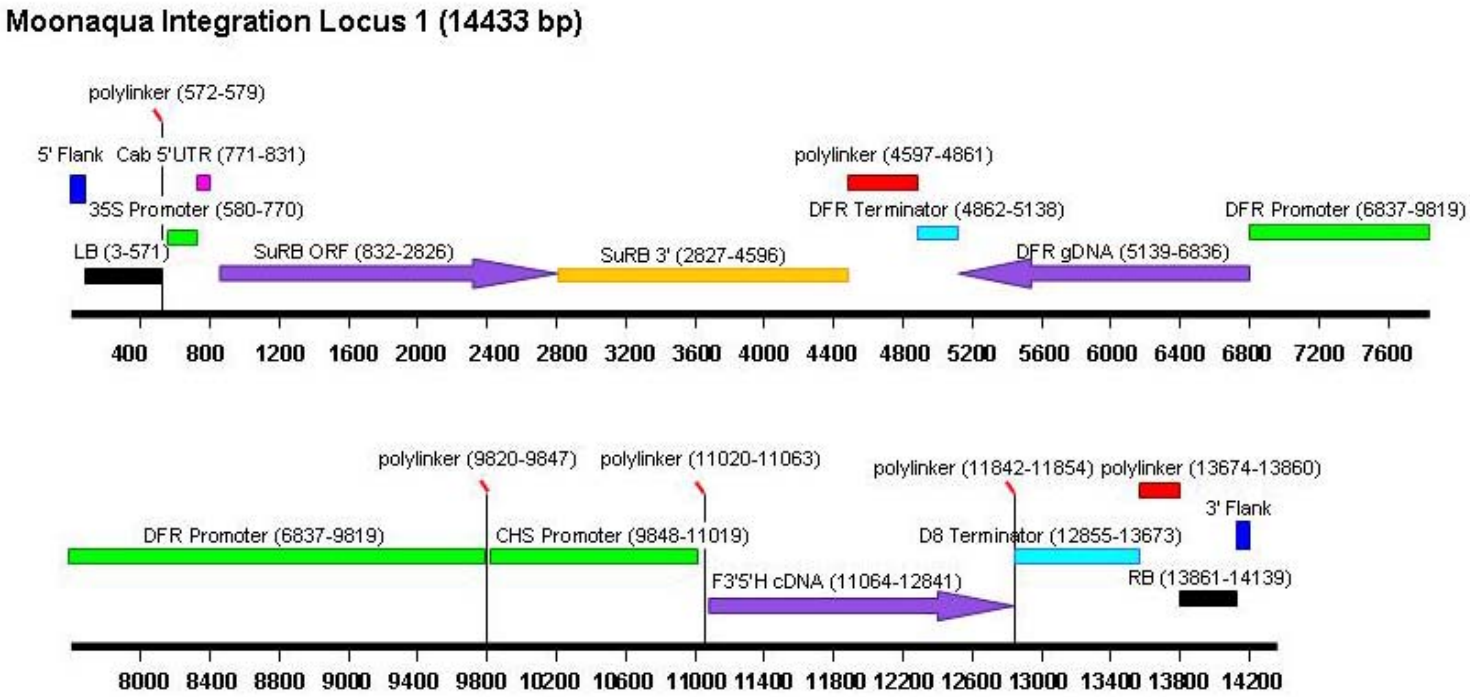
ATTACHMENT A7

The nucleotide sequences of the insert(s) and of the associated flanking regions

FLORIGENE Moonaqua™ contains three integration loci. The sequences of the three loci are provided in this attachment, at figures 1, 2 and 3.

The nucleotide sequence is colour coded for direct reference to schematic diagrams of the inserts, provided at Attachment A6.

Figure 1. FLORIGENE Moonaqua™ Locus 1 schematic and sequence (page 2).



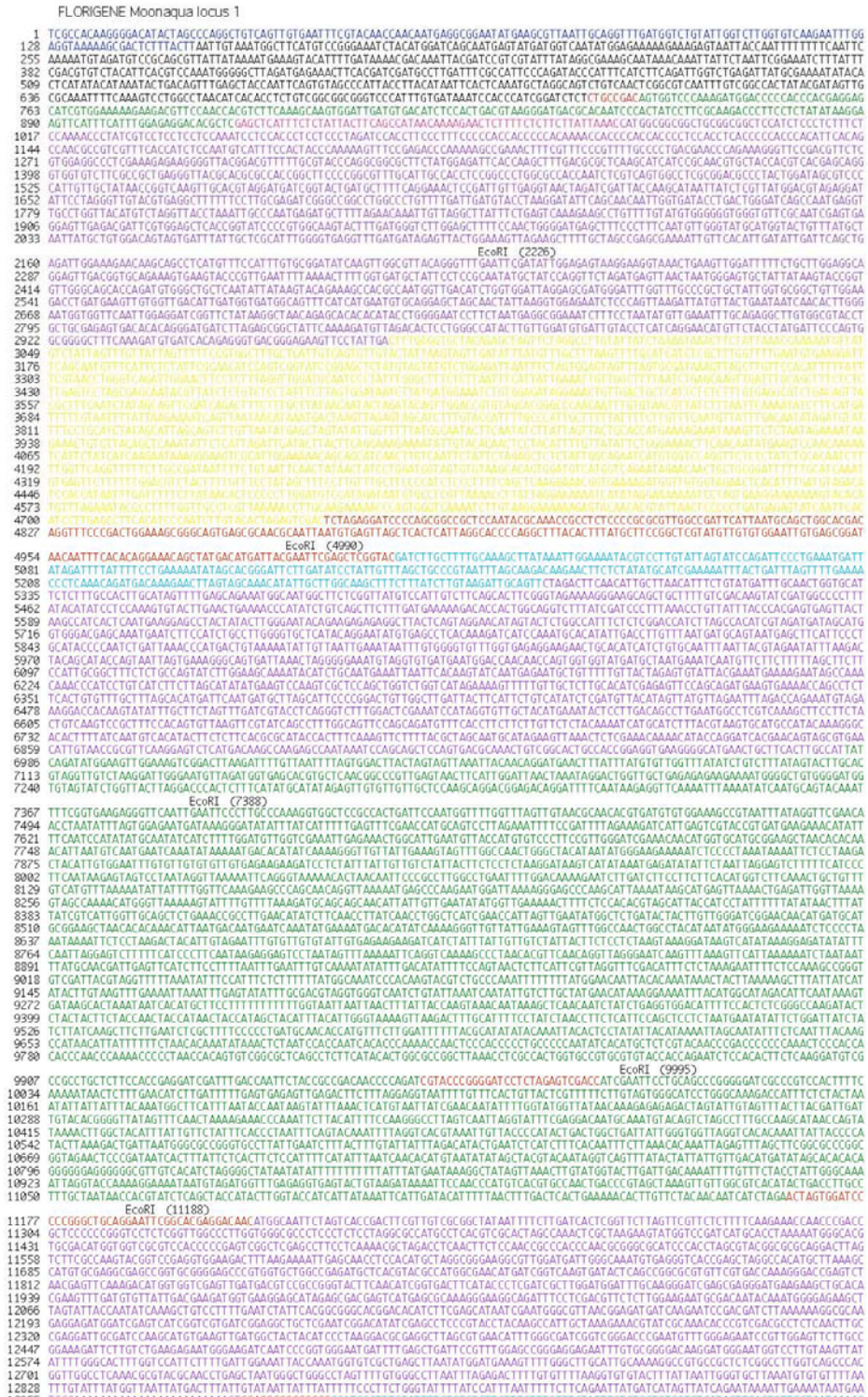
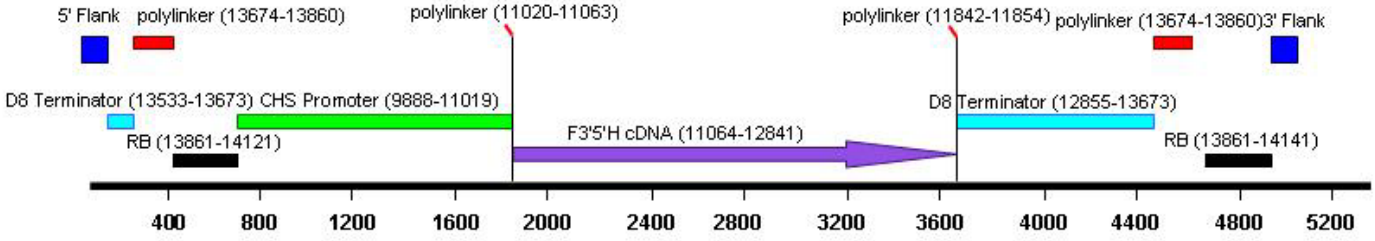

FLORIGENE Moonaqua locus 1
EcoRI (2226)
EcoRI (4990)
EcoRI (7388)
EcoRI (9995)
EcoRI (11188)
EcoRI (14333)

Figure 2. FLORIGENE Moonaqua™ Locus 2 schematic and sequence.

Moonaqua Integration Locus 2 (5140 bp)



FLORIGENE Moonaqua locus 2

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 275 ATCCTCGACCTAGAGCGGCCGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG
 412 AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCAGATCCCTGAAAGCGACGTTGGATGTTAACATCTACAAATTGCCTTTTCTTATCGACCATGTACGTAAGCGCTTACGT
 549 TTTTGGTGGACCCTTGAGGAAACTGGTAGCTGTTGTGGGCCTGTGGTCTCAAGATGGATCATTAATTTCCACCTTCACCTACGATGGGGGGCATCGCACCGGTGAGTAATATTGTACGGCTAAGAGCGAATTTGGCC
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 823 TTTCTTGTAGTGGGCATCCTGGGCAAAGACCATTTCTCTACTAAATATTATTATTTACAAATGGCTTCATTTAATACCAATAAGTATTTAAACTCATGTAATTATCGAACAATATTTTGGTATGGTTATAACAAAGA
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1097 AGCCTTTGCCAAGCATAACCAGTATAAAACTTGGCTACATTTATTGTTCTATTTCACCCTAATTTCAGTACAAATTTTAGGTCACGTAAATTGTTACCCCATACTGACTGGCTGATTATTGGGTGGTTAGGTCACAC
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                                                                                                                                                EcoRI (1894)
1782 ACCATACTTGGTACCATCATTATAAATTCATTGATACATTTTTAACTTTGACTCACTGAAAAACACTTGTTCTACAACAATCATCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGACAACATGG
1919 CAATTCTAGTCACCGACTTCGTTGTCGCGGCTATAATTTTCTTGATCACTCGGTTCTTAGTTCGTTCTCTTTTCAAGAAACCAACCCGACCGCTCCCCCGGGTCCTCTCGGTTGGCCCTTGGTGGGCGCCCTCCCT
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3015 TCTCAACTTGCCGAGGATTGCGATCCAAGCATGTGAAGTTGATGGCTACTACATCCCTAAGGACGCGAGGCTTAGCGTGAACATTTGGGCGATCGGTCGGGACCCGAATGTTTGGGAGAATCCGTTGGAGTTCTTGC
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4111 TTCGCACATTAATAATACTCCTATTATTATTAACCAACTCCATACTTGGAGAAAAAGTCAGCTTGTCTACACAAGACGAAAAAAAAATTAGATAGAATAAACTATATAGAAACAAGGAATTAAGAACAATATCAAGA
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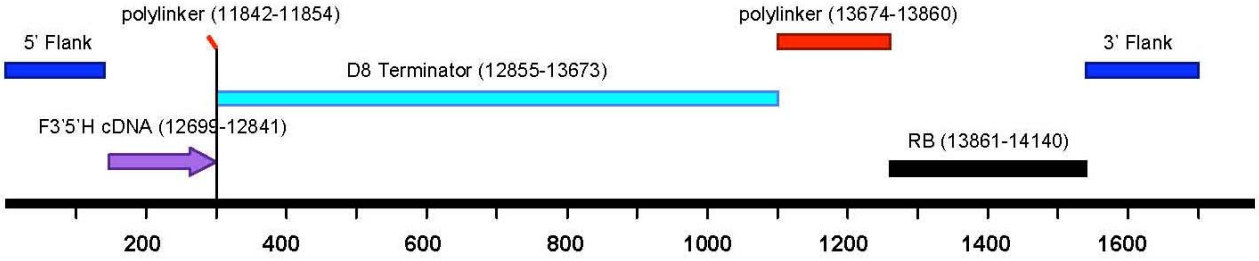
Figure 3. FLORIGENE Moonaqua™ Locus 3 schematic and sequence.

Moonaqua Integration Locus 3 (1741 bp)



FLORIGENE Moonaqua locus 3

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 781 ACACAAGACGAAAAAAAAATTAGATAGAATAAACTATATAGAAACAAGGAATTAAGAACAATATCAAGAAGAACACTCTTCAATTATCTGTACTAGATGTCAACGTTAACAACATCCAAATAAATTAGTC
 911 CTAATGAGGGGAAGCCATACGTTAAAATTGATGCTAAAGTTCTTAAATAGTTCATTATAGAATAGAGTGGACTACTTACCACTGTTCTAATTTATTGGCTCACTCATTTATAAATACTTATACTTAATTA
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1691 GTTAAAACATACTAAAAATAGAAAGGGGACAAATAATTTGGGAAAAATTTT
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ATTACHMENT A8

Bioinformic analysis of coding sequences; Summary of BLAST searches for T-DNA coding region and flanking sequences; ORF homology

Summary
The flanking sequences of the integrations (loci 1 to 3 in Attachment A6) were analysed for new open reading frames. We looked for open reading frames larger than 50 amino acids and starting with a methionine in each of the 150 bp flanking sequences. No open reading frames (ORFs) fitting these criteria were found at the 6 junctions of integrated DNA and carnation genomic DNA.
Blast searches using the translated nucleotide sequence of the open reading frames contained in the T-DNA of the binary vector pCGP1991 (tobacco ALS, petunia DFR, pansy F3'5'H (BP40)) revealed highest identities to the same enzymes from various plant species. Under the conditions used there were no obvious homologies to known toxic proteins or allergenic proteins.

Aim
To provide up-to-date searches of the GenBank and SwissProt database using the translated sequences of the coding regions contained in the T-DNA of the binary vector, pCGP1991.

Methods
The sequences analyzed were the (1) selectable marker enzyme tobacco acetolactate synthase (ALS), (2) petunia dihydroflavonol 4-reductase (DFR), derived from 6 exons within the genomic petunia DFR nucleotide sequence contained in pCGP1991 and (3) pansy flavonoid 3'5'hydroxylase (F3'5'H). The sequences are shown in table 1.

Searches were performed against the GenBank and SwissProt databases using:

- the translated amino acid sequence (the longest open reading frame with an AUG start codon and a stop codon) derived from the nucleic acid sequence.
- the search program BLAST2.2.9 located within the NCBI (The National Centre for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov).

The alignments were saved as the following HTML files, provided with this application;

- Oct06ALSBlast.htm
- Oct06 DFRBlast.htm
- Oct06 F3'5'HBlast.htm

Results
(1)*SuRB* (ALS) (664 amino acids)
Highest identities observed with acetolactate synthase (ALS) proteins (also described as acetohydroxyacid synthase) from various plant species. To our knowledge, there appeared to be no homology to known toxic proteins or allergens.
(2) Petunia DFR (genomic) (380 amino acids)
Highest identity was with the dihydroflavonol 4- reductase from *P. hybrida* followed by DFR sequences from other plant species. To our knowledge, there appeared to be no homology to known toxic proteins or allergens.
(3) Pansy F3'5'H (BP40) (506 amino acids)
Highest identity with the *Gossypium hirsutum* putative F3'5'H amino acid sequence followed by flavonoid 3'5' hydroxylases from other plant species (74 to 71% identity). Lower level identities

(below 51%) were observed with various flavonoid 3' hydroxylases from plants. To our knowledge, there appeared to be no homology to known toxic proteins or allergens.

Conclusion

The translated nucleotide sequences of tobacco ALS, petunia DFR and pansy F3'5'H were not homologous to any known toxic or allergenic proteins found in the GenBank and SwissProt databases using the BLAST searching program located within the NCBI website.

Literature cited

1. (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) Appendix 1.

Table 1 Translated nucleotide sequences used in BLAST searches

Code	Sequence
SuRB (ALS)	MAAAAAAPSPSFSKTLSSSSSKSSTLLPRSTFPFPHHPHKTTPPPLHLTPTHIH SQRRRFTISNVISTTQKVSETQKAETFVSRFAPDEPRKGSDVLVEALEREGVT DVFAYPGGASMEIHQALTRSSIIRNVLPRHEQGGVFAAEGYARATGFPGVCI ATSGPGATNLVSGLADALLDSVPIVAITGQVPRRMIGTDAFQETPIVEVTRSI TKHNYLVMDVEDIPRVVREAFFLARSGRPGPVLIDVPKDIQQQLVIPDWDQ PMRLPGYMSRLPKLPNEMLLEQIVRLISESKKPVLYVGGGCSQSSEELRRFV ELTGIPVASTLMGLGAFPTGDELSLSMLGMHGTVYANYAVDSSDLLLAFGV RFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSICADIKLALQGLNSI LESKEGKLKLDFSAWRQELTVQKVKYPLNFKTFGDAIPPQYAIQVLDELTN GSAIISTGVGQHQMWAAQYYKYRKPRQWLTSGGLGAMGFGLPAAIGAAV GRPDEVVVDIDGDGSFIMNVQELATIKVENLPVKIMLLNNQHLGMVVQWE DRFYKANRAHTYLGNPSNEAEIFPNMLKFAEACGVPAARVTHRDDLRAAI QKMLDTPGPYLLDVIVPHQEHVLPMIPSGGAFKDVITEGDGRSSY
gpetdfr (petunia DFR)	MASEAVHAPSPPVAVPTVCVTGAAGFIGSWLVMRLLERGYNVHATVRDPE NKKKVKHLLELPKADTNLTLWKADLTVEGSFDEAIQGCQGVFHVATPMDF ESKDPENEVIKPTVRGMLSIIESCAKANTVKRLVFTSSAGTLDVQEQQKLFY DQTSWSDLDFIYAKKMTGWMYFVSKILAEKSAMEETKKKNIDFISIIPPLVV GPFITPTFPPSLITALSLITGNEAHYCIIKQGQYVHLDDLCEAHIFLYEHPKAD GRFICSSHHAIIYDVAKMVREKWPEYYVPTEFKGIDKDLPVVSFSSKKLTDM GFQFKYTLEDMYKGAIETCRQKQLLPFSTRSAADNGHNREAIAISAQNYAS GKENAPVANHTEMLTNVEV
pansyf3'5'h (pansy F3'5'H)	MAILVTDFVVAAIIFLITRFLVRSLFKKPTRPLPPGPLGWPLVGALPLLGAMP HVALAKLAKKYGPIMHLKMGTCDMVVASTPESARAFLKTLDLNFSNRPPN AGASHLAYGAQDLVFAKYGPRWKTLRKLSNLHMLGGKALDDWANVRVT ELGHMLKAMCEASRCGEPVVLAEMLTYAMANMIGQVILSRRVFVTKGTES NEFKDMVVELMTSAGYFNIGDFIPSIAWMDLQGIERGMKKLHTKFDVLLTK MVKEHRATSHERKGKADFLDVLLEECDNTNGEKLSITNIKAVLLNLFTAGT DTSSSIIEWALTEMIKNPTILKKAQEEMDRVIGRDRRLLESDISSLPYLQAIAK ETYRKHPSTPLNLPRIAIQACEVDGYYIPKDARLSVNIWAIGRDPNVWENPL EFLPERFLSEENGKINPGGNDFKLIPFGAGRRICAGTRMGMVLVSYILGTLV HSFDWKLPNGVAELNMDESFGLALQKAVPLSALVSPRLASNPYAT

ATTACHMENT A9

Bioinformic analysis of flanking sequences; BLAST searches for T-DNA coding region and flanking sequences; homology to allergens

Introduction
The flanking sequences of the integrations (loci 1 to 3 in Attachment A6) were analysed for new open reading frames. We looked for open reading frames larger than 50 amino acids and starting with a methionine in each of the 150bp flanking sequences. No open reading frames (ORFs) fitting these criteria were found at the 6 junctions of integrated DNA and carnation genomic DNA. Public databases were therefore searched for short identical stretches of contiguous amino acids within sequences of known allergens using the deduced amino acid sequences of the three transferred coding sequences (ALS, DFR and F3'5'H).

Method
The amino acid sequences for the three transferred coding sequences of ALS, DFR and F3'5'H were used to search databases of known allergens using BLAST2.2.13 (Altschul et.al, 1997) found on the webpage of the U.S. National Library of Medicine's Entrez website at http://www.ncbi.nlm.nih.gov/BLAST.

The protein BLAST algorithm was selected to search for short nearly exact matches. The following search parameters were used; expect 20000; word size: 2; matrix: PAM30; gap costs: existence 9, extension 1. The Entrez query was limited by the word 'allergen' and both 500 descriptions and alignments were requested.

Results
Blast output results are shown in three files attached with this application;

- AllergenALSBLASTQ4.htm
- AllergenDFRBLASTQ4.htm
- AllergenF3'5'HBLASTQ4.htm

The BLAST results were scanned for identical peptide sequences of 6 or more contiguous amino acids. Identical peptide sequences from proteins that are not allergens but were retrieved by use of the query limit 'allergen' were excluded from further consideration. These were, for example, antibodies that react with allergens and putative proteins from open reading frames identified in genome projects that share similarities with allergens.

Antigenicity predictions for both the transgenic protein and the allergen were then carried out by constructing a hydrophilicity plot by the Hopp and Woods method using a window size of 6. The height of the peak surrounding the identical sequence identified by the BLAST search in either the transgenic protein or the allergenic protein was checked to see if it coincides with the highest peak in the plot. If it does then the probability for antigenicity of the identical peptide sequence is high. A schematic overview of the steps taken to assess allergenic potential of the stretches of 6 identical amino acids is shown in Figure 1. The example used is the sequence, 'EGQRVV' is found in both F3'5'H and the allergen Can f 1 from *Canis familiaris*.
Scientific literature was then checked for data on IgE-binding epitopes within the allergens identified.

Summary of results

Results are summarized in the three tables below. In these, the following abbreviations are used;

* = allergenic protein listed by WHO/IUIS (http://www.allergen.org)
aa = amino acid
gi: = GenBank accession number (retrievable from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi)

Antigenic? (prediction)

"yes' = the highest point of the antigenicity plot falls within the identical sequence.
"-" = the highest scoring point does not fall within the identical sequence.

ALS

Identical sequence	Allergen (1)			Antigenic? (prediction)		Part of linear IgE epitope?
	name	Length aa	source	Transgenic protein	Allergen	
AAAAAA	Asp f 4*	286	Fungus *Aspergillus fumigatus*	- (2)	-	-
ELTGIP	Pen c 19*	503	Fungus *Penicillium citrinum*	-	-	-
IIRNVL	Gal d 2*	386	Chicken *Gallus domesticus*	-	-	-
PRKGSD	Amb a 1.4*	392	Ragweed *Ambrosia artemisiifolia*	-	-	-
YLGNPS	Chloro-phyll a/b binding protein	264	Celery *Apium graveolens*	-	-	-
EALERE	Fus c 3	450	Fungus *Fusarium culmorum*	-	-	-
GSDVLV	SMIPP-S	257	Mite *Sarcoptes scabiei* type hominis	-	-	-
AAAAAP	Tri a 4	518	Wheat *Triticum aestivum*	- (2)	-	-
IIRNVL	Chain D, S-ovalbumin	385	Chicken Gallus domesticus	-	-	-
VLYVGG	Expansin-like protein	250	Arabidopsis thaliana	-	-	-
VLYVGG	Allergen-like protein	260	Arabidopsis thaliana	-	-	-
LEREGV	Alkyl hyperoxide reductase	173	Anaeromyxobacter dehalogenans	-	-	-

(1) Accessions: Asp f 4: gi:83300369, gi:3005839; Pen c 19: gi:14423733, gi:1498496; Gal d 2: gi:129293, gi:15826578, gi:15826579; Chlorophyll a/b binding protein: gi:1769849, gi:14423661; Amb a 1.4: gi:113478, gi:166445; Fus c 3: gi:74654406; SMIPP-S: gi:38202333; Tri a 4: gi:55859458; Chain D, S-ovalbumin: gi:34811333, gi: 34811332; gi: 34811331, gi: 34811330; expansin-like protein: gi: 29028846; gi: 110736418; allergen-like protein: gi: 2245060, gi: 7268454, gi: 7488049; alkyl hyperoxide reductase: gi: 86157572, gi: 85774083.
(2) Calculation not possible because sequence is N-terminal of acetolactate synthase.

F3'5'H

Identical sequence	Allergen (1)			Antigenic? (prediction)		Part of linear IgE epitope?
	name	length aa	source	Transgenic protein	Allergen	
EGQRVV	Can f 1*	174	Dog *Canis familiaris*	-	-	-
SEENGK	sbpA	1268	Bacillus sphaericus	-	-	-
PGGNDF	Ag5-2	227	Aedes aegypti	-	-	-
ALAKLA	Salivary Ag 2	264	Ctenocephalides	-	-	-

(1) Accession: Can f 1: gi:2598974; sbpA gi: 6665712; Ag5-2 gi: 137395; alivary Ag2: gi: 7638032.

DFR

Identical sequence	Allergen (1)			Antigenic? (prediction)		Part of linear IgE epitope
	name	length aa	source	Transgenic protein	Allergen	
EGSFDE	Gad c 1*	113	Baltic cod *Gadus callarias*	-	-	-
VVSFSS	Cuc m 1*	731	Muskmelon *Cucumis melo*	-	-	-
GMLSII	FcER1	244	Homo sapiens	-	-	-
KDLPVV	Il5R	415	Mus musculus	-	-	-

(1) Accession: Gad c 1: gi:131112; Cuc m 1: gi:71153243; FcER1: gi: 4503677; Il5R: gi: 6680431.

Conclusion

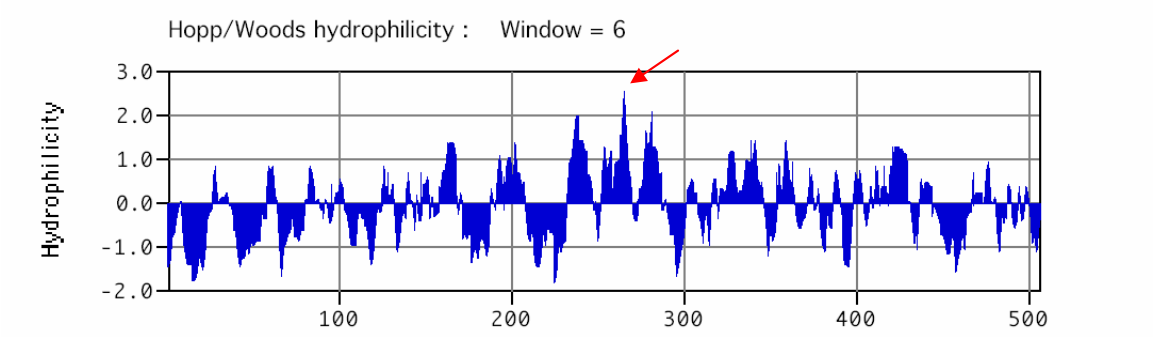
None of the transferred coding sequences inserted into FLORIGENE Moonaqua™ contain any sequences of high antigenicity comparable to those of allergenic proteins with short identical amino acid sequences.

Figure 1 Overview of steps taken to assess antigenic potential of the stretch of 6 identical amino acids found in F3'5'H and the allergen Can f 1 from *Canis familiaris*.

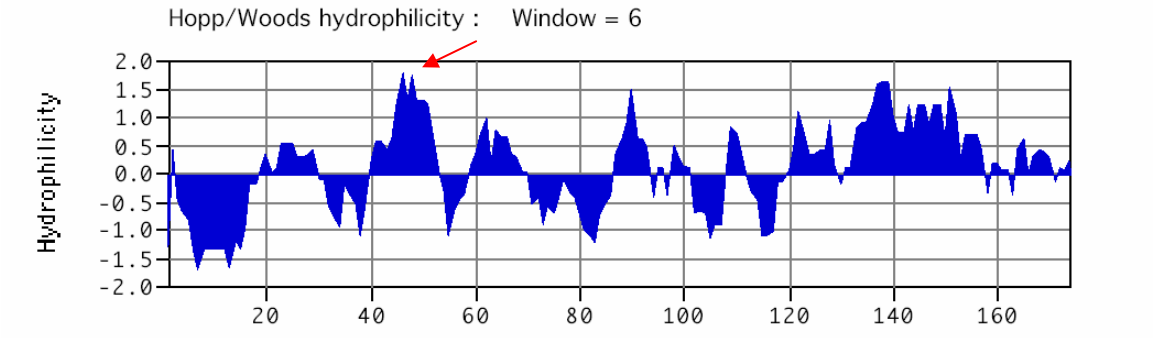
Step 1
6 contiguous identical amino acids 'EGQRVV' were identified in the amino acid sequence for F3'5'H and known allergen Can f 1 (Database Accession gi:2598974).

Step 2
Hydrophilicity plots for prediction of antigenicity for the amino acid sequences for F3'5'H and Can f 1 were constructed by the Hopp and Woods method using a window size of 6. (Program can be accessed from the following website http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html)
Plots for F3'5'H and Can f 1 are shown below.
The location of the highest peak in each plot was determined (shown by the red arrows). These regions correspond to the sequence having the highest allergenic potential.

F3'5'H (the highest point in the plot is identified by the red arrow)



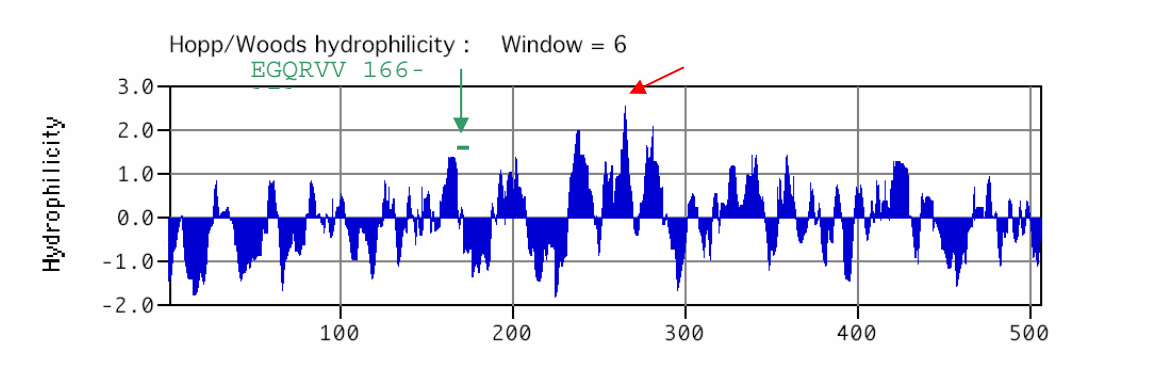
Can f 1 (the highest point in the plot is identified by the red arrow)



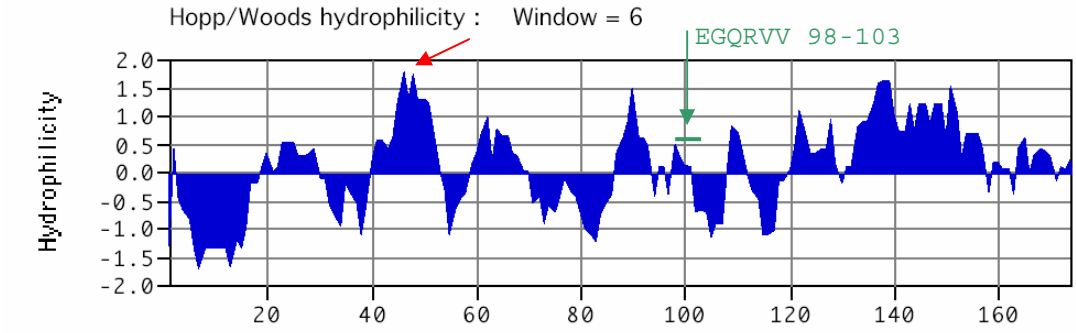
Step 3

The amino acid motif 'EGQRVV' occurs at position 166-171 of F3'5'H and 98-103 of Can f 1. These regions are located on the hydrophilicity plots (see below, shown in green).

F3'5'H (red arrow = highest point in the plot; green arrow = location of identical sequence)



Can f 1 (red arrow = highest point in the plot; green arrow = location of identical sequence)



Step 4

The highest point of each hydrophilicity plot does NOT fall within the 6 identical amino acids in either protein sequence. Therefore we may conclude that the amino acid sequence EGQRVV would have low allergenic potential.

ATTACHMENT A10

Description of the genetically modified product - FLORIGENE Moonaqua™ (123.8.12)

Flower colour

The primary difference between FLORIGENE Moonaqua™ (123.8.12) and the recipient plant is in the colour of the flowers, because of the production of delphinidin in the GMO. The colour of the transgenic flowers, matched as closely to actual colour as possible, as shown in the digital image below.

FLORIGENE Moonaqua™ (123.8.12)

Colours of the recipient and transgenic are also documented in the Table 1 using RHS colour codes. The codes are a set of colours published by the Royal Horticulture Society (London), used as a standard colour reference in the horticulture industry.

Table 1.RHS colour codes

Character	Control	FLORIGENE Moonaqua™ (123.8.12)
Petal main colour	159D	84B

PBR based comparison between recipient plant and transgenic plant

For Plant Breeders Rights purposes, UPOV (Union for the Protection of Plant Varieties), the International agency coordinating registration of new varieties has published a set of character measurements that may be used to distinguish between different carnation varieties. Measurement of many of these characteristics has been incorporated into our field trial design. Table 2 summarises the observations made for the control and the GMO FLORIGENE Moonaqua™ (123.8.12) derived from it.

Table 2. Comparison of key morphological characteristics

UPOV number	**Description**	**Control**	***Florigene Moonaqua ™* (123.8.12)**
1	Stem – laterals without flower buds or flowers	Present	Present
5	Plant – arrangement of individual flowers	One flowered	One flowered
6	Stem total length of 7 internodes (mean)	64 cm, long	66 cm, long
6	Stem total length of 7 internodes (Range)	59 – 68 cm	62 – 70 cm
7	Stem – thickness	medium	medium
8	Stem – length of 5th internode (average)	11.3cm, long	9.6cm, medium
8	Stem – length of 5th internode (range)	11-12cm	9-10.5cm
9	Stem–cross section	edged	edged
10	Stem-hollowness	absent	absent
11	Leaf shape	linear	linear
12	Leaf length (average)	11.9cm, long	12.8cm, long
12	Leaf length (range)	11 –13	12.5 – 13.5
13	Leaf width (average)	0.8cm, medium	0.8cm, medium
13	Leaf width (range)	0.7 – 0.9cm	0.7 – 0.9cm
14	Leaf- longitudinal axis	recurved	recurved
15	Leaf cross section	straight	straight
16	Leaf colour	green	green
17	Leaf – waxy layer	medium	medium
18	Leaf – margin	absent	absent
19	Bud shape	elliptical	elliptical
20	Bud- style extrusion	absent	absent
21	Flower diameter (average)	6.7cm	6 cm
21	Flower diameter (range)	6 –7 cm, large	5.5 – 6.5 cm large
22	Height of Corolla	Tall	Tall
23	Corolla – upper profile	Convex	Convex
24	Corolla – lower profile	flat	flat
25	Flower fragrance	Present little	Present little
26	Position of outer epicalyx leafs in relation to calyx	adpressed	adpressed
27	Apex of outer epicalyx lobes	acuminate	acuminate
28	Length of outer epicalyx lobes (average)	1.4cm	1.4cm average (medium)
29	Apex of inner epicalyx lobes	acuminate	acuminate

UPOV number	Description	Control	*Florigene Moonaqua™* (123.8.12)
30	Length of inner epicalyx lobes	Short	Short
31	Calyx length (average)	3.7 cm	3.5 cm, medium
31	Calyx length (range)	3 – 4cm	3 –4cm
32	Calyx shape	cylindrical	cylindrical
33	Calyx – longitudinal axis of lobes	convex	convex
37	Shape of calyx lobe	Long acute	Long acute
38	Length of calyx lobe	medium	medium
39	Flower type	double	double
40	Number of petals (average)	49	45
41	Predominant petal shape	Type 3	Type 3
42	Surface of petal blade	undulating	undulating
43	Margin of petal blade	Serrate	Serrate
44	Depth of incision on petal blade	shallow	shallow
45	Petal length (average)	5.4cm, long	4.9 cm, medium
45	Petal length (range)	5 – 6cm	4.5 – 6 cm
46	Petal width (average)	4.1 cm, broad	2.8 cm,medium
46	Petal width (range)	3.5 – 4.2cm	2.5 – 4 cm
52	Ovary shape	rhomboid	rhomboid
53	Ovary – main colour of lower part	whitish	whitish
54	Ovary surface	ribbed	ribbed
55	Number of styles	Only four	Three and four
56	Style length	Medium	Medium
57	Style shoulder	present	present

Note: The characteristics are numbered the same as the UPOV list of characters for carnation. Absence of number indicates an additional measurement made by Florigene.

ATTACHMENT A11

Trial data – FLORIGENE Moonaqua™ (123.8.12)

Introduction

Two data files containing morphology measurements are provided with this application, in excel format;

- **AQUA morphology data 2000.xls**
- **AQUA morphology data 2005.xls**

These provide the results from two block trials; one carried out in the Netherlands in 2000, and one in Australia in 2005. In each field trial measurements were taken for 18 morphological characteristics. T-TEST and single-factor ANOVA analysis was carried out on data collected from each trial.

Results

Year 2000 field trial

Results are shown in Table 1.

Table 1.Summary of 2000 trial. For details see attached file "AQUA morphology data 2000.xls. Characters were there was a significant difference between control and transgenic at the 1% level are highlighted in blue.

	Means		One –way ANOVA P-value	Percentage difference between means
CHARACTER	123	123.8.12		
Plant height (cm)	100	99	0.6803	1
Number of internodes per stem	14	13	0.0000	11
Length of 5th node, mm	113	96	0.0000	15
Thickness of 5th Node (mm)	8	7	0.0240	11
Flower Diameter (mm)	64	60	0.0285	6
Leaf Length of 3rd node from top (mm)	119	128	0.0210	7
Height of Corolla (mm)	27	26	0.7458	1
Calyx Diameter (mm)	18	18	0.4512	3
Calyx Length (mm)	37	35	0.0317	4
No. of Lobes per Calyx	7	6	0.0047	10
Number of petals per flower	49	45	0.0053	9
Petal Length (mm)	54	50	0.0001	7
Petal Width (mm)	41	28	0.0000	30
No. of Stamens	4	4	0.4000	12
No. of Styles	4	3	0.0007	19
No. of Anthers	1	0	0.0127	91
Style Length (mm)	23	25	0.0616	10
Stamen Length (mm)	22	19	0.0162	16

Year 2005 field trial

Results are shown in Tables 2 and 3. Figure 1 shows the appearance of the trial.

Table 2 provides information on time to flowers, which was very similar for the control and transgenic lines. Table 3 provides data for the same morphological characters that were measured in the year 2000 trial in the Netherlands.

Table 2.Time of first flowering. Plants were planted July 1 2005.

	Days after planting	
	FE123	**123.8.12**
First plant flowered	80	84
Last plant flowered	143	143
Average days to flowering	109	113

Table 3.Summary of 2005 trial. For details see attached file "AQUA morphology data 2005.xls. Characters were there was a significant difference between control and transgenic at the 1% level are highlighted in blue.

	Means		One –way ANOVA P-value	Percentage difference between means
CHARACTER	**123**	**123.8.12**		
Plant height (cm)	137	136	0.8684	1
Number of internodes per stem	19	19	0.9534	0
Length of 5th node, mm	87	85	0.5287	3
Thickness of 5th Node (mm)	5	5	0.0174	10
Flower Diameter (mm)	65	62	0.0853	5
Leaf Length of 3rd node from top (mm)	88	84	0.3663	4
Height of Corolla (mm)	25	24	0.1283	5
Calyx Diameter (mm)	25	24	0.1483	4
Calyx Length (mm)	32	31	0.1855	3
No. of Lobes per Calyx	6	6	0.1382	4
Number of petals per flower	47	46	0.6279	3
Petal Length (mm)	52	51	0.8046	3
Petal Width (mm)	36	33	0.8549	7
No. of Stamens	4	2	0.0112	49
No. of Styles	4	3	0.0016	26
No. of Anthers	2	0	0.0102	76
Style Length (mm)	33	29	0.0297	13
Stamen Length (mm)	25	20	0.0045	19

Anther number and pollen viability

In both trials, there was highly significant difference in anther number between line 123.8.12 and the control. In June 2006 anther number was again measured, in a sample of 8 flowers grown in Colombia. It was again observed that the number of anthers in the control line was significantly lower than the control (Table 4).

Table 4. Presence of anthers in flowers grown in Colombia

	FE 123	**FE123.8.12**
Number of flowers dissected	8	8
Number of flowers with anthers	7	1
Range of anther number per flower	0-5	0-1
Average (all flowers)	2.4	0.1
Average (anther carrying flowers only)	2.7	1

In September flowers from the Ecuador production of line 123.8.12 were also examined. Three out of ten flowers examined had anthers; two flowers had one anther, and one flower two anthers. A repeat measure of Colombia grown flowers of the variety was also made in September; three out of

ten flowers examined had a single anther in all cases.

Due to the low number of anthers, pollen viability has not been systematically measured. However, in 2000 pollen was extracted from one anther of line 123.8.12 and placed on a pollen germination medium (25% sucrose, 150 mg/L boric acid, 0.25% gelrite and 1g/L magnesium sulphate). Germination percentage was 8%, compared to 6% for pollen from anthers from control plants.

Discussion

There was relatively little variation between plants within treatment, and the variation was lower in the 2005 trial than in the 2000 trial. This low variation results in statistical differences between control and transgenic liens, even though the percentage difference (and so apparent difference) between means was sometimes less than 10% (for example calyx length and flower diameter in the 2000 trial). We consider significant morphological differences to be those where there was a statistical difference between control and transgenic in both trials, and where the difference between means for control and transgenic lines was greater than 10%. On this definition significant morphological differences are;

- Thickness of stem at 5th node
- Number of stamens
- Number of styles
- Number of anthers (confirmed in flowers grown in Colombia)
- Stamen length

For all these characters the value for the control line was higher than the transgenic line.

Anecdotally, the commercial trade generally accepts that the variety FLORIGENEMoonaqua™ has smaller flowers than other Florigene varieties derived from the same parental variety. This is clearly a valid observation, as for all flower size measurements, the transgenic flowers had lower or the same values as flowers from the control (Table 4).

Table 4. Difference in means for flower characters. The data shows the value calculated from mean of FE123/mean of 123.8.12). Any number above 1.0 indicates a smaller measurement in the transgenic line.

	Mean 123/ Mean 123.8.12	
CHARACTER	**2000 trial**	**2005 trial**
Flower Diameter (mm)	1.07	1.04
Height of Corolla (mm)	1.04	1.04
Calyx Diameter (mm)	1.00	1.04
Calyx Length (mm)	1.06	1.03
Number of petals per flower	1.09	1.02
Petal Length (mm)	1.08	1.02
Petal Width (mm)	1.46	1.09

Conclusion

The transgenic line FLORIGENE Moonaqua™ (123.8.12) produces smaller flowers than the parental line it is derived from, and these flowers have a significantly reduced reproductive capacity, measured by a much reduced number of anthers, styles and stamens. The styles and stamens are also significantly shorter in the transgenic line.

Figure 1. Appearance of 2005 trial. Trial was planted in Melbourne in July 2005.

ATTACHMENT B1

Potential toxicity and allergenicity of the ALS protein

This attachment is divided into three sections. The first provides a literature review of the ALS gene and gene product, the second provides an assessment of the potential toxicity of the ALS protein and the third an assessment of the potential allergenicity of the ALS protein.

1. Literature review

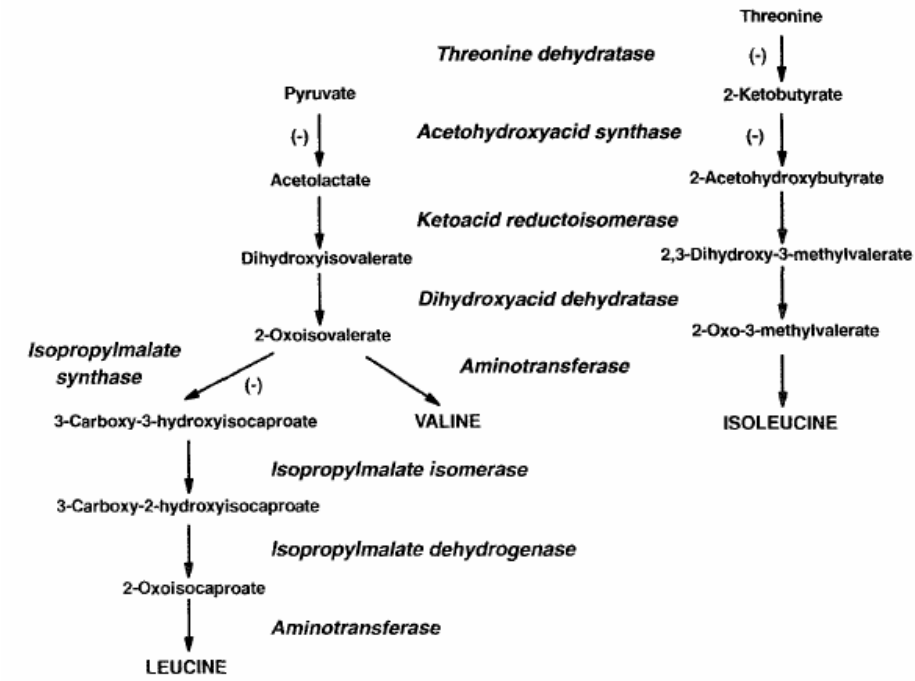
1.1 Nomenclature

Acetohydroxyacid synthase (EC 4.1.3.18) is an enzyme that has two metabolic roles (Duggleby and Pang, 2000). The anabolic role is in the early steps of branched chain amino acid biosynthesis. The catabolic role, confined to certain micro-organisms only, is in the formation of Butanediol. In the plant literature, acetohydroxyacid synthase is very often referred to as acetolactate synthase. For ease of reference to cited literature, this is the name used for the enzyme in this document. Throughout this text the abbreviated name of the enzyme and gene is used (ALS).

1.2 Biochemical function of the ALS enzyme

The review by Duggleby and Pang (2000) provides an excellent overview of ALS function in micro-organisms and plants.

- Acetolactate synthase is found in all plants, in bacteria and in fungi. Animals do not synthesize the branch chain amino acids.
- In plants, ALS catalyses the biosynthesis of branch chain amino acids leucine, valine and ioleucine through the reactions shown in the figure below (extracted from Singh and Sander (1995);



- Co-factors required for ALS activity are thiamin diphosphate (ThDP), metal ions (generally satisfied with 0.1 to 10mM Mg^{2+}) and flavin (FAD).
- ALS activity is subject to feedback inhibition. Valine , leucine and isoleucine are all feedback inhibitors, but to different degrees in different species.

ALS enzyme is very labile, and is difficult to purify, as it occurs at low concentrations.
Southan and Copeland (1996) have purified and characterized the enzyme in wheat but carnation ALS has not been isolated.

1.3 Inhibition of ALS activity by herbicides

The ALS enzyme is inhibited by four classes of herbicides. The inhibition caused by herbicides can be reversed by provision of valine and isoleucine to pea cultures, restoring the ALS function (Ray,1984). ALS-inhibiting herbicides are the sulfonylureas (Chaleff and Ray, 1984; Ray, 1984), imidazolinines (Shaner et.al.,1984), triazolopyrimidines (Subramanian et.al.,1990) and pyrimidines.
The mechanism of inhibition is through the binding of these herbicides to a relic quinone-binding site in the ALS enzyme (Schloss et.al., 1988), rather than to the substrate binding site. ALS-inhibiting herbicides have no structural similarities to the substrates (pyruvate and α-ketobutyrate) or cofactors (thiamine pyrophosphate, FAD and magnesium) necessary for ALS function (Schloss et.al., 1988).

1.4 Characterization of ALS gene(s)

In plants, ALS is nuclear encoded but chloroplast localized (Mazur et.al.,1987). Duggleby and Pang (2000) indicate that in all species at least one ALS gene is expressed constitutively. Depending on species there may be between 1 and 7 copies of the ALS gene (White et.al., 2003). Sugar beet and Arabidopsis only have one gene (Mazur et.al.,1987; Keeler et.al., 1993). Ouellet et.al. (1992) investigated the ALS gene in *Brassica napus.* Five genes were identified, which were expressed constitutively in some cases, or in a tissue specific fashion in other cases. Cotton has six ALS genes. Fang et.al (1992) showed maize has two ALS genes, which in common with all ALS genes studied in plants has no introns.

Conservation
Within a species the ALS genes are highly conserved – the two genes in maize are 94% identical in the coding region, for example (Fang et.al., 1992). Between species there is also a high degree of conservation. Based on aligned amino acid sequences, two of the genes in cotton, which were constitutively expressed, were collinear to the genes from both *Brassica napus* and tobacco (Grula et.al., 1995).
Le et.al. (2003) has shown that seven methionine residues (numbered 332, 347, 350, 489, 512 and 569 in tobacco) were conserved in all ALS sequences they examined.

Tobacco ALS
Mazur et.al. (1987) first isolated the ALS gene, from Arabidposis and tobacco. In tobacco there are two unlinked ALS genes , SuRA and SuRB (Chaleef and Mauvais, 1984; Chaleef and Bascomb, 1987). The tobacco ALS gene has been studied in great deal, primarily through site-directed mutagenesis. This research has found amino acid residues 141, 219,372, 376 and 512 and 652 are located at the active site, and mutations inactivate the enzyme and prevents binding of the cofactor FAD (Chong and Choi, 2000;Yoon et al, 2002; Le et.al.., 2005).Chong and Choi (2000) determined that amino acid pro187 was essential for maintaining the resistance of the enzyme to fragmentation and oxidation. Using site-directed mutagenesis Yoon et al (2002) identified a lysine residue (position 255) which was substituted in mutations showing broad herbicide resistance, and so likely to be in a domain at which all herbicides bind.

ALS gene expression in tobacco has been studied by Keeler et.al. (1993). SuRA and SuRB are both expressed in tissues with the highest metabolic activity (presumably due to the need for amino acids for protein biosynthesis), with a 3 to 4 range in expression in different tissues. The SuRB gene is expressed at higher levels than the SuRA gene. The SuRA and SuRB genes are highly conserved, with a predicted amino acid divergence of 0.7% (Lee et.al., 1988).

1.5 Isolation of ALS gene mutations conferring herbicide resistance

Herbicide resistant ALS gene-mutants have been isolated in many plants (Table 1).

Table 1. Mutations of the ALS gene conferring herbicide resistance

Mutation	Characteristic of mutant	Species/citation
Single amino acid (557 Trp to Leu)	Multiple herbicide resistance. Feedback inhibition unaffected.	*Brassica napus* Hattori et.al.(1995)
Single amino acid (552 Trp to Leu)	Multiple herbicide resistance.	*Xanthium sp.* (Bernasconi etal, 1995)
Single amino acid (197 Pro to His)	Resistant to sulfonylurea. Specific activity of ALS reduced less sensitive to feedback inhibition	*Lactuca sativa* (Eberlein et.al., 1999)
Single amino acid (197 Pro to Ser)	Chlorsulfuron resistance. *Csr1-1* allele from mutant locus *csr1*	*Arabidopsis thaliana* (Haughn et.al. 1988)
Single amino acid (196 Pro to Gln)	Sulfonylurea resistance. C3 mutation of SuRA gene.	*Nicotiana tabacum* (Lee et.al., 1988)
Double amino acid substitution (196 Pro to Gln and 573 Trp to Leu)	Sulfonylurea resistance. S4-Hra mutation of SuRB gene.	*Nicotiana tabacum* (Lee et.al., 1988)
Single amino acid (573 Trp to Leu)	Highly resistant (Tested on chlorsulfuron only)	*Nicotiana tabacum* (Kochevenko and Willmitzer, 2003)
Single amino acid (196 Pro to Ala, Thre, Glut or Ser)	Highly resistant (Tested on chlorsulfuron only)	*Nicotiana tabacum* (Kochevenko and Willmitzer, 2003)
Single amino acid (196 Pro to Leu)	Resistant (Tested on chlorsulfuron only)	*Nicotiana tabacum* (Harms et.al., 1992; Kochevenko and Willmitzer, 2003)
Single amino acid (255 Lys to Phe)	Multiple herbicide resistance.	*Nicotiana tabacum* (Yoon et.al., 2002)
Single amino acid (350 Met to Cys)	Multiple herbicide resistance. Enzyme more sensitive to pH change	*Nicotiana tabacum* (Le et.al., 2003)
Single amino acid (569 Met to Cys)	Multiple herbicide resistance.	*Nicotiana tabacum* (Le et.al., 2003)
Single amino acid (121 Ala to Thr)	Multiple herbicide resistance.	*Nicotiana tabacum* (Chong and Choi, 2000)
Single amino acid (214 Ser to Leu)	Valine resistance.	Hervieu and Vaucheret (1996)

Isolation of mutants has been achieved through selection with and without mutagenesis and through site directed mutagenesis. Typically, resistance to ALS-inhibiting herbicides is due to gene mutation rather than amplification, duplication or changes in regulation. By selection in cell culture, Harms

et.al. (1992) were able to select a tobacco line resistant to sulfonylurea herbicides in which ALS was amplified 20 fold. However, they also demonstrated that this gene was mutated. Frequency of mutation to the ALS inhibiting enzymes is much higher than for glyphosate (Jander et.al.,2003). Jander et.al. (2003) estimated that in arabidpopsis only 50,000 mutations are required to have a 95% probability of generating an ALS-inhibitor resistant line.

Characterization of mutations

In *Lolium* there is some evidence for resistance mechanism involving increased herbicide metabolism (Christopher et al., 1992) but in all other cases resistance to ALS-inhibitors is due to single, and sometimes double, amino acid substitutions (Table 1). These mutations are thought to alter the herbicide binding site, and the enzymes consequently retain normal function. Multiple herbicide resistance as a result of a single mutation suggests an overlapping domain essential for herbicide binding (Hattori et al., 1995). In different species, amino acid substitutions or mutations at sites 193, 197 and 205 confer resistance to ALS inhibitors (White et.al., 2003)

The recent review by Tan et.al. (2005) provides an excellent overview of ALS-inhibiting mutations conferring resistance to imidazolinone herbicides in plants and more detail is also available in Duggleby and Pang (2000).

Feedback inhibition in ALS mutants

In the majority of cases, the enzymes of ALS-inhibiting mutants display normal feedback kinetics. However, in lettuce Eberlein etal (1999) showed that the levels of valine, leucine and isoleucine were higher in seeds and leaves from herbicide resistant lines, suggesting altered feedback sensitivity. Plants of the *csr1-4* double mutant line *of Arabidopsis* also displayed altered feedback inhibition and were less sensitive to added valine and leucine (Mourad et.al.,1995).

ALS gene mutations in weed species

As a result of the ease of mutation for resistance, and the selection pressure exerted by widespread use of ALS-inhibiting herbicides, many resistant weed species have now been identified in the wild. Examples are given in Table 2.

Table 2. Examples of resistance mutations to ALS-inhibiting herbicides in weeds

Species	**Amino acid substitution**	**Reference**	**Type of resistance**
Xanthium	Trp552leu	(Bernasconi etal, 1995; Tranel et.al, 2004)	Multiple
Kochia scoparia	Pro173Thr	(Guttieri et.al., 1992).	Clorsulfuron
Helianthus spp. (common sunflower)	Ala205Asp	(White et.al., 2003)	Multiple
Bromus tectorum	Pro197Ser	(Park and Mallory-Smith, 2004).	Multiple
Amaranthus retroflexus	Pro248Leu	(Sibony et.al.,2001).	Sulfonylurea
Amaranthus hybridus	Leu574Try	Maetens et.al. (2004)	Sulfonylurea and imidazolinone

1.6 S4-Hra; ALS gene mutation in tobacco

The mutation of the SuRB gene that has been used as the selectable marker gene in the transformation vector pCGP1991 is S4-Hra. This mutation was derived from a line (S4) isolated from a mutagenized haploid cell culture (Chaleef and Ray, 1984) selected on the sulfonylurea herbicide chlorsulfuron. A diploid herbicide resistant plant homozygous for a single dominant gene was regenerated, which was

then subjected to further selection (leading to the double mutant line S4-Hra) for increased resistance to chlorsulfuron (Creason and Chaleff, 1988). Lee et.al.(1988) characterized and cloned the S4-Hra mutant of the SuRB gene, showing there were three nucleotide substitutions in the S4-Hra line, when compared to the wild type. One was silent and two lead to amino acid substitutions (196 Pro to Gln and 573 Trp to Leu). Full details of the characterization of the mutant S4-Hra are provided in Bedbrook et.al. (1991). Tobacco lines resistant to ALS-inhibitors have been isolated by independent researchers, who showed amino acid substitutions at the same amino acid positions (Table 1).

1.7 Crops resistant to ALS-inhibiting herbicides

ALS mutations can also be selected in the important crop plants. For example in corn, plants regenerated from cell cultures selected on imidazolinine herbicides were shown to be a series of mutations which conferred resistance to all or some of ALS-inhibiting herbicides tested (Newhouse et.al.,1991).The relative ease with which plants resistant to ALS-inhibiting herbicides can be selected has therefore been exploited by plant breeders to develop herbicide resistant crop plants (Tan et.al., 2005). There are now many other crops available, and Table 3 provides a list of varieties registered in Canada, as an example.

Table 3. Examples of crop varieties resistant to ALS-inhibiting herbicides. Data extracted from the Canadian Food Inspection Agency website.

Product	Proponent	Decision Date
Imidazolinone resistant maize (3417R)	Pioneer Hi-Bred International	May 30, 1994
Imidazolinone resistant canola (lines NS738, NS1471, NS1473)	Pioneer Hi-Bred International	April 25, 1995
Sulfonylurea tolerant flax - CDC Triffid	Crop Development Centre, University of Saskatchewan	February 16
Imidazolinone Tolerant Corn (Mutation of AHAS Enzyme)	Pioneer Hi-Bred International	June 8, 1998
Imidazolinone tolerant (IT) wheat (SWP 965001)	Cyanamid Crop Protection	November 12, 1999
Imidazolinone tolerant rice lines CL121, CL141, and CFX51 (ClearfieldTM rice)	BASF Canada Inc.	February 11, 2002
Imidazolinone Tolerant (Clearfield) PWC16 Rice	BASF Canada	January 10, 2003
Imidazolinone Tolerant ClearfieldTM Wheat (AP602CL)	BASF Canada	March 21, 2003
Imidazolinone Tolerant ClearfieldTM Wheat (AP205CL)	BASF Canada	November 14, 2003
Clearfield TM Imidazolinone tolerant wheat (Teal 11A)	BASF Canada	June 25, 2004
Clearfield^{TM} Imidazolinone tolerant lentil (RH44)	BASF Canada	June 25, 2004
Clearfield^{TM} Sunflower line X81359	BASF Canada	October 25, 2005

With the exception of Flax, all the varieties shown in Table 3 are non-transgenic and have been developed by either mutagenesis of commercial varieties, or introgression of naturally occurring mutations, sometimes discovered in related weed species (this was the case in sunflower, for example). The mutations in the commercial varieties listed in Table 3 are single or double amino acid substitutions.

Clearfield™ technology is becoming increasingly important, and these crops are not subject to regulation as GMO's in Europe. The CLEARFIELD™ corn grown in the USA, for example, does not face the same international grain market restrictions that glyphosate tolerant genetically modified varieties do.

1.8 Biosafety evaluation of food crops containing ALS gene mutations

Canadian assessments of imidazolinone-tolerant Clearfield™ crops

As representative examples, we have reviewed the decision documents for three imidazolinone-tolerant Clearfield™ crops; rice, wheat and sunflower (Canadian Food Inspection Agency, 2002, 2004, 2005), all of which were single amino acid substitution mutations for ALS. Though not transgenic, the Canadian government assesses the varieties for food safety in a similar way to transgenic varieties. The following conclusions were specific to the ALS gene;

- Regulation of the levels of valine, leucine and isoleucine by feedback inhibition was not affected in any of the three varieties.
- Unlike known food allergens, ALS is a minor protein in plant tissue (~0.001% of total protein in rice seed) and is heat sensitive and trypsin susceptible. Specific data is given in Table 4

Table 4. Stability of ALS from imidazolinone-tolerant varieties of three species

	Time for complete loss of activity		
	Sunflower	**Wheat**	**Rice**
Heat sensitivity	1 min at 100ºdegree	1 min at 100ºdegree	1 min at 100ºdegree
Trypsin	60 min	30 min	5 min

- The unmodified form of the ALS protein in all three species showed no amino acid similarity to known allergens.
- ALS from none of the three species is a known toxin or allergen.
- Difference in levels of ALS activity in imidazolinone-tolerant and imidazolinone-susceptible leaf tissue is not biologically significant as the ALS enzyme in both still has the same physicochemical properties and functional activity.

Food crops containing the SuRB gene mutation S4-Hra

To our knowledge, no food crop variety has been commercialized in which the SuRB gene mutation we have used in the carnation line 123.8.12 (FLORIGENE Moonaqua™) has been used in the transformation vector. A transgenic cotton line resistant to sulfonylurea was developed, but never commercialized. This line was developed by DuPont, who did prepare a petition for deregulation to the US government (DuPont, 1995).In that petition, levels of cotton toxicants were measured, but no direct measurements of the toxicity or allergenicity of the ALS gene or ALS enzyme were included (that data is included in a petition to FDA, which we do not have). It was pointed out in the petition that;

- Even though herbicide resistant, the ALS gene was still functionally identical to the numerous ALS genes found in plants and bacteria in nature.
- ALS enzymes tolerant to sulfonylurea herbicides occur in bacteria naturally.

In reviewing the application USFDA (1996) took into consideration, given the high homology in ALS, that animal and humans eating plant food would have been exposed to a wide variety of very similar enzymes.

The University of Queensland has developed a transgenic pineapple line using the S4-Hra gene as the selectable marker. The potential toxicity and allergenicity of the gene was reviewed by the Australian government (OGTR, 2003) who concluded there was no potential risk on the basis that the enzyme is

not a known toxin or allergen and related enzymes are expressed in a variety of edible plants (e.g. soybean and rice). The review also pointed out the high homology to ALS from other species, and lack of sequence homology with known allergens.

Transgenic flax

The *csr1-1* gene from Arabidopsis, which confers resistance to sulfonylurea herbicides, has been used as a selectable marker in a number of transgenic plants, and in the development of a flax variety resistant to these herbicides. Agronomically, the transgenic flax line is not significantly different to the parent (McSheffrey et al, 1992). In the USFDA response to a petition for use as animal feed and food (USFDA, 1998) it was stated that the ALS activity was 56.3 nanomoles (nmol)/mg/hr for the parent cultivar and 88.8 nmol/mg/hr for the transgenic. The difference was attributed to the combined activity of the endogenous and inserted ALS, not altered feedback inhibition. There was no difference in seed amino acid composition between the two lines.

2. An assessment of the potential toxicity of the ALS protein from the ALS gene S4-Hra used in the transformation vector pCGP 1991

2.1 Plant toxins

The chemical nature of plant derived compounds toxic to animals and man is extremely diverse, and potential toxicity is of course determined by dose, exposure time, the age and general health of the animal or person exposed, the combination of toxins and the way the plant is prepared before being consumed. The majority of plant toxins are non-protein secondary products, such as alkaloids and glycosides of alkaloids (Goetz et.al., 2006). Plants may also accumulate minerals such as selenium and calcium oxalate to very high concentrations, making the plants toxic.

Some plant toxins are proteins based. Excluding mushroom derived polypeptide toxins, the most toxic peptide based plant toxins are **ribosome-inactivating proteins** (Nielsen and Boston, 2001). This includes the lectins ricin, abris and concanavalin (Rudiger and Gabius, 2001) which are some of the most toxic compounds known to man. Lectins are largely found in beans and some grains. Two other groups of protein based toxins are;

Prolamins. These are storage proteins found in cereal seed and they cause toxicity symptoms in people suffering coeliac disease (McLachlan et.al., 2002).

Thionins. These are low-molecular weight polypeptides that are found in the Gramineae (Bohnlman and Apel, 1991) and some other species (Romagnoli et.al., 2000; Fung et.al., 2003).

There are many databases and textbooks describing toxic plants The one we refer to here is from Indiana, USA (Goetz et.al., 2006), which provides a rank of toxicity and identifies the chemicals responsible for toxicity, where this is known. This reference source identifies the following plants poisonous to animals, where the causative agent is protein based;

- *Abrus precatorius*. The toxins in this plant are the protein abris, and one seed has sufficient protein to kill an adult human.
- The Aroid family (i.e. Anthurium and *Dieffenbachia)*. These plants contain proteolytic enzymes which release histamine and kinins.
- *Ricinus communis*. The toxin in castor bean is ricin, a water soluble protein
- *Pteridium aquilinum* The toxin in bracken fern is thiaminase, an enzyme that destroys thiamine (Kenton, 1957).
- *Equisetum arvense* and *Equisetum hyemale* The cause of toxicity is also thiaminase.

None of the peptide based classes of plant toxins described above are functionally or structurally related to the ALS gene product, and the ALS gene product can not be implicated as a cause of

toxicity. Furthermore, classes of known plant peptide toxins are localized in seed, or associated with cell walls, while ALS is not.

2.2 The toxicity of tobacco

The toxicity of smoked and chewed tobacco to man is very well known. In fresh plants consumed by animals the toxin in tobacco is nicotine. Nicotine is an alkaloid, and is produced on a biosynthetic pathway unrelated to branched chain amino acid biosynthesis.

Tobacco can also cause occupational dermatitis (McBride et.al.,1998; Mitchell and Rook, 2006).Tobacco workers are exposed to tobacco during processing, when leaves are harvested and dried. Contact is through touch, and through tobacco dust carried in the air (Mitchell and Rook, 2006). Many other chemicals are used in tobacco processing and these may also contribute to, or be responsible for some dermatological reactions. Dermatitis in the tobacco industry is uncommon (Mitchell and Rook, 2006). However, nicotine is water soluble, and long term exposure to wet tobacco leaves can cause nicotine poisoning (so-called “green tobacco disease”, see McBride et.al., 1998). .

The ALS gene product can not be implicated as a cause of toxicity in tobacco.

2.3 Exposure to carnation

An important consideration in assessing the potential toxicity of the S4-Hra gene product is a consideration of possible routes of contact. For carnation flowers imported into Europe the only realistic route of exposure is through handling.

Unlike an allergic response, which requires sensitization, toxin-mediated contact urticaria (chaffing, blistering, rash etc.) is not selective, and occurs to most people with whom a plant may come into contact. McGovern and Barkley (2006) provide a detailed summary of plants causing urticara as a result of toxins. Most plants belong to the Urticaceae (nettle) family, but also the Hydrophyllaceae, Loasaceae and Euphorbiaceae. Common toxins include histamine, acetylcholine, and 5-hydroxytryptamine. Phytophotodermatitis disease (excessive darkening of the skin) is caused by the presence of furocoumarins such as bergapten and xanthotoxin (McGovern and Barkley, 2006). Tobacco is not a known source of any of these toxins, and neither ALS, nor the products of ALS are involved in their biosynthesis.

Dermatological response to plants can also be through mechanical (thorns or oxalate crystals for example) and chemical irritants. There is no evidence that tobacco or ALS are chemical irritants (McGovern and Barkley, 2006).

2.4 Conclusions

There is sufficient evidence to suggest that the ALS gene product S4-Hra, is not toxic.

1. ALS is a well conserved protein found in all plants, and S4-Hra is functionally identical to the numerous ALS enzymes found in plants and bacteria in nature.
2. Single amino acid substitution in ALS mutations conferring herbicide resistance are found in several food crops and weed species and occur naturally in bacteria. Humans eating plant food would have been exposed to a wide variety of enzymes very similar (even the same amino acid change) to the protein encoded by S4-Hra.

3. ALS activity in widely grown imidazolinone-tolerant food crops is not biologically significant to parental varieties; the ALS enzyme still has the same physicochemical properties and functional activity.
4. ALS mutations, including S4-Hra, have been assessed as non-toxic by US and Canadian regulatory agencies.
5. There is no evidence that amino acid metabolism is affected in ALS mutant lines.
6. None of the known peptide based classes of plant toxins are functionally or structurally related to the ALS protein.
7. Tobacco is not a known source of phytophotodermatitis causing toxins, and neither ALS, nor the products of ALS are involved in the biosynthesis of compounds which are.
8. Neither tobacco or the ALS protein (from any species) are known chemical irritants
9. The toxic chemical in fresh tobacco is nicotine, not the ALS protein. Nicotine is produced on a biosynthetic pathway unrelated to that in which ALS functions.

The inserted S4-Hra gene product in carnation will not, in any event, be consumed as a food. In addition, the transgenic carnation has a long history of large-scale, safe use, with no reports of any toxic effects.

3. An assessment of the potential allergenicity of the ALS protein from the ALS gene S4-Hra used in the transformation vector pCGP1991

3.1 Plant allergens

Allergens of plant origin are found in food and drinks, in the atmosphere (pollen and dust borne plant particles) and in situations where direct contact is made with plant or processed plant material. Allergens are almost always proteins (Mills et.al, 2004), but not always (Table 5)

Table 5. Plant families with the most commonly encountered contact allergens* (*extracted from McGovern and Barkley, 2006).

Family	Examples	Allergen
Anacardiaceae	Poison Ivy. Poison oak,	Catechols and resorcinols (1,2 and 1,3--dihydroxybenzenes)
Anacardiaceae	mango skin, cashew husk	Phenols and resorcinol
Ginkgoaceae	Gingko seed pulp	anacardic acid,
Proteaceae	*Grevillea*	pentadecylresorcinol
Compositae (Asteraceae)	chrysanthemum, dandelion ragweeds, worts etc.	sesquiterpene lactones
Compositae (Asteraceae)	Chicory, sunflower	lactucin and lactopicrin, 1-0-methyl 1-4,5-dihydroniveusin A
Jubulaceae	Liverwort, magnolia	sesquiterpene lactones
Alliaceae	Garlic, onion	diallyl disulfide, allylpropyl disulfide, and allicin
Alstroemeriaceae	Alstromeria	Tuliposide A
Liliaceae	Tulips	Tuliposide A
Primulaceae	Primula	primin

Most food allergens are a cross-reaction; the result of initial contact with allergens that sensitizes individuals to similar allergens later found in food. Allergens may be initially contacted through inhalation, of pollen for example (van Ree, 2002), or touch. The Hev series of allergens in natural rubber latex, for example, cause cross reactivity to a large number of fruits and vegetables (Wagner and Breitender, 2002). True food allergens capable of sensitization after ingestion, typically tolerate

low pH and proteolytic degradation (Mills et.al., 2004).

There a large number of characterized allergens, which have been grouped into families, based on the structural and functional similarities of the proteins. Groups of plant food allergens have been recently summarized by Mills et.al. (2004) and Jenkins et.al. (2005). Major groups include the prolamins, profilins, Bet v 1 family, Lol p1 family, Papain family and cupins. The profilins are also a major group of food allergens that also occur in pollen and natural rubber latex, along with pathogenesis related proteins such as chitinases and glucanases, which may also be allergens (Wagner and Breitender, 2002).

A large number of pollen allergens have now been cloned (de Weerd et.al., 2002). As a major source of health problems grass pollen allergens are a well studied group of and have recently been grouped by function (Andersson and Lidholm, 2003; see summary Table 6).

Table 6. Immunological classification of major grass pollen allergen groups (Andersson and Lidholm, 2003)

Group	Comment	Allergen characteristic
1	Major allergen.90% of sensitized individuals are cross reactive	Glycoproteins
2,3	Minor allergen. *Lolium* and *Dactylis*	Non-glycosylated proteins of 95 -98 amino acids
4	Major allergen.80% of sensitized individuals are cross reactive	Highly basic glycoproteins. Possibly flavoproteins.
5	Dominant allergen in Poodieae family.	Glycoproteins, similar to group 1
6	*Poa* species only. 60 - 70% of sensitized individuals are cross reactive	Acidic protein of ca. 13kD
7	Also found in *Brassica*, olive and birch	Small proteins of ca. 8o amino acids
11	*Lolium*	Similar structure to soybean trypsin inhibitor
12	Minor allergens in grasses	Profilins
13	*Phleum pratense*	Probable polygalacturonase

Radauer and Breiteneder (2005) have recently provided a summary of pollen allergens, grouped by abundance and taxonomy. The families with the most allergens were the grasses (Poaceae), followed by the Cupressaceae. The tobacco family (Solanaceae) was not represented in the survey The most important classes of allergens were identified as the prolamins (Shewry et.al., 2002). These are a large group of sulphur-rich seed proteins, including trypsin/ α-amylase inhibitors and lipid transfer proteins (van Ree et.al., 2002). Other classes of allergens listed were the expansins, which are common in grass allergens (Andersson and Lidholm, 2003), profilins (also found in natural rubber latex) and thaumatin-like proteins. Thaumatin-like proteins are a class of pathogenesis-related proteins (Breitender, 2004). Other examples of protein allergens found in pollen are EF-hand, Ole e1-like, pectate lyases and ribonucleases.

The ALS protein

ALS has never been identified as an allergen despite of the presence of this protein in all plants. A search of the plant food-allergens, aero-allergens and contact allergens in the Faarp protein database of known allergens (www.allergenonline.com) did not list ALS from any source.

3.2 Allergenicity of tobacco

Cases of hypersensitivity to tobacco, and the statement that the leaf contains both heat-stable and heat-labile allergens are cited in Mitchell and Rook (2006).

Tobacco pollen has been shown to contain an allergenic profilin (Mittermann et.al., 1995). A search of the aero-allergens in the Faarp protein database of known allergens (www.allergenonline.com) identified Calcium-binding proteins, villin 1 and 2 and Beta expansin-like protein from *Nicotiana tabacum.* None of these are functionally related to ALS.

The antigen in tobacco leaf is cross-reactive to mugwort pollen (Ortega et.al., 1999; Armentia et.al., 2005), tomato extract (Ortega et.al., 1999) and *Lolium* pollen (Armentia et.al., 2005). There is no evidence that the ALS gene product is an antigen in tobacco.

3.3 Allergens in flowers

Carnation is not a food. Contained in carnation flowers imported into Europe the only realistic route of exposure to potential allergens is therefore through handling.

Along with food processors, florists and flower growers have one of the highest rates occupational asthma, as they experience long-term, high-duration exposure to allergens in pollen, through handling plant material and through dust (deJong et.al., 1998; Goldberg et.al., 1998; Akpinar-Elci et.al., 2004; Emberlin et.al., 2004; Mapp et.al, 2005). In a comparison of different types of European crop growers Monso et.al. (2000) identified flower growing as a high risk factor for asthma. In situations where large numbers of flowers are grown or sold there are also secondary sources of allergens. This can include insect allergens from pest and predatory mites (Cistero-Bahima et.al.,2000; Johnasson et.al., 2003) and fungal allergens such as *Aspergillus* (Monso et.al., 2002; Emberlin et.al., 2004). Sensitizing chemicals are also found in the florist's workplace. Allergenicity to flowers is a minor medical problem. Symptoms usually disappear away from the workplace DeJong (1998) and change of profession offers a permanent solution (Goldberg et.al., 1998; Monso et.al., 2002). The high intensity exposure by florists may also result in sensitization not seen in the general population (Bolhaar and van Grinkel et.al., 2000).

Flowers of the Compositae (Asteraceae), such as chrysanthemum (De Jong et.al.,1998; Groenewoud et.al. ,2002) are known to be major sources of aero- allergens. Other species are listed in Table 7. Carnation is insect pollinated and any pollen produced is not wind-borne.

Table 7. Flower species known to exhibit air-borne allergenicity

Species	Reference	Species	Reference
Chrysanthemum leucanthemum	Monso et.al., 2002	*Narcissus pseudonarcissu*	Monso et.al., 2002
Solidago canadiensi	Monso et.al., 2002	*Hyacinthus orientalis*	Monso et.al., 2002
Helianthus annuus	Monso et.al., 2002	Molucella	Miesen et.al., 2003
Gladiolus spp	Monso et.al., 2002	Easter lily	Piiriala et.al.,1999
Stephanotis floribunda	Van der Zee et.al., 1999	*Rosa rugosa*	Demir et.al., 2002

Emberlin et.al. (2004) identified cyclamen, solidago, lisianthus, lily, hypercium and alstromeria pollen as the most abundant in flower shops.

The causative agents of the allergic reactions to flowers are largely unidentified, but where known (Table 8) does not include the ALS gene product.

Table 8. Adverse contact reactions through handling floricultural products

Plant	Allergen	Reference
Bishops weed	Psoralens	Kiistala et.al., 1999
Easter lily	Not identified	Piiriala et.al.,1999
Tulip	Tuliposide-A	Piiriala et.al.,1999
Hydrangea	Hydrangenol	Rademaker, 2003
Alstromeria	Tuliposide-A	Kristiansen and Christense, 1998
Carnation	IgE bands at 15 and 17kDa	Vidal and Polo, 1998
Lily	IgE bands at 19 and 22 kDa	Vidal and Polo, 1998
Chrysnathemum	arteglasin-A	McGovern and Barkley, 2006
Lilium	profilins	Faarp protein database; DeWeerde et.al., 2002

3.4 The presence of contiguous amino acid sequences in S4-Hra homologous to sequences in known allergens

Kleter and Pejnenburg (2002) showed that a 6 contiguous amino acid sequence in the S4-Hra gene shares homology with a single known allergen, the Amb a 1.4 from the pollen of ragweed, *Ambrosia artemisiifolia* (see also Attachment A9 of this application). Amb a 1.4 is a minor allergen in ragweed, most likely encoding a pectate lyase (Wopfner et.al., 2005).The short amino acid sequence (PRKGSD) identified by Kleter and Pejnenburg (2002) occurs at positions 96 to 101 in tobacco (Lee et.al, 1988) and so does not include the two amino acid substitutions that distinguish S4-Hra from the wild type ALS gene SuRB. Both SuRA and SuRB therefore also carry this motif (Lee et.al, 1988). The 6 amino acid sequence also excludes the herbicide binding site. Because ALS is a relatively well conserved gene, a very large number of other plants therefore most probably have the same contiguous sequence of 6 amino acids. A BLAST search (see files referenced at Attachment A8) identified it in sunflower, maize and rice. In several species where there is not an exact match to the sequence PRKGSD, it is consistently matched to PRKGAD. This includes certain maize varieties (Fang et.al., 1992), cotton (Gruda et.al., 1995), sunflower and a herbicide resistant line of *Sinapis arvensis*.

Recognizing the limitations of sequence comparison alone, Kleter and Pejnenburg (2002) employed an additional screen on positive matches, the hydrophilicity plot of Hopps and Woods. Proteins (either the target protein or the homologous allergen) where the region of maximum hydrophilicity coincided with the 6 amino acid sequence would then qualify for further examination s potential allergens. In the case of ALS, an allergen-homologous sequence (KVLENR) that was found to occur in the ALS from Arabidopsis **(**Kleter and Pejnenburg, 2002) fell into this category. The sequence (KVLENR) does not occur in S4-Hra (Lee et.al., 1988 and BLAST search, file attached at Attachment 8). It does however, occur in the ALS of the food crop *Brassica napus* (Bekkaoui et.al., 1991).

The sequence PRKGSD was not sufficiently hydrophilic to be indicative of an allergen (Kleter and Pejnenburg (2002). As discussed above, the related sequence, PRKGAD (a serine to alanine substitution from PRKGSD), also occurs in many food crops. This substitution would reduce the total hydrophilicity of the 6 amino acid sequence from +9.3 to +8.8 (Hopp and Woods, 1981), meaning it also is not sufficiently hydrophilic.

<u>Amino acid sequence as a predictor of allergenicity</u>

Sequence comparison is one tool for screening for potential allergens in proteins. In addition to the work of Kleter and Pejnenburg (2002), comparison programs have been published by Riaz et.al. (2005), Stadler and Stadler (2003) and Bjorkland (2005). These authors have criticized the use of 6 contiguous amino acids as an indication of potential allergenicity, as recommended by the FAO/WHO (FAO/WHO, 2001). Stadler and Stadler (2003) found a very high percentage of "false positives" using such a technique, with two-thirds of all proteins in the Swiss-prot database determined to be allergens, and recommended the use of allergen motifs for screening. Hileman et.al. (2001) concluded the use of 6 amino acid windows produced many irrelevant matches, and this was also acknowledged by Kleter and Pejnenburg (2002).
The amino acid sequence may have no prediction value without knowledge of secondary protein structure, such as the case with B-type epitopes (Bjorkland et.al., 2005).

Lack of sequence similarity does not mean a protein can never be an allergen and short commonality of sequence is not necessarily related to similar function. In fact, there is ample evidence that sequence similarity does not necessarily translate to structural similarity of a folded protein because sequence differences do not generate predictable structural differences. Sequence alone does not provide any information on possible post translational modifications. The link between antigenicity and allergenicity and the presence of certain motifs is tenuous, because it would suggest that more allergens would be found and could readily be engineered. In the case of the double amino acid substitution in S4-Hra, if the protein then became an allergen there would be many similar allergens in many, many plants, and it is very likely that they would have been identified by now, if they existed. It might also be expected that if S4-Hra was an allergenic protein far more homologies with sequences in other allergens (the entire Amb series for example) might have been identified.

There are many proteins that have strong characteristics of an allergen, including sequence homology, but do not elicit an allergenic response (Stadler and Stadler, 2003; Lehrer and Bannon, 2005).

Factors in determining whether a protein is allergenic or not, that should be considered in addition to sequence homology include;

- A high number of disulphide bridges, conferring stability (Mills et.al., 2004)
- For true food allergens, high concentration, enhancing the probability of protein resistance to digestion
- Ability to bind to membranes (Mills et.al., 2004)
- Proteolytic activity of the allergen (Jenkins et.al., 2005)
- Three dimensional structure, including folding, tunnels and total surface area, of the mature protein (Aalberse et.al., 2000; Jenkins et.al., 2005). The conservation of specific surface residues and main chain conformations was found by Jenkins et.al (2005) to play an important role in conservation of IgE-binding epitopes.

3.5 Conclusions - Allergenicity of the S4-Hra mutant SuRB gene

There are strong reasons to suggest that the S4-HRa gene does not encode an allergen.

1. The ALS protein is highly conserved and S4-Hra is very similar to the protein found in all plants. Though the sequence, and so degree of variation, of native carnation ALS is unknown it is likely the sequence of S4-Hra is not significantly different to the sequences endogenous to carnation or to those found in a very wide range of plant foods and other plants.
2. The ALS protein is ubiquitous, but there is no evidence of cross-reactivity as a result of sensitization to the ALS protein
3. Short amino acid sequences from ALS, with homology to sequences in an allergen, are known to occur in some plants, and probably occur in many others, yet there is no evidence the ALS protein is allergenic.

   a. Wild type tobacco has exactly the same short amino acid sequence but allergenic response in tobacco is not due to ALS.
   b. The short amino acid sequence has insufficient hydrophilicity to warrant further study as an allergen (Kleter and Pejnenburg, 2002).
4. ALS mutations similar to S4-Hra are now present in many food crops, and the pollen of weed species. However, ALS has never been identified as an allergen despite this widespread opportunity for exposure.
   a. ALS mutations in food crops, including S4-Hra, have been assessed as non-allergenic by US, Australian and Canadian regulatory agencies.
5. Allergens are typically abundant and stable (Clare Mills et al, 2004). ALS is not found at high concentration and is not stable. The ALS protein is heat labile and sensitive to trypsin and highly unlikely to be a true food allergen
6. ALS proteins are not structurally or functionally similar to any known allergen groups, in food or pollen.
7. An important consideration in assessing the potential allergenicity of the S4-Hra gene product is also a consideration of possible routes of contact. Carnation is not a food, and does not produce wind borne pollen, so in carnation flowers imported into Europe the only realistic route of exposure is through skin contact during handling within the floral industry.
   a. Occupationally, the people most likely to be regularly exposed to the carnation carrying the S4-Hra gene are florists. Allergies in this work group have never been attributed to the ALS protein, or structurally or functionally similar proteins

The genetically modified carnations are not a food, and have not been assessed for food or animal use by any other regulatory body that has approved the commercial release of the flowers. It is also unrealistic to expect that any of the imported carnation flowers will be adopted for use as food or animal feed. The inserted S4-Hra gene product in carnation will therefore not, in any event, be consumed as a food.

The transgenic carnation now has a long history of large-scale, safe use, with no reports of any allergenic effects.

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ATTACHMENT B2

Ames test

Data is included with this application in electronic format. The file name is;
AQUA data Ames.xls

Introduction

The mutagenic activity of leaf and petal extracts FLORIGENE Moonaqua™123.8.12 was evaluated using four different strains of *Salmonella typhimurium* grown with or without a metabolic activator, rat liver microsomal fraction (S9mix). TA97 and TA98 are frame shift strains. TA 100 and TA102 are base change strains. The tests were carried out by an accredited laboratory in Japan.(Dr. Wataru Fujii, Quality Assurance Division, Suntory Ltd., 1-1-1 Wakayamadai, Shimamoto, Mishima,Osaka 618-8503). The procedure used was approved by the Ministry of Health, Labour and Welfare, Japan. The strains and replication used are consistent with the recommendations for conducting the Ames test by both the US FDA (FDA, 2000) and the OECD (1997).

Materials and methods

Petals and leaves were freeze-dried and extracted in distilled water at a concentration of 50 mg freeze dried material per mL water. This solution was filtered through the 0.45 µm syringe filter for use in the assay. Table 1 provides the extraction weights.

Table 1. Extraction weights

	Tissue	Fresh weight (g)	Weight after freeze drying (g)	mg/mL distilled water for extract preparation	Effective fresh weight (mg) extracted per mL
FE123	Leaf	25	5.2	50	240
	Petal	20	2.35	50	426
123.8.12	Leaf	25	4.6	50	272
	Petal	20	1.95	50	513

The raw extract was serially diluted with sterile distilled water to a ten-fold dilution. Giving solutions equivalent to 5, 12.5, 25, 37.5 and 50 (undiluted) mg freeze dried material per mL

Salmonella typhimurium strains TA97, TA98, TA100 and TA102 (provided by the National Institute of Hygienic Sciences in Japan) cultured in nutrient broth at 37°C overnight were used for mutation tests. The pre-incubation method was used, in which. 0.1 ml of sample (i.e.0.5, 1.25, 2.5, 3,75 or 5 mg freeze dried material per plate) was mixed with 0.5 ml of S9 mix in 0.1 M sodium phosphate buffer (pH 7.4) or 0.5ml buffer only (-S9) , and 0.1 ml of bacterial culture. The mixture was incubated at 37°C for 20 min with shaking, then 2ml of soft agar was added and the mixture poured over minimal glucose agar plate. After incubation at 37°C for 2days, the number of revertants was counted. Distilled water was used as a negative control. Positive controls used were;

Without S9mix

TA97: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) 0.1µg/plate
TA98:2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide ((AF-2) 0.1µg/plate
TA100: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide ((AF-2) 0.02µg/plate
TA102:tert- butylhydroperoxide solution 70% (t-BuOOH) 0.5µg/plate

With S9mix

TA97:2-aminofluorene (2-AF)	5µg/plate
TA98: benzo[*a*]pyrene (B[a]P)	5µg/plate
TA100: benzo[*a*]pyrene (B[a]P)	5µg/plate
TA102:2-aminoanthracene (2-AA)	10µg/plate

There were two replicate plates for each treatment except the negative controls, where there were three. Samples giving revertants more than 200% of the negative control (the number of spontaneous revertants) were regarded as positive.

Results

There was no effect of either control or transgenic carnation leaf or petal extracts on the number of revertants per plate, with or without rat liver microsomal fraction, at any concentration tested. All positive control mutagens were effective. The observations are summarized in table 2, which identifies the extract concentrations in which the difference in revertant colony number against negative control was greatest, as percentage of colony reversion on distilled water. In many cases, all extract concentrations in a particular strain/mix combination had a lower revertant rate than the negative control. Otherwise, reversion rate was at most 45% greater than the negative control, so indicative of no mutagenic activity in any extract.

Table 2. Summary of revertant rate of extracts relative to the negative control (i.e.100% is the same as the control). "none" indicates all extracts tested gave a revertant rate less than 100% Rate = extract concentrations at which the maximum difference to negative control was observed. Diff =number of colonies in test extract/ number of colonies in negative control.

	FE123 leaf		**FE123 petal**		**123.8.12 leaf**		**123.8.12 petal**	
	Rate (mg)	**Diff.**	**Rate (mg)**	**Diff.**	**Rate (mg)**	**Diff.**	**Rate (mg)**	**Diff.**
TA100 +	5	115%	2.5	107%	1.25, 5	103%	3.75	107%
TA102 +	None		2.5	106%	None		5	105%
TA97 +	0.5	103%	5	117%	1.25	108%	3.75	115%
TA98 +	0.5, 3.75	116%	3.75	145%	3.75	134%	5	116%
TA100 -	None		5	108%	None		3.75	104%
TA102 -	None		None		2.5	108%	None	
TA97 -	3.75	114%	2.5	116%	0.5	103%	None	
TA98 -	None		3.75	126%	0.5	106%	None	

Conclusions

Based on the results, it is concluded that the extract from the genetically modified carnation FLORIGENE Moonaqua™ (123.8.12) has no mutagenic activity, measured in an Ames/*Salmonella* test. Brown and Dietrich (1979) found delphinidin was inactive in the Ames assay system using 5 different strains of *Salmonella typhimurium*. In fact, delphinidin may have anti-mutagenic properties, as Huang et.al. (1983) found that 5nmol delphinidin chloride was sufficient to reduce by 50% the mutagenic effect of BP 7,8-diol-9,10-epoxide-2 in the TA100 strain. There is also evidence delphinidin may have anti-cancer (Kobori, 2003) and anti-neoplastic effects (Middleton et.al., 2000). The health properties of anthocyanins, including delphinidin, are further described in Sterling (2001) and Lila (2004).

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ATTACHMENT B3

Acute toxicity test

Data from these experiments is included in this application as an electronic file;
AQUA data acute toxicity 2006.xls

Introduction
An acute toxicity study was conducted in ICR male mice, administered a single dose of extract at 4g/kg. Animals were observed for 14days after dosage and autopsied at the end of the experiment. The experiments were carried out by the Safety Science Institute, Suntory Ltd., Japan, using extracts from leaf and from petal of the transgenic line FLORIGENE Moonaqua™ (123.8.12), in comparison to the extracts from leaf and petal of the control (FE123), and a water supplemented diet (control group). The procedure used was approved by the Ministry of Health, Labour and Welfare, Japan. Replication, dosage and duration are consistent with EPA guidelines (EPA, 1998), which are themselves consistent with OECD chemical testing guideline 420 (Acute Oral toxicity – Acute Toxic).

Methods
Petals and leaves of control (Line 123) and line FLORIGENE Moonaqua™ (123.8.12) were frozen in liquid nitrogen and ground with a pestle and mortar in distilled water. Weights after freezing are given in table 1 below;

Table 1. Fresh weights of material used for samples

Line	Source material	Fresh weight (g)	Weight after freeze drying (g)
FE 123	Leaf	25	5.2
FE 123	Petal	20	2.35
123.8.12	Leaf	25	4.6
123.8.12	Petal	20	1.95

To prepare samples for feeding 4g freeze dried material was dissolved in 10mL distilled water, after which extracts were filtered through a 0.45µm membrane. Animals were then administered at a rate of 10mL per initial body weight (i.e. equivalent to 4g freeze dried material per kilogram body weight) Four-week-old ICR male mice (SPF) were purchased from CLEA Japan Inc. and used for the test after 6 days acclimation. Mice were held in polyisopentene cages 5 mice in a cage, in an air-conditioned room (temperature of 23.5ºC±2.0°C, humidity of 55±5%, lighting of 12hours/day). Food (CE-2, CLEA Japan Inc.) and water were taken freely. Mice were divided into groups of 5 animals for each treatment. Observations were carried out for 14 days after the administration of the extract. Clinical observation was conducted 0, 1 and 3 hours after administration of the test material, and regularly thereafter. Body weight was measured immediately before administration of the materials and regularly thereafter. All mice were killed by cervical vertebrae dislocation after 14 days of the administration, and were observed macroscopically.

Results
No deaths occurred in mice throughout the experimental period, and no apparent abnormalities were observed in any groups throughout the experimental period. The body weight and terminal autopsy showed no treatment-related changes. Mean body weight with time is shown in Figure 1.
For petal extracts, the Student T-TEST was performed for the transgenic line, on all dates of data. Data comparison was against water, and against recipient variety. On all dates there was no significant difference between groups, reflected in a comparable mean body weight (figure 1).

For leaf extracts, the Student T-TEST was performed for the transgenic line, on all dates of data collection. Data comparison was against water, and against recipient variety. On day 1 there was a significant difference between the FE123 group and the FE 123.8.12 group. On all other dates there was no significant difference between groups, reflected in a comparable mean body weight (figure 1).

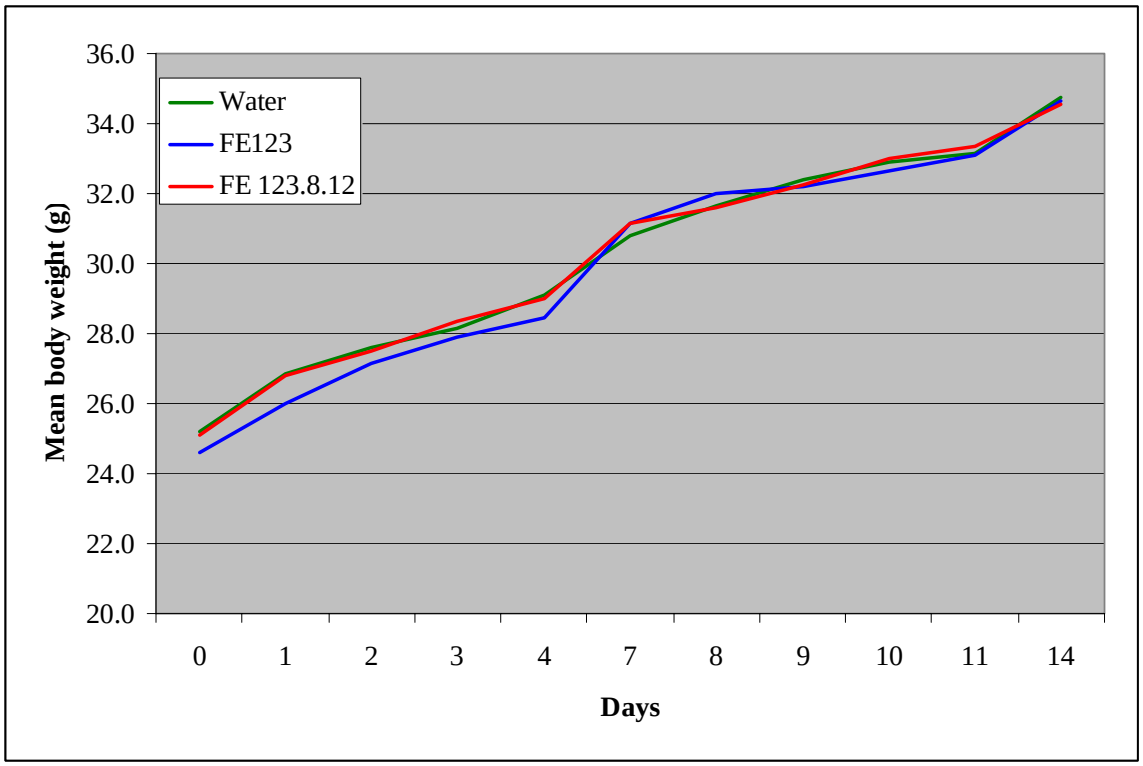
Conclusions

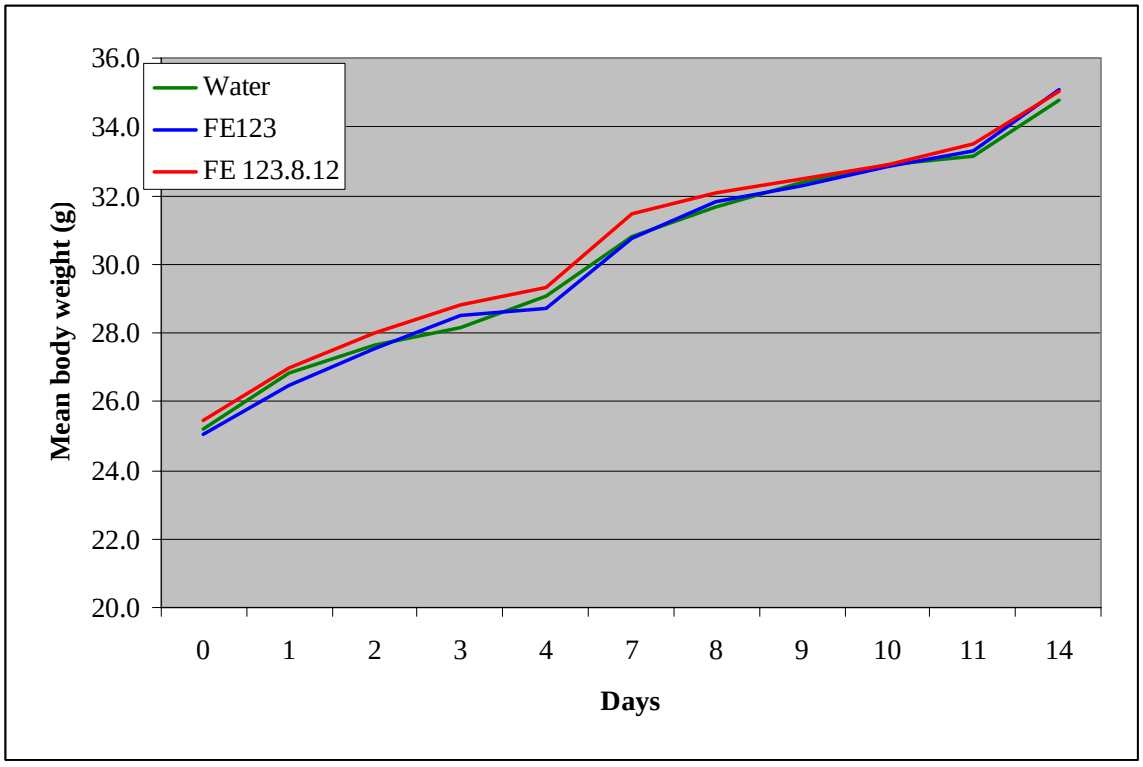
No apparent abnormalities were observed in either group throughout the experimental period and no abnormalities were observed in either control or test group during autopsy.
As no significant change to body weight was detected it was concluded that consumption of petal or leaf extract from either the transgenic line FLORIGENE Moonaqua™ (123.8.12), or the parental line it is derived from, exerted no toxic effects.

References

1. EPA Health Effects Test Guidelines EPA 712–C–98–190 OPPTS 870.1100
Acute Oral Toxicity, August 1998

Figure 1. Mean body weights. Upper graph is for petal extract and lower graph is for leaf extract





ATTACHMENT B4

Phytotoxicity

Introduction

A plant bioassay was used to determine whether FLORIGENE Moonaqua™ (123.8.12) leaf exhibited any phytotoxic effects. In this test lettuce seed germination and seedling growth was used as a bioassay in soil to which material from the transgenic line, and the parental line FE123 were added. To supplement the plant Bioassays, microflora counts were made from soil in which transgenic or recipient plants had been grown.

Methods

(i) Assessment of potential phytotoxicity. Lettuce seed (cv.green mignonette) was germinated in 100g soil (control), and 100g soil to which had been added leaf material from recipient or transgenic plants. The leaf material had been previously frozen in liquid nitrogen, and was mixed into the soil at the equivalent rate of 5g fresh leaf per 100g soil. For each treatment there were five pots, and each pot was sown with 20 seed. The shoot weight, shoot length and root length of all germinated seedlings was weighed, and the data for the first ten germinated seedlings (to provide an even replication) analysed by ANOVA. Data is provided in electronic format with this application;
AQUA seed germination.xls

(ii) Microflora in used soil. Three grams of soil was mixed well in 27 ml of a 0.85% solution of NaCl. A set of serial dilutions was made (10, 100, 1000 and 10000 times) and 0.1 ml of diluted samples was spread on the surface of 9cm agar plates. In addition, thirty grams of soil was desiccated at 80 degrees for 24 hours to measure percentage of moisture content, for dry weight calculations (results are indicated per1 gram of dry soil).Two different media were used;

- PDA; Potato dextrose agar powder 39 g/L, Rose Bengal 100mg/L and Chloramphenicol 170 mg/L . This medium is specific for fungal colony growth.
- TSA; Trypticase Soy Broth powder 30 g.L, Cycloheximide 100 mg/L and Bacto agar 15g/L. This medium is specific for bacterial growth.

TSA medium was inoculated with 0.1mL of the 1000 and 10000 dilutions and incubated at 29 ºC for 3 days.PDA medium was inoculated with 0.1mL of the 10 and 100 times dilutions and incubated at 29 ºC for 4 days. At the end of the experiment, colonies per plate were counted.
Three pots were sampled per soil type (grown with FE123 or grown with the transgenic line), and five replicate plates set up per sample.

Results – lettuce seed germination

Percentage germination was approximately 85% (Table 1).

Table 1. Percentage germination

Treatment	Germination (%)
Seed grown in fresh soil	88 ± 6
Seed grown in soil with leaf from FE123	86 ± 8
Seed grown in soil with leaf from 123.8.12	82 ± 13

Table 2 provides a summary of means per treatment for each of the three growth parameters. There was no significant difference between treatments with each parameter.

Table 2. Effect of leaf supplements on the seedling weight of lettuce. Data shows mean values.

	Control	**Recipient (123)**	**FLORIGENE Moonaqua ™ (123.8.12)**
Shoot weight (mg)	23	21	24
Seedling length (mm)	26	26	23
Root length (mm)	19	19	17

Results - soil microflora

Despite very significant variation between extracts, there was no significant difference in soil microflora (Table 3) when soil from pots in which the transgenic line was grown was compared to soil from pots in which the recipient plant was grown.

Table 3. Microflora counts in soil from pots in which the transgenic line FLORIGENE Moonaqua ™ (123.8.12) and the recipient plant was grown

Medium	**Extract number**	**(cfu/g dried soil)**	
		Recipient (123)	**FLORIGENE Moonaqua ™ (123.8.12)**
PDA	1	2.9×10^2	2.5×10^2
	2	2.3×10^2	2.3×10^2
	3	1.9×10^2	1.6×10^2
	Mean	**2.0**	**2.1**
TSA	1	146×10^6	212×10^6
	2	70×10^6	15×10^6
	3	18×10^6	19×10^6
	Mean	**78**	**79**

Each data point is the mean of 5 plates from a single extraction

Conclusions

On the basis of the plant bioassays leaf extracts added to soil mix, from either the recipient plant or the transgenic line, there was no evidence that there were any phytotoxic compounds leached into the soil by either the recipient plant or FLORIGENE Moonaqua™ (123.8.12). There was no significant difference in the microflora profile of soil taken from around the roots of the recipient and transgenic plant.

ATTACHMENT B5

An assessment of the probability of gene dispersal from cut-flowers of the cultivated carnation (*Dianthus caryophyllus*) imported into Europe

CONTENTS

SUMMARY

The attached document assesses the probability of the dispersal of genes from carnation flowers imported into Europe. It provides background information on the biology of carnation supported by an extensive literature review.

On the basis of this background information we conclude that the probability of unintentional gene dispersal from an imported carnation flower is nil.

This is because possible avenues for gene dispersal are not available. There are three possible mechanisms for gene dispersal:

1. Vegetative spread of the imported cut flowers, leading to the formation of wild clonal populations.
2. Formation and dispersal of seed from the imported cut flower as a result of self fertilization or fertilization with pollen from an external source.
3. Formation of seed by a recipient plant, fertilized by pollen dispersed from the imported cut flower.

The paragraphs below briefly outline why the probability of gene dispersal from a carnation flower by any of these routes is nil.

1. Vegetative spread. Carnation does not spread vegetatively, i.e. the plant does not produce organs such as stolons, rhizomes, root-borne shoots, tubers, bulbs, corms or runners. Roots will not form on discarded or old cut-flowers. Florigene has experience of large scale production of carnation in Australia, Japan, Colombia and Ecuador. Carnation has never been found growing wild, even in the

immediate vicinity of carnation growing areas where waste material has been discarded or has been left for composting.

2. Formation of seed on a cut flower. For gene dispersal by seed formation to occur from a cut carnation flower, the following events would all need to occur successfully; arrival of viable pollen on the stigma of the carnation, pollen germination, pollen tube growth to the ovule of the carnation, fertilization, seed formation and seed dispersal. Notwithstanding the fact that successful pollination of a carnation flower in a vase is highly unlikely, no seed set could occur. This is because the process of seed development takes at least 5 weeks on a plant – where the growth of any developing embryo could be sustained. A cut flower will remain in a consumers hand for three weeks at most before dying.

3. Pollen dispersal from a cut flower leading to a successful hybridisation event. There are several mutually exclusive facts that, in combination, indicate that potential pollen spread is not a feasible avenue for gene dispersal.
Firstly, the potential for pollen spread from a cut flower is only theoretically possible;

- In general, production of viable pollen by carnation is much lower than that of wild *Dianthus* species.
- Hybridization of *Dianthus* in nature is facilitated by insect pollination and is only effectively achieved by the Lepidoptera (butterflies, moths). Pollen is not spread by wind.
- The only point in the chain where insects could be reasonably expected to access flowers is when on display or in consumers hands. The physical barrier of the multiple petals presents a significant obstacle to any potential pollinating insects in less open flowers.
- As a carnation flower opens out in the vase, any anthers are likely to have fallen from the stamens and any pollen would have significantly reduced viability.

Secondly, were viable pollen to actually be produced and successfully dispersed by an insect vector, the realistic chance of a successful fertilization resulting in a wild hybrid population is also extremely unlikely.

- *Dianthus caryophyllus* is very rare and only found wild in coastal Mediterranean areas.
- In commercial carnation production, flowers are removed. The high concentrations of carnation production in Europe are therefore not available as potential recipients.
- While interspecific hybridization is know in *Dianthus*, hybridization is restricted to local regions where two species grow in high density and freely intermingle and is particularly common in the Pyrenees. In these wild conditions there may be 4-5 flowering plants of any one species per square metre.
- The majority of *Dianthus* species are not widely spread in Europe, and are confined to one or two countries, to specific mountain regions or to alpine areas.
- The flowering period of wild *Dianthus* species is limited to summer in Europe. When wild *Dianthus* species are not in flower, which is for 8 – 11 months, there is NIL risk of gene dispersal from carnation as a result of fertilization and seed set in recipient plants.
- In the extremely remote case that a seed was formed as a result of fertilization of carnation pollen onto a wild *Dianthus* species, there are very limited environments where a plant could become established. In nature, *Dianthus caryophyllus*, like many *Dianthus species* is only found in calciferous soils in coastal regions. The tops of sand or limestone hills and outcrops could also be suitable habitats. The seed of the putative hybridization event would probably have to germinate in such environments in order to become established in the wild.

The long history of cultivation and consumption of carnation flowers in Europe strongly supports the assertion that there is nil risk of gene dispersal.

- Carnation is not a weed in Europe. 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. Each year over 10 billion flowers are produced for the world's flower markets.
- No hybrid between carnation and any other *Dianthus* species has ever been recorded in the wild.

A. BACKGROUND

A1. The genus *Dianthus*

The *Dianthus* genus is a member of the Caryophyllacea, or pink, family and contains about 300 species. The genus is native to Europe, Asia, North Africa and the Arctic region, where one species is found (Hickey and King, 1981; Tutin and Walters, 1993). The second edition of *Flora Europaea* (Tutin, and Walters, 1993) lists 115 species, and 91 sub-species within 32 of these species. Seventy seven of the species listed are endemic to Europe. The centre of biodiversity for *Dianthus* is southern Europe and the greatest range of *Dianthus* species are found in the south eastern European countries. Table 1 from Tutin and Walters (1993) lists the number of species recorded in countries with the greatest diversity of *Dianthus* species.

Table 1. European countries with the greatest diversity of Dianthus species

Country	Number of *Dianthus* species
Former Yugoslavia	44
Bulgaria	39
Greece	37
Romania	32
Central + S.W. European Russia	31
Spain	26
Italy	24
Albania	21
France	20

In Europe, most *Dianthus* species are found in the Balkan region and in the Mediterranean countries. In North Europe *Dianthus* species are far less common, or even absent. Only six species are known in the British flora (Clapham *et al*., 1987), and five in Holland (Tutin and Walters, 1993). The majority of *Dianthus* species are not widely spread in Europe, and are confined to one or two countries, to specific mountain regions (Strid and Tan, 1997) or to alpine areas (Schwegler, 1979). There are six species that have been found throughout the world. They are described below using information from Tutin and Walters (1993):

- *D. barbatus.* Not a European species exclusively, *D. barbatus* is native to all Balkan countries and Eastern Europe. It has naturalized elsewhere after escape from cultivation.
- *D. armeria.* The most widely distributed *Dianthus* species in Europe, as far north as Southern Sweden. This species has a world-wide distribution.
- *D. sylvestris*. This species is highly polymorphic and closely related to *D. caryophyllus,* the wild species from which the carnation was developed (De Langen *et al.,* 1984). This species is found in southern Europe and Mediterranean islands. There are six sub-species, three of these which are endemic to Europe, though the species itself is not.
- *D. superbus*. This species, which is not endemic to Europe, is found in all areas except much of the west and south (Tutin and Walters, 1993). There are three sub-species.
- *D. deltoides*. This species is not endemic to, but is distributed in, most of Europe. It is rarer in the south (Tutin and Walters, 1993).

- *D. carthusianorum.* The only one of the six species which is possibly endemic, this species is found in south, central and western countries and has a very variable form.

In northern European countries the majority of *Dianthus* populations, if present, will be represented by one or more of the above six species (e.g. Perring and Walters, 1976; Van der Meijden, 1990). The distribution of these species in the North of Europe may be sporadic (Berten, 1990; Schonfelder and Ahlmer, 1990; Benkert *et al.*, 1996). *D. deltoides* and *D. carthusianorum* are more common than the other four species listed above (Schonfelder and Ahlmer, 1990; Benkert *et al*., 1996).

The genus *Dianthus* contains several species which have been cultivated for hundreds of years for their ornamental value (Ingwerson, 1949). Table 2 lists some of the more commonly grown 'pinks' species.

Table 2. Dianthus species commonly grown for their ornamental value

Species	Common name
D .plumarius	Cottage pink, grass pink
D. alpinus	Alpine pink
D. sylvestris	Wood pink
D. chinensis	Chinese pink, Rainbow pink, Indian pink, Japanese pink
D. deltoides	Maiden pink
D. gratianopolitanus	Cheddar pink
D.carthusianorum	Carthusian pink
D. superbus	Fringed pink
D. armeria	Deptford pink

A2. *Dianthus caryophyllus*

Carnations are double-flowered cultivars and in the general trade, botanical and horticultural literature carnation cultivars are considered to belong to the species *Dianthus caryophylllus.* The common name for *Dianthus caryophyllus* is carnation. However, the exact taxonomic and breeding history of carnation is not known (Hughes, 1991) and it is almost certain that carnation is a hybrid involving two or more *Dianthus* species, one of which is likely to be *Dianthus caryophyllus* (Hughes, 1991; Allwood, 1954). It is believed that carnation breeding began in the 1500's in France (Holley and Baker, 1963). More than one hundred years ago carnation breeding was well established in the USA, and today there are half a dozen large breeders in the world. The second edition of the International *Dianthus* register (1983) lists over 30,000 cultivars.

In its unimproved, single flower form, *Dianthus caryophyllus* is called the clove pink or Grenadine (Britannica, 1999). Clove pink was grown in the middle ages for its clove like perfume. Clove pink was named by Linneaus in *Species Plantarum* (De Langen *et al.,* 1984) and may be taken as the type species for *Dianthus*.

Dianthus caryophyllus is only found wild in coastal Mediterranean areas, and is rare. Whilst this area encompasses Spain, France, Greece, North Africa and Italy, Tutin and Walters (1993) state that the species is possibly only native to Sicilly, Sardinia, Italy and Greece. Polunin and Huxley (1967) suggest the species occurs in France, Algeria and Morocco. In European floras the species is listed in Italy, Sicily and Sardinia (Zangheri, 1996), but not in recent floras of Greece (Strid, 1986; Strid and Tan, 1997; Turland *et al*., 1993), France (Guinochet and Vilmorin, 1973) and Andalucia (Valdes *et al*., 1987). In Mallorco *Dianthus caryophyllus* is only listed as a garden species (Barcelo, 1978). According to Ingwersen (1949) wild *Dianthus caryophyllus* can only be found commonly in specific coastal regions of Corsica. The flower of some cut-flower carnation varieties is now quite different to a flower from a wild Dianthus species, such as *Dianthus barbatus*, shown in Figure 1.

Figure 1. Comparison of a flower from Dianthus barbatus (left hand side) to a flower from a cut-flower "standard" carnation variety (right hand side).

Cut flower varieties of carnation grow to 60 – 120 cm high, depending on variety, and produce flowers with a diameter up to 60 mm. Carnation can have from 30 – 100 petals per flower and the reproductive tissues of the flower are enclosed by the petals. In contrast, wild Dianthus species have an open flower, with the stigma and style easily accessible. The distinctive calyx of the genus Dianthus is seen in both flowers shown in Figure 1. The long tubular calyx is a morphological adaptation to pollination by moths and butterflies in the wild. In wild Dianthus species the calyx ranges from 5 – 30 mm (Strid, 1986) and in cut flower carnation varieties from 25 - 40 mm. The calyx is relatively thick in the larger cut flower varieties.

A3. Types of cultivated carnation

There are hundred's of cut-flower varieties of cultivated carnation. These are divided into groups based on plant form, flower size and flower type. The two dominant groups, accounting for 96% of sales in the Dutch auctions, are standards and sprays.

Standards. These cultivars are grown under conditions in which a single large flower is produced per stem. Side shoots and buds are removed (a process called disbudding) to increase the size of the terminal flower.

Sprays (also called miniatures in the USA market). These cultivars are intended for cultivation to give a large number of smaller flowers (one per side shoot) per stem. Only the central flower is removed, allowing the laterals to form a 'fan' of stems.

The midi is a less widely grown type of carnation. Midi varieties have the form of a standard

carnation flower in that they do not exhibit extensive branching, and have a single dominant flower per stem. However, the flower has a smaller diameter and the stem length is typically 45 – 60 cm, compared to 55 – 80 cm for a standard carnation flower. Midi varieties also produce twice as many flowers, per plant as standards. Midi carnations are not disbudded and so small, unopened flower buds may occur around the main stem or on short side branches. Figure 2 compares a midi carnation to a standard carnation.

Figure 2. Two of Florigene's transgenic carnation varieties. FLORIGENE Moonvista™(left) a standard variety, and FLORIGENE Moondust™(right) a midi type.

In addition to carnations, cut flower breeders have released varieties of other *Dianthus* species for sale to cut flower growers. Examples of these types of cultivars are the Sole Mio series and the Gypsy series, which are derived from *Dianthus barbatus*. The appearance of these varieties is distinct from carnation in that the flowers are singles, like pinks, whereas the carnation has many.

A4. Carnation cultivation methods

The cultivated carnation is vegetatively propagated and to produce plants for cut flower production cuttings are taken from vegetative 'mother plants' which are continually pruned to produce a high number of vegetative cuttings from axillary buds. These cuttings are rooted in conditions of high humidity, after treatment with rooting powder.

Rooted plants may be planted in soil or grown hydroponically, and are kept for 1-2 years. Flowers are produced in flushes, beginning 3-5 months after rooted cuttings are planted. Picking of all flowers is essential and flowers must be harvested in tight bud (or closed bud for spray types) for distribution and marketing. The correct pick stage is strictly enforced by the Dutch auction system, to ensure a satisfactory vase life in the hands of the consumer.

A major problem for growers is the fungal wilt disease *Fusarium oxysporum.* The occurrence of this fungus in untreated soil has led to relocations of growing areas in countries such as Spain, and adoption of cleaner cultural practice by the majority of European growers. Major pests of carnation are thrips, aphids and mites.

A5. Carnation consumption in Europe

Approximately 8 billion carnation flowers are consumed in Europe each year (Heinrichs and Siegmund, 1998). Approximately 20% of the European supply of cut carnation flowers is imported (largely into Holland, United Kingdom and Germany) from Colombia, Ecuador, Kenya, Israel, Morocco, Turkey and Zimbabwe (Heinrichs and Siegmund, 1998). As Table 3 indicates, most of the flowers produced in Europe are grown in Italy and Spain. The data suggests that there have been in excess of 800 million carnation plants in cultivation on an annual basis in Europe for more than 30 years. On the basis of an average yield of 8 flowers this represents 6 to 8 billion flowers a year, the vast bulk of which would be consumed in Europe.

Table 3.Historical amounts of carnation production in Europe. Carnation is planted at a density of 200,000 to 250,000 plants per ha.

Country	Number of plants in production		
	Year	ha	Millions of plants
Spain	1975	1,739	391.2
	1991	3,651	821.3
France	1970	427	159.3
	1990	142	31.9
Greece	1986	246	55.3
	1992	179	40.2
Italy	1975	2,679	602.6
	1994	1,280	287.9
Netherlands	1975	428	96.3
	1994	213	47.9
UK	1980	40	9.0
	1993	9	2.0
Germany	1978	34	7.7
	1992	23	5.2

For countries with available data, the majority of cultivated carnation is grown under cover. The percentage of carnation under cover is, for Holland, France, Italy and Spain respectively, 100%, 100%, 85% and 79% (Heinrichs and Siegmund, 1998).

A6. Carnation importation and distribution

Information on the actual number of carnation flowers imported into Europe can be accessed from EUROSTATS (http://fd.comext.eurostat.cec.eu.int/xtweb/submitformatselect.do). Table 4 overleaf provides an extract of the data for the major producing and importing countries.

Carnations are imported into Europe by air freight, or by truck from Turkey and Morocco. Typically imports are handled by specialist importers, who provide distribution to wholesale and retail flower outlets. At the wholesale level these outlets could be wholesale florists or flower markets, such as Covent Garden in the UK. There are many specialist importers affiliated to the Dutch auctions. Wholesale florists and markets provide access to flowers for florists. Importers may also be, or may forward to, companies that specialize in provision of flowers for supermarket/ grocery/ garage chains. In these case final product (perhaps assembled in bouquets with other flowers), would be sleeved, labeled and distributed to individual stores. Flower longevity in this chain is summarized in Table 5 overleaf.

Table 4. Number of carnation flowers imported into several EU countries from several production countries from 2000 -2003

Importing country	Year	Colombia	Turkey	Kenya	Morocco	Israel	Ecuador	Total
Germany	**2000**	50,765,268	359,664	5,356,078	8,198,212	3,858,990	4,952,972	73,491,184
	2001	48,902,440	451,874	2,317,981	5,115,067	1,576,500	2,961,638	61,325,500
	2002	52,677,256	412,552	62,760	2,892,839	944,850	1,382,891	58,373,148
	2003	50,761,280	647,482	3,000	4,345,319	138,880	1,355,470	57,251,431
United kingdom	**2000**	307,234,912	64,353,136	99,251,968	21,624,688	1,394,100	1,181,611	495,040,415
	2001	255,149,728	77,593,696	102,245,400	19,361,512	658,200	291,748	455,300,284
	2002	243,859,264	81,239,352	74,903,128	18,394,280	278,142	194,642	418,868,808
	2003	284,695,264	81,649,392	36,575,208	13,943,995	415,800	2,498,762	419,778,421
Netherlands	**2000**	72,195,368	13,720,400	15,733,225	4,773,235	41,338,272	7,686,663	155,447,163
	2001	96,347,656	17,742,966	16,243,393	2,856,530	24,660,408	13,322,383	171,173,336
	2002	121,064,904	4,682,580	11,241,201	3,420,528	19,270,770	9,400,649	169,080,632
	2003	146,683,984	11,456,981	8,377,228	2,942,700	17,001,572	8,350,561	194,813,026
Total	**2000**	452,988,906	78,433,200	120,341,271	34,596,135	46,591,362	13,821,246	746,772,120
	2001	427,499,097	95,788,536	120,806,774	27,333,109	26,895,108	16,575,769	714,898,393
	2002	439,628,832	86,334,484	86,207,089	24,707,647	20,493,762	10,978,182	668,349,996
	2003	497,550,354	93,753,855	44,955,436	21,232,014	17,556,252	12,204,793	687,252,704

Table 5. The process chain for cut carnation flowers imported into Europe.

Step in process	Duration (days)	Maximum total duration (days)
Harvest of flowers at farm	0	0
Processing, storage for flying	1- 14	14
Distribution to importers	1-3	17
Processing and distribution to arrival at final retail destination	1-3	20
Display at retail	5	25
Consumption “in the vase” –e.g. display and use by consumer before discarding	3- 21	46

Until the time flowers are displayed for consumers, distributors maintain flowers dry in boxes, or in buckets with a small amount of water. During this time flowers are refrigerated, typically in large walk in cool rooms, and the flowers are closed, as the refrigeration prevents flowers opening. In the consumers hands, at ambient temperature, and in water, flowers will hydrate, fully open and eventually senesce and die.

Carnation vase life is determined by the age of the flowers from harvest, variety, and how well the flowers have been treated after harvest with preservative chemicals. Correct treatment prevents damage by ethylene, the compound that triggers senescence in carnation flowers. Ethylene is produced by carnation flowers 2 – 7 days after flowers are fully open. Even well treated, very fresh carnation flowers will eventually dehydrate, senesce and die in the vase.

A7. Weediness

Dianthus caryophyllus is not a weed (Tutin and Walters, 1993). Despite decades of cultivation, and plantings in parks and gardens, it has not become a weed, or escaped from cultivation, anywhere in the world. Each year over 10 billion flowers are produced for the world’s flower markets, and we have studied the floras of several areas with a significant area of cultivated carnation. There are no reports of naturalization of carnation in these floras:

- **Japan**. Flora-Kanagawa, 1988; Ohwi, 1965.
- **Andean mountains, Ecuador**. Jorgensen and Ulloa Ulloa, 1994.
- **Uplands of Kenya**. Agrew, 1974.
- **Michoacan, Mexico.** Garcia and Jimenez, 1993, Jimenez and Garduno, 1995.
- **Israel.** Weissmann-Kollmann, 1965; Zohary, 1966.
- **Victoria and New South Wales, Australia**. Harden, 1992; Willis, 1988

The cultivated carnation has no capacity to escape from cultivation as the crop possesses no vegetative propagation mechanisms and there are no opportunities for seed-set.

In Europe, there have been escapes from cultivation of some *Dianthus* species (Tutin and Walters, 1993). These populations could be considered weeds because of their appearance in disturbed lands. However, they cannot be considered ecologically or economically important weeds (Holm *et al*., 1979; Guillerm and Maillet, 1982; Holzner and Immonen, 1982). Many *Dianthus* species are adapted to very specific geographical and climatic regions, such as alpine, rocky or sandy areas. This restricts their capacity for weediness.

B. ASSESSMENT OF THE PROBABILITY OF GENE DISPERSAL

B1. Introduction

The dispersal of genes from a flower can be by three routes;

- Through vegetative spread of the discarded flower, leading to the formation of adjacent clonal populations. This possibility is discussed for carnation in Section B3.
- Through the formation and dispersal of seed from the flower in question, as a result of self fertilization or fertilization with pollen from an external source. This possibility is discussed for carnation in Section B4.
- Through the formation and dispersal of seed by a recipient plant, fertilized by pollen transferred from the flower. This possibility is discussed for carnation in Section B5.

As there are known to be weeds in the Caryophyllaceae family in Europe, the probability of intergeneric hybridization is also discussed, in Section B6.

B2 Hybridization in *Dianthus*

In nature the *Dianthus* genus is characterized by a capacity for interspecific hybridization (Pax and Hoffmann, 1934; Ingwersen, 1949; Demmink, 1978; Castroviejo *et al.,* 1990). Hybrids are possible between different species of *Dianthus* and the resulting ploidy levels are not related to fecundity (Gatt M.K. *et al.,* 1998).

Dianthus sylvestris is found “from SE Spain to Greece and Northwards to the Swiss Jura and the Alps”. The typical habitat is rocky places (Polunin, 1980). There are also isolated pockets of garden escapes. For example, Clement and Foster (1994) describe a single population of *Dianthus sylvestris* in the UK, established on the rocks near Whitby harbour, in Yorkshire.Carnation has been grown and traded in Europe on a large scale for decades but there are no reports of the existence of hybrids between *D.caryophyllus* and *D. sylvestris* in the wild in Europe (Tutin et al., 1993).

Hybridization of *Dianthus* in nature is facilitated by insect pollination (Knuth, 1908; Frankel and Galun, 1977; Erhardt, 1988; Jennersten, 1983 and 1984) and is only effectively achieved by the Lepidoptera (butterflies, moths). These insects are the only ones with proboscis long enough (up to 2.5 cm) to reach the nectaries, which are located right at the base of the flower in all Dianthus species (Hickey and King, 1981). *Dianthus* flowers are tubular (Figure 1), with strong bracts and calyx to

exclude other insects (Knuth, 1908). Table 6 lists pollinators of *Dianthus* observed in Europe. *Dianthus* species are protrandous, i.e. the anthers and pollen mature before the pistils (Knuth, 1908; Buell, 1952; Keane, 1989). When *Dianthus* flowers first open, the styles remain short and smooth. At this time the flower sheds pollen, and the styles are non receptive. As the flowers age the styles elongate and become covered on their inner surface with many hairs. If a flowers is not successfully pollinated the styles continue to grow and curve. This can be seen in some cut carnation flowers, when the styles of some varieties protrude beyond the petals when they are left in the vase. If fertilization in *Dianthus* has been successful the flower collapses and the styles quickly shrivel. Protrandy largely prevents self-pollination. However, *D. deltoides* and *D.barbatus* are known to readily self seed, and volunteer in this way (MSU, 1996; Anon, 1999).

Table 6. Reported Lepidoptera insect pollinators of Dianthus. From Knuth (1908), Jennersten (1983,1984) and Erhardt (1988)

***Dianthus* species**	**Moth genera**	**Butterfly genera**
deltoides	-	*Hesperia, Aphantopus, Aporia, Cyaniris, Ochlodes, Mesoacidalia, Polyommatus,Thymelicus*
superbus	*Macroglossum*	-
carthusianorum	-	*Hesperia, Plusia*
chinensis	-	*Plusia*
barbatus	*Macroglossum*	*Pieris*
sylvestris	*Macroglossum*	-
atrorubens	-	not identified
monspessulanus	*Macroglossum*	-
caryophyllus	-	-

According to Tutin and Walters (1993) hybridization is restricted to local regions where two *Dianthus* species grow in high density and is particularly common in the Pyrenees. In these wild conditions there may be 4-5 flowering plants of any one species per square metre (Jennersten, 1984).

Carnation breeding

Efforts to hybridize *Dianthus caryophyllus* and other *Dianthus* species have been made to introduce useful horticultural genes into the cultivated carnation. Table 7 lists the species reported to hybridize to *D. caryophyllus*.

Table 7. Dianthus species reported to hybridize with D. caryophyllus

Species	**Reference**
arenarius	Holley and Baker, 1963; Umiel *et al*., 1987
barbatus	Pax and Hoffman, 1934; Umiel *et al*., 1987
carthusianorum	Demmink, 1978; Segers, 1987; Sparnaaij and Koehorst, 1990
chinensis	Mehlquist, 1945; Demmink, 1978; Sparnaaij and Koehorst, 1990
deltoides	Umiel *et al*., 1987
gallicus	Holley and Baker, 1963
giganteus	Demmink, 1978; Sparnaaij and Koehorst, 1990
knappii	Holley and Baker, 1963; Segers, 1987; Sparnaaij and Koehorst, 1990
monspessulanus	Holley and Baker, 1963
plumarius	Gatt *et al*., 1998
sinensis	Holley and Baker, 1963; Umiel *et al.*, 1987
sylvestris	Holley and Baker, 1963; Umiel *et al*., 1987; Demmink, 1987
seguieri	Holley and Baker, 1963
versicolor	Sparnaaij and Koehorst, 1990

D. knappii has been used as a genetic resource for yellow flower colour, *D.superbus* for its long feather like petals and *D.barbatus* for its multiple flower head.

Interspecific crosses can only be made in the glasshouse using manual intervention. Where carnation is the female parent this entails preliminary petal removal, manual pollination, calyx opening, final petal removal and fruit ripening on the plant (Sparnaaij and Beeger, 1973; Keane, 1989, Gatt et al, 1998). Carolin (1957) made 108 different interspecific crosses of *Dianthus*, and found 22% of crosses were fertile or sub-fertile, possibly due to embryo abortion (Buell, 1953).

Some carnation cultivars are self-sterile (Darwin, cited in Knuth, 1908 first observed this phenomenon in D. *caryophyllus)* and selfing, even under controlled conditions, produces either no seed or fewer viable seeds per capsule than cross-pollination (Mehlquist and Geissman, 1947). Whilst there are some female sterile cultivars, (e.g. Copareve, Eolo) the cultivated carnation is not usually completely sterile (Silvy, 1978) and poor 'selfer' lines may produce seed after cross-pollination (Mehlquist and Geissman, 1947).

It has been possible to achieve a successful cross of *D. caryophyllus* to the widely spread European species *D. barbatus, D. carthusianurom* and *D. sylvestris* (this species is closely related *to D. caryophyllus*). All four of these species are diploid (2n = 30).

B3. Probability of gene dispersal from carnation by vegetative propagation

Carnation is vegetatively propagated (by cuttings) but the species does not spread vegetatively, i.e. the plant does not produce organs such as stolons, rhizomes, root-borne shoots, tubers, bulbs, corms or runners. Cuttings have to be struck in optimized conditions, and roots will not form on discarded materials, i.e. cut-flowers or old plants disposed of by growers or florists. Cultivated clove pink (single flowered *D.caryophyllus*) is not winter hardy (MSU 1996), as expected from the species natural distribution range on the coastal regions of the Mediterranean. The carnation will not survive outdoors in northern Europe. Florigene have spoken to many carnation growers and have our own experience of the large scale production of transgenic carnation in Israel, Holland, Australia, Japan and Ecuador. Carnation has never been found growing wild, even in the immediate vicinity of carnation flower growing areas.

B4. Probability of gene dispersal from carnation by seed set and seed distribution

For gene dispersal by seed dispersal to occur in a carnation flower, the following events must all occur:

- Arrival of viable pollen on the stigma.
- Pollen germination and pollen tube growth to the ovule.
- Fertilization
- Growth and maturation of the embryo and seed maturation on the cut flower.
- Seed dispersal.
- Seed germination and plant establishment

Fertilization

Whilst fertilization of carnation, even with pollen from other *Dianthus* species, is theoretically possible, the probability of natural fertilization in carnation, even under cultivation conditions is extremely low. This is because of the physical barrier of the multiple petals. This barrier presents a significant obstacle to any potential pollinating insects that might cross pollinate within the flower growing area or be carrying pollen from external sources. During production flowers are picked closed, and exported to Europe at this stage.

Seed set

Notwithstanding the fact that successful pollination is unlikely to occur, no seed set could occur on the cut flower. This is because the process of seed development takes from 5 weeks (Sparnaaij and Beeger, 1973; Gatt et.al, 1998) to 2 months (Arthur, 1981). Cut flowers of carnation, even if treated with silver for increased vase-life cannot be kept in the vase for longer than 3 weeks. Separation of the flower from the plant would in any event deprive any developing embryos of essential hormones and nutrients, preventing maturation.

B4. Probability of gene dispersal by pollen distribution from carnation

For gene dispersal to be successful by pollen dispersal, viable pollen would have to be transmitted to a recipient plant and fertilisation, seed set and seed dispersal occur. This sequence of events is extremely unlikely.

Pollen production by carnation

Standard and midi type cultivars of the cultivated carnation produce little or no pollen (Mehlquist *et al*., 1954; Kho and Baer, 1973; Nichols, 1976) because in most commercial cultivars of this type anthers are converted to petals early in flower development (Arthur, 1981). This is illustrated in Figure three. Spray type carnations produce more pollen than standards or midis. Amongst the spray types there are cultivar to cultivar differences in pollen production, but in general production of viable pollen by carnation is much lower than that of wild *Dianthus* species. It is known that water and nutrient stress can improve anther production in some standard cultivars and that temperature controls stamen and pollen production (Kho and Baer, 1973 Mehlquist *et al*., 1954). Carnations may be subject to high temperatures during the summer in southern Europe and this will reduce the potential for pollen production. Carnation pollen can be stored for one week (Sparnaaij and Beeger, 1973; Otten, 1991), placing a limit on the time available for transfer to a potential recipient. In enclosed environments such as glasshouses and greenhouses high humidity reduces pollen longevity.
The carnation flower opens out in the vase, increasing accessibility to the reproductive structures. In practice this is the only realistic opportunity for insect mediated pollen dispersal. However, at this stage of flower development any anthers are likely to have fallen from the stamens (Spaarnaaij and Beeger, 1973) and pollen would have significantly reduced viability, if any (Buell, 1952; Keane, 1989). If there were any viable pollen, it would still need to be dispersed to a suitable recipient plant.

Figure 3. Left. Dissected flower of flowers from a midi carnation, the Florigene variety FLORIGENE Moonshadow™. Functional anthers are replaced by petaloids (arrow). In FLORIGENE Moonvista™(right), a standard carnation, anthers are present in some flowers (arrowed).

Pollen dispersal

Carnation pollen cannot be spread by wind. Any pollen produced is heavy and sticky (Jennersten, 1983) and buried deep in the flower. A survey of the atmosphere in the Netherlands, a country with a large carnation industry, failed to detect any carnation pollen (Driessen *et al*., 1988).

Only insects have the capacity to disperse carnation pollen, and only *Lepidoptera* could facilitate pollen transfer from carnation flowers to potential recipient plants. Common pests like thrips, aphids and spider mites are unlikely to move pollen and though ants can be found in flowers of all types, ants are not likely to move much further than a few metres (Armstrong, 1979). It is well established that ants are also typical `nectar robbers' and their secretions usually kill pollen (Herrera *et al*., 1984; Gottsberger, 1989; Harriss and Beattie, 1991; Gomez and Zamora, 1992). Bees and wasps can be observed foraging in carnation flowers, but there is no evidence that this foraging may lead to cross pollination. As a precaution, bee movement is controlled in breeding houses, because petals are usually removed from flowers.

Assuming, theoretically, an insect were to access a carnation flower in a vase in someone's home, and carry away any viable pollen that might be present, the probability of subsequently fertilizing a recipient, flowering, *Dianthus* plant is limited. There are several studies suggesting insect pollinators only move pollen hundred's of metres from source when feeding (Price, 1984; Nilsson *et al*., 1992).

Ecological factors

The flowering period of wild *Dianthus* species is limited to summer in Europe (Table 8). When wild *Dianthus* species are not in flower, which is for 8 – 11 months, there is NIL risk of gene dispersal from carnation as a result of fertilization and seed set in recipient plants.

Table 8. Flowering period for several Dianthus species in the wild. Alpine flowering periods are from Schwegler (1979) and Mediterranean data is from Strid and Tan (1997)

Alpine		**Mediterranean**	
Species	**Flowering months**	**Species**	**Flowering months**
armeria	June - August	*armeria*	June - August
arenarius	June - September	*deltoides*	June - September
barbatus	June - September	*elegans*	May - July
carthusianorum	June - September	*giganteus*	June - August
glacialis	July - August	*gracilis*	June - August
monspessulanus	June – September	*integer*	July - August
pavonius	July	*superbus*	July - August
sylvestris	June - September	*sylvestris*	July - September

Many *Dianthus* species are found at high altitude on mountains (Strid, 1986) or on islands, not in carnation growing areas. These species are very unlikely to receive pollen from carnation plants.

In the extremely remote case that a seed was formed as a result of fertilization of carnation pollen onto a wild *Dianthus* species, there are limited environments where a plant could become established. In nature, *Dianthus caryophyllus,* like many *Dianthus species* is only found in calciferous soils in coastal regions. The tops of sand or limestone hills and outcrops could also be suitable habitats. The seed of the putative hybridization event would probably have to germinate in such environments in order to become established in the wild.

B6. Probability of intergeneric hybridisation

There are several species in the Caryophyllaceae which are widespread throughout Europe, and which are also considered serious weeds. These include *Arenaria serpyllifolia, Polycarpon tetraphyllum,*

Sagina apetala, Stellaria media, Silene gallica, Silene vulgaris and *Spergularia rubra*. However, as far as we are aware, there are no reports of intergeneric hydridisation in the Caryophyllaceae.

B7. Conclusions

There is no risk of gene dispersal by vegetative spread:

Carnation does not spread vegetatively, i.e. the plant does not produce organs such as stolons, rhizomes, root-borne shoots, tubers, bulbs, corms or runners. Roots will not form on discarded or old cut-flowers. Florigene has experience of large scale production of carnation in Australia, Japan, Colombia and Ecuador. Carnation has never been found growing wild, even in the immediate vicinity of carnation growing areas where waste material has been discarded or has been left for composting.

It could be possible, using tissue culture or other propagation techniques, to eventually deliberately cultivate plants from imported flowers. IF this were to occur, the propagated plant material could be ordered to be destroyed by Florigene, as its production would be in breach of our intellectual property protection in Europe, and we believe identification in the market place would be possible. If propagation was on a small scale, and we were unaware, we do not believe this would have an environmental impact. This is because the cultivated carnation has no means for natural gene dispersal. Unless cultivated, any propagated plant material would therefore remain in the area it was illegally propagated, and eventually die if not deliberately re-propagated.

There is no realistic possibility that seed could form on an imported cut flower

Notwithstanding the fact that successful pollination of a carnation flower in a vase is highly unlikely, no seed set could occur. This is because the process of seed development takes at least 5 weeks on a plant – where the growth of any developing embryo could be sustained. A cut flower will remain in a consumers hand for three weeks at most before dying.

There is no possibility that pollen could disperse from a cut flower and create a viable hybrid population

There are several mutually exclusive facts that, in combination, indicate that potential pollen spread is not a feasible avenue for gene dispersal.

Firstly, the potential for pollen spread from a cut flower is only theoretically possible;

- In general, production of viable pollen by carnation is much lower than that of wild *Dianthus* species.
- Hybridization of *Dianthus* in nature is facilitated by insect pollination and is only effectively achieved by the Lepidoptera (butterflies, moths). Pollen is not spread by wind.
- The only point in the chain where insects could be reasonably expected to access flowers is when on display or in consumers hands. The physical barrier of the multiple petals presents a significant obstacle to any potential pollinating insects in less open flowers.
- As a carnation flower opens out in the vase, any anthers are likely to have fallen from the stamens and any pollen would have significantly reduced viability.

Secondly, were viable pollen to actually be produced and successfully dispersed by an insect vector, the realistic chance of a successful fertilization resulting in a wild hybrid population is also extremely unlikely.

- *Dianthus caryophyllus* is very rare and only found wild in coastal Mediterranean areas.
- In commercial carnation production, flowers are removed. The high concentrations of carnation production in Europe are therefore not available as potential recipients.
- While interspecific hybridization is know in *Dianthus*, hybridization is restricted to local regions where two species grow in high density and freely intermingle and is particularly common in the Pyrenees. In these wild conditions there may be 4-5 flowering plants of any one species per square metre.
- Most *Dianthus* species are not widely spread in Europe, and are confined to one or two countries, to specific mountain regions or to alpine areas.

- The flowering period of wild *Dianthus* species is limited to summer in Europe. When wild *Dianthus* species are not in flower, which is for 8 – 11 months, there is NIL risk of gene dispersal from carnation as a result of fertilization and seed set in recipient plants.
- In the extremely remote case that a seed was formed as a result of fertilization of carnation pollen onto a wild *Dianthus* species, there are very limited environments where a plant could become established. In nature, *Dianthus caryophyllus*, like many *Dianthus species* is only found in calciferous soils in coastal regions. The tops of sand or limestone hills and outcrops could also be suitable habitats. The seed of the putative hybridization event would probably have to germinate in such environments in order to become established in the wild.

We have provided evidence for a lack of hybrids between cut flower carnation varieties and native *Dianthus* species in the wild in Europe, and for a lack of naturalized carnation in Europe. This is after decades, even centuries, in which very large numbers of carnation flowers have been imported into Europe. Large numbers of carnation are also grown in Europe. Compared to an imported cut flower, established plants of cut flowers varieties would theoretically be a more likely source material for gene dispersal than cut flowers. **However, no hybrid between carnation and any other *Dianthus* species has ever been recorded in the wild**. Specific information on the historical scale of production in Europe is available from the International Association of Horticultural producers (Heinrichs and Siegmond, 1995). Data extracted from this reference shows that there have been in excess of 800 million carnation plants in cultivation on an annual basis in Europe for more than 30 years.

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D. EXPERT OPINIONS

At page 20 of this attachment is a letter from Dr. Keith Hammett, an acknowledged expert on *Dianthus* breeding. He has concluded ***"that the likelihood of gene dispersal from cut flowers of fully double carnations to be highly improbable, if not inconceivable"***

At pages 21 to page 25 of this attachment is an expert opinion provided by Dr. Flavio Sapia. In it he states;
"The areas of the Coté d'Azur in France and of Riviera dei flora in Italy has been from 1920 to 1976 the biggest producer of carnation cut flowers in Europe. Even if this area has seen the growing of millions of plants and thousands of different varieties, never we had a cultivated carnation capable to survive in wild conditions"
" for this reason the dispersal of a gene inserted in a carnation variety is, from my point of view, impossible.."

DR KEITH HAMMETT

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5 October 2004

My name is Dr. Keith Hammett. I am an experienced plant breeder. I have bred cultivars of various *Dianthus* species and hybrids over a period of more than thirty years. During this time I have supervised a MSc. study concerning interspecific hybridization between various *Dianthus* species. I have written scientific papers on the genus, popular articles and co-authored a book on Carnations and Pinks.

I have been asked to critique a manuscript titled "An assessment of the probability of gene dispersal from cut-flowers of the cultivated carnation (*Dianthus caryophyllus*) imported into Europe".

The title and first sentence of the Summary clearly limit the scope of this assessment to cut flowers imported into Europe. It does not address the question of possible gene dispersal from plants of any specified cultivar of carnation that might be grown in Europe or elsewhere.

I concur with the three key claims made in the document. These can be paraphrased as:

1. The establishment of wild self-maintaining populations of carnation as escapes from cultivation is unknown.

2. Formation of and dispersal of seed from a cut carnation flower is extremely unlikely.

3. Pollen transfer from an imported cut carnation flower to a recipient plant, which might then produce seed, is also highly improbable.

The arguments presented in the Executive Summary are sound and I consider that the likelihood of gene dispersal from cut flowers of fully double carnations to be highly improbable, if not inconceivable.

K.R.W. Hammett

Hybrida S.R.L

Relation for FLORIGENE EUROPE

Possibility of gene dispersal in carnation (*Dianthus sp.*)

by HYBRIDA S.r.l

Dr. Flavio Sapia (Breeder)

Diffusion and spreading of a genotype in natural conditions.

Every genotype in nature, can reach a different diffusion according to its capability to survive in different climates and in unfavorable meteorologic circumstances, as well as to its competitiveness with the other species living in the same habitat and its fastness in self reproducing. In nature we have species with wide diffusion, species limited to a specific ecosystem and rare species that survive just in some micro-climates. The genus *Dianthus* presents species with good competitiveness only in few climatic areas and also there, they cannot be defined as common species. Many of them like *D. superbus*, *acer*, *knappi*, *subacaulis*, *caryophillus*, *deltoides* etc. are very rare and have a good competitiveness only in specific micro-climates.

Characteristics that could influence the diffusion of a specie.

It is of course difficult to say what really are the most important characters for a vegetal species in order to survive in nature; every ecosystem requests different features and, as consequence, different genes codifying them. The adapting of a plant to a particular condition mostly is due to a winning inter-action between many single characters linked to different genes. For instance, a resistance to dry condition could be due to a wider rooting apparatus, to different stomas size and shape, to different byo-chemical approach to photosynthesis, to the different waxiness of leaves etc...

Nowadays the cultivation of many species also outside their natural habitat is a normal procedure, but just in few cases for vegetal we had been an adaptation to the wild life in areas different from the original one. The possibility of adaptation to a place that is not the original area is anyway always linked to a similarity of the two ecosystems, and to the capability of the new comer to be enough competitive with the

existing flora and to reproduce itself in the new condition. The vegetal species used in floriculture and horticulture are only in few cases close to their ancestors, and always the competitiveness in wilderness is a lot lower in the cultivated forms.

The possibility of dispersal in nature of a variety with a new gene inserted are depending by the characters of the host variety, by the capability of the engineered genotype to reproduce itself and by the new characters brought by the inserted gene.

Possibility of dispersal of a gene from a cultivated carnation variety.

The insertion of a new gene by genetic engineering in the genome of a carnation variety could influence the spreading of the variety. Such influence may be bigger or lower according to modifications brought to the phenotype of the host variety by the new gene.

Carnation is one of the most cultivated ornamental plants in the world, was used by Greeks and Romans more than 2000 years ago. Ever since many varieties have been bred and grown by amateurs and professional growers; its use is not limited to the cut flower production but many varieties are commercialized as pot plant for gardens. The area of Cotè d'Azur in France and of Riviera dei fiori in Italy has been from 1920 to 1975 the biggest producer of carnation cut flowers in Europe. Even if this area has seen the growing of millions of plants and thousands of different varieties, never we had a cultivated carnation capable to survive in wild conditions. Considering that in this area are living many wild species of carnation, surely we have to exclude that this cultivated varieties had not the possibility to cross with the existing flora. and that their spreading was limited by the unfavorable climatic condition. The same consideration can be done for millions of garden varieties cultivated every year in many different climatic conditions. The inability of a cultivated variety to survive in wilderness has to be researched in the characters that make a

carnation variety attractive for the market. Generically speaking, we can observe that a good carnation cut flower must have a big flower , many petals, a well filled shape, an attractive color, a good vase life and so on. These attractive things for the human eye are big barriers for the pollination, are wasting water in dry condition and are using a remarkable part of the energy of the plant to build structures unuseful for surviving in the wild life. Almost all the traits improved by breeding in the carnation varieties get away the possibility for them to come back to the wild life. All the actual carnation varieties seem not to have this possibility, but what about inserting a new gene? automatically comes the question: "what gene?"

All the genes that could seem attractive for the market are again not useful to promote the competitiveness in wild of the engineered variety. A longer vase life could only bring more problem to the self pollination, a blue color could cause an head ache to some insect and the resistance to an herbicide is not a good assurance for surviving in a not treated meadow. I can imagine that more resistance to some diseases can give to a variety some more possibility to spread, but many times these diseases are dangerous for the species only in an industrial production. In wilderness a resistance to *Fusarium oxysporum* , one of the most important limitation to carnation growing, is less important, since this fungus is typical of overworked lands. The same for other fungi typical of the high humidity of the greenhouses as *Fusarium roseum, Alternaria, Heterosporyum* and so on.

All the modern carnation varieties are hybrids, this condition assures them a good thriving and make them suitable for industrial cultivation, they are obtained by specialized breeding firms using hundreds of genetical lines improved and selected in many years of work.

Breeding carnation varieties is always difficult and the production of seeds is never high; many varieties are not producing pollen, many others have sterile female apparatus, inter-specific crosses rarely are

successful for more than two-three generations and sometimes the varieties from different breeding lines have incompatible genotypes. For these and other reasons is very difficult to see a self crossed variety but also when it occurs the surviving of the obtained seeds is compromised in part by the unfavorable circumstances and in part by the generated condition of inbreeding. In fact more we press these hybrid varieties to homozygosis and more are raising up killer genes, fertility barriers and weak growing habit.

For an engineered genotype the possibility of self re-production without human's help looks lower than for a not modified variety, for this reason the dispersal of a gene inserted in a carnation variety is, from my point of view, impossible.

ATTACHMENT C1

Details of PCR based identification method

Introduction
This report provides a method enabling simple PCR-mediated positive and specific identification for FLORIGENE Moonaqua™ (123.8.12). Data is presented here showing that the test is able to distinguish this line from different transgenic and non-transgenic carnation lines, including the parent carnation line used as the transformation target.

This report also provides the sequence of a unique PCR primer pair that can be used to amplify a product of a designated size when genomic DNA isolated from FLORIGENE Moonaqua™ (123.8.12) is used as template. The product has been identified by visualization under UV illumination in an agarose gel stained with ethidium bromide. No products are detected when genomic DNA from non-specific transgenic lines is used as template in the PCR (transgenic or non-transgenic). Primers amplifying a carnation anthocyanidin synthase (ANS) gene fragment (1300bp) are included as an internal positive control.

Methodology for identifying and detecting the GMO product.
(i) Unique PCR primers **-** Sequences adjacent to transformation vector-derived RB sequences integrated into the carnation genome of FLORIGENE Moonaqua™ (123.8.12) were generated using procedures as described in Liu *et al*. (1995) or Zhou *et al*. (1997). PCR primers designed to these sequences are shown in Table 1.

Table 1. PCR primers designed to specific sequence generated at the RB flanking region of FLORIGENE Moonaqua™ (123.8.12) carnation line.

Primer Set	Line	Primer name	Primer sequence (5' to 3')
1	all lines-positive control	ANS.F	CTAGATCGGAGGTCACCATACC
		ANS.R	GAAACCGTGACCATGGTCTCG
2	FLORIGENE Moonaqua™ (123.8.12)	rRB(7-26)	GAATAGAGTGGACTACTTAC
		123.8.12-2.1R	GGAGGTGTACTATGGGAATCC

ANS = primers designed to the anthocyanidin synthase gene from Carnation; RB = Right Border; F = Forward, R = Reverse.

In order to show that the PCR reaction conditions were optimal and that the DNA was amplifiable, primers designed to the promoter fragment of the carnation *ANS* gene were included in each reaction (Table 1). A 1300bp fragment was therefore expected in each reaction where genomic DNA isolated from carnation was used as template.
PCR primers were subsequently designed to the specific RB flanking sequence generated for FLORIGENE Moonaqua™ (123.8.12) and then tested on several transgenic carnation lines to show that the specific primers amplified a product only when the appropriate, specific genomic DNA from FLORIGENE Moonaqua™ (123.8.12) was used as template. Negative controls were;
a) no DNA

b) genomic DNA isolated from non-transgenic carnation lines including the parent carnation line used as the transformation target (Table 2).

Table 2. Summary of carnation lines described in this report.

Line	Trade Name	Transformation Vector
123	-	Parent carnation line, non-transformed
145	-	Carnation line, non-transformed
18	-	Carnation line, non-transformed
123.8.12	FLORIGENE Moonaqua™	pCGP1991
123.8.8	FLORIGENE Moonvista™	pCGP1991
123.2.2	FLORIGENE Moonshade™	pCGP1470
123.2.38	FLORIGENE Moonlite™	pCGP1470
11363	FLORIGENE Moonshadow™	pCGP1991

(ii) PCR conditions - Crude genomic DNA used in PCRs as template was isolated from leaf tissue essentially as described by Edwards *et al*. (1991). The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). PCR reaction solution included 2.5 µL 10 x Qiagen DNA Polymerase buffer (Qiagen), 1 µL 10 mM dNTPs, 2 µL each primer (50ng/µL), 2 µL genomic DNA template, 0.5 µL Qiagen Hotstar Taq DNA Polymerase (Qiagen) and pure water to a total volume of 25 µL. The PCR was incubated at 95ºC for 15 minutes, followed by 35 cycles of 94ºC for 1 minute, 52ºC for 1 minute and 72ºC for 1 minutes and then a final incubation at 72ºC for 10 minutes with subsequent storage at 4ºC. The reactions were set up according to Table 3 using the following genomic DNA samples as templates: no DNA (negative control), parent variety line 123 (non-transgenic negative control), carnation variety line 145 (non-transgenic negative control), carnation variety line 18 (non-transgenic negative control), FLORIGENE Moonaqua™ (123.8.12), and the transgenic carnation lines developed by Florigene (123.2.2, 123.2.38, 123.8.12 and 11363).

Table 3. Primer sets and template DNA from carnation lines included in each reaction.

Reaction #	Primer set	DNA template
1	1 and 2	No DNA - negative control
2	1 and 2	Parent carnation line (line 123)
3	1 and 2	Carnation line - Cerise Westpearl (line 145)
4	1 and 2	Carnation line – Rendezvous (line 18)
5	1 and 2	Transgenic line 123.8.12 – FLORIGENE Moonaqua™
6	1 and 2	Transgenic line 123.8.8 - FLORIGENE Moonvista™
7	1 and 2	Transgenic line 123.2.2 - FLORIGENE Moonshade™
8	1 and 2	Transgenic line 123.2.38 - FLORIGENE Moonlite™
9	1 and 2	Transgenic line 11363 - FLORIGENE Moonshadow™

The reaction products were electrophoresed through a 1% (w/v) agarose gel alongside 10 µL 100 µg/µL standard DNA markers *Eco*RI digested SPPI (Geneworks) and visualised under UV light.

Experimental data demonstrating the specificity of the methodology.
A photograph of the agarose gel under UV light conditions was taken by Polaroid camera and is shown in Figure 1. A summary of the results is described in Table 4.

Table 4. Summary of the sizes (bp) of products detected in PCR reactions shown in Figure 1.

Reaction No.	Template DNA	PCR Product Sizes (bp)
1	No DNA	-ve
2	Parent variety line 123	1300
3	Carnation variety line 145	1300
4	Carnation variety line 18	1300
5	FLORIGENE Moonaqua™ 123.8.12	1300+750
6	123.8.8	1300
7	123.2.2	1300
8	123.2.38	1300
9	11363	1300

bp = base pairs, −ve = negative (i.e. no amplified bands)

The data presented demonstrates that we have provided a unique set of primers that are able to detect and differentiate the transgenic carnation line FLORIGENE Moonaqua™ (123.8.12) from other transgenic carnation lines (123.2.2, 123.2.38, 123.8.8 and 11363), and non-transgenic carnation varieties 123, 145 and 18. A product of the expected size (750bp) was only detected in the transgenic line FLORIGENE Moonaqua™ (123.8.12) when the unique primer set was included in the PCR. No products were detected in reactions that did not contain genomic DNA. All reactions contained primer set 1 (primers to an endogenous carnation *ANS* gene) which resulted in amplification of the expected 1300bp product showing that the PCR conditions were optimal for product amplification.

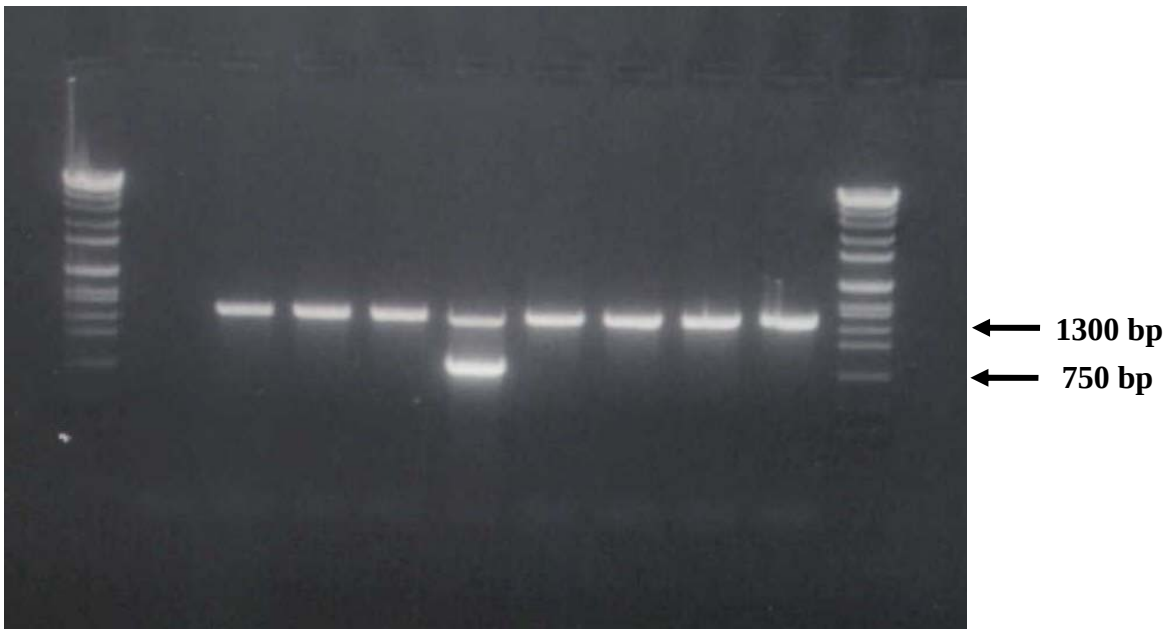

Figure 1. Scanned photograph of an agarose gel containing the PCR products of reactions set up according to Table 3. M = standard marker (i.e. EcoRI digested SPP1 DNA).

Literature cited

1. Edwards K, Johnstone C and Thompson C. A simple and rapid method for the preparation of plant DNA for PCR. Nucleic Acids Research 19: 1349, 1991

2. Liu et al., Efficient Isolation and mapping of *Arabidopsis thaliana* T-DNA insert junction by Thermal asymmetric interlaced PCR. Plant J 8: 457-463, 1995

3. Zhou, Y., Newton, R. and J.H. Gould. A simple method for identifying plant/T-DNA junction sequences resulting from *Agrobacterium*-mediated DNA transformation. Plant Molecular Biology Reporter 15:246-254, 1997

Florigene have contacted the Community Reference Laboratory of the Joint Research Centre in order to arrange a validation of the above PCR-based identification test.

ATTACHMENT D1

Distribution list

Herbaria and Botanical gardens

	Country	Organization Name	Address
1	Austria	Herbarium of the Institute of Botany, University of Vienna	Prof. Dr. Kiehn Michael, Herbarium of the Institute of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, AUSTRIA
2	Austria	Herbarium, Karl-Franzens-Universität Graz	The Curator, Herbarium, Karl-Franzens-Universität Graz, Institut für Botanik, Holteigasse 6, A-8010 Graz, AUSTRIA
3	Belgium	National Botanic Garden of Belgium	Director - Jan Rammeloo, National Botanic Garden of Belgium, Domein van Bouchout, B-1860 Meise, BELGIUM
4	Croatia	Department of Botany and Botanical Garden	Director, Department of Botany and Botanical Garden, Faculty of Science, University of Zagreb, Marulićev Trg 20/II, HR-10000 Zagreb. CROATIA
5	Cyprus	Environment Service	Ministry of Agriculture, Natural Resources and Environment, Loukis Akritas Ave., CY-1411 Nicosia CYPRUS
6	Cyprus	Environmental Studies Centre	P.O. Box 74, Kritou Terra, CY-8724 Paphos. CYPRUS
7	Czech republic	Institute of Botany	Botanicky ustav, Akademie ved Ceske republiky, Pruhonice, CZ-252 43. CZECH REPUBLIC
8	Denmark	Botanical Museum & Library	Associate Professor Henrik Ærenlund Pedersen, Curator, Botanical Museum, Gothersgade 130, DK-1123 Copenhagen K, DENMARK
9	Denmark	Herbarium, University of Aarhus	The Curator, Herbarium, University of Aarhus, Bygning 137 Universitetsparken , DK-8000 Aarhus C., DENMARK
10	Estonia	Herbarium, Institute of Zoology and Botany	Dr Tiiu Kull, Herbarium, Institute of Zoology and Botany, Riia 181, EE51014 Tartu, ESTONIA
11	Finland	Herbarium, University of Turku	Dr Seppo Huhtinen, Head Curator, Herbarium, University of Turku, FI-20014 TURKU FINLAND
12	Finland	Botanical Museum - Finnish Museum of Natural History	Professor Pertti Uotila, Director, Botanical Museum, PO Box 7, FI-00014 University of Helskinki FINLAND
13	France	Herbarium, Université de Montpellier II	Joel Mathez , Director, Herbier, Institut de Botanique, Université de Montpellier II, 163 rue Auguste Broussonet, F 34090 MONTPELLIER FRANCE
14	France	Natural History Museum	J. Prud'homme, Curator, Natural History Museum, 5 Bardineau Place, 33000, Bordeaux, FRANCE

15	France	Conservatoire Botaniqu National du Bassin Parisien	Conservatoire Botaniqu National du Bassin Parisien, Muséum national d'Histoire naturelle, 61 rue Buffon, 75005 Paris FRANCE
16	France	Herbiers, Universite de Lyon I (Claude Bernard)	Georges Barale, Herbiers, Universite de Lyon I (Claude Bernard), Campus de la Doua - Batiment 406,, 43, Bd du 11 novembre 1918 - 69622 VILLEURBANNE Cedex, FRANCE
17	Germany	Herbarium Dresdense, Technische Universitat	Dr. rer. nat. Frank Müller, Institut für Botanik, Herbarium Dresdense, Technische Universitat, Zellerscher Weg 22, 01062 Dresden GERMANY
18	Germany	Herbarium der Universität Bayreuth	Dr. Ulrich Meve, Herbarium der Universität Bayreuth, Ökologisch-Botanischer Garten, Universität Bayreuth, 95440 Bayreuth GERMANY
19	Germany	Herbarium, Universitat Bielefeld	The Curator, Herbarium, Universitat Bielefeld, Fakultät für Biologie, Abteilung 3 - Ökologie, Postfah 100131, 33501 Bielefeld GERMANY
20	Germany	Herbarium, Freie Universitat Berlin	Dr. Harald Kürschner, Herbar, Institut für Biologie der Freien Universität Berlin, Systematische Botanik und Pflanzengeographie, Altensteinstr. 6, 14195 Berlin, GERMANY
21	Germany	Herbar, Georg-August-Universitat Gottingen	Prof. Dr. Stephan Robbert Gradstein, Herbar, Georg-August-Universitat Gottingen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Abt. Systematische Botanik, Untere Karspüle 2, 37073 Göttingen GERMANY
22	Germany	Herbarium, Martin-Luther-Universitate Halle	Dr. Uwe Braun, Institut für Geobotanik und Botanischer Garten, Herbarium, Martin-Luther-Universitate Halle, Neuwerk 21, 06108 Halle GERMANY
23	Germany	Herbarium, Universitat Essen	Prof. Dr. Martin Heil, Head of Department, Herbarium, Universitat Essen, Arbeitsgruppe Botanik/Pflanzenphysiologie und Botanischer Garten, Universitätstraße 5, 45117 Essen GERMANY
24	Germany	Herbarium des Botanisches Institut (GFW), Ernst-Moritz-Arndt-Universitat Grefswald	Prof. Dr. Michael Succow, Herbarium des Botanisches Institut (GFW), Ernst- Moritz-Arndt-Universitat Grefswald, Grimmer Straße 88, 17487 Greifswald, GERMANY
25	Germany	Botanische Staatssammlung Munchen	Prof. Dr. Susanne Renner, Director, Botanische Staatssammlung Munchen, Menzinger Straße 67, D-80638 München GERMANY
26	Greece	Herbarium, Natural History Museum of Crete	Charikleia Papadaki, Herbarium, Natural History Museum of Crete, University of Crete, P.O. Box 2208, 71409 Heraklion CRETE
27	Greece	Botanical Museum, Siatista	Dr. Vlasis Vlasidis, Botanical Museum, Siatista, I. Trabadzi Square, Siatista, Kozani prefecture, Macedonia, GREECE
28	Hungary	Debreceni Egyetem	Department of Botany, Faculty of Science, University of Debrecen], Egyetem tér 1, 4010 Debrecen. HUNGARY
29	Hungary	Magyar Termeszettudomanyi Muzeum Novenytara	Hungarian Natural History Museum, Department of Botany, Könyves Kálmán krt. 40, 1087 Budapest. HUNGARY

30	Ireland	Trinity College Herbarium	Associate Professor John A. N. Parnell, Botany Department, Trinity College Dublin, Dublin 2 IRELAND
31	Italy	Herbarium Aquilanum (AQUI)	F. Tammaro, Curator, Dipartimento di Scienze Ambientali, Sezione Botanica, Universita' degli Studi di L'Aquila , Via Vetoio Loc. Coppito I - 67100 L'Aquila ITALY
32	Italy	Florence Natural History Museum, Botanic Gardens	Dr Paolo Luzzi, Florence Natural History Museum, Botanical Section, Via G. La Pira, 4, 50121 Florence, ITALY
33	Italy	Herbarium and Botanical Museum	Dott.ssa Annalisa Managlia, Curator - Herbarium and Botanical Museum, University of Bologna, Via Irnerio, 42, 40126 Bologna, ITALY
34	Italy	Biological Museum of the University of Trieste	Prof. Pier Luigi Nimis, Biological Museum of the University of Trieste, Via Giorgieri 10, Trieste ITALY
35	Latvia	The Museum of Botany of the University of Latvia	The Director, The Museum of Botany of the University of Latvia, Kronvalda bulvaris 4, Riga, LV-1586 LATVIA
36	Lithuania	Herbarium of the Institute of Botany (BILAS)	Dr. Romas Pakalnis, Director- Herbarium, Institute of Botany, Žaliųjų ežerų 49, LT-2021 Vilnius, Lietuva, LITHUANIA
37	Luxembourg	Musée national d'histoire naturelle	Musée national d'histoire naturelle Centre de recherche scientifique Section Botanique 25, rue Muenster L-2160 LUXEMBOURG
38	Malta	Department of Biology	Department of Biology ,University of Malta, Msida MSD 06. MALTA
39	Netherlands	Herbarium, University of Wageningen	Professor Dr. Jan Johannes Wieringa, Scientific Curator Herbarium, Wageningen University, Biosystematics Group, Generaal Foulkesweg, 37, 6703 BL Wageningen, THE NETHERLANDS
40	Netherlands	Nationaal Herbarium Nederland	G. Thijsse, Chief Collection Manager, Nationaal Herbarium Nederland, P.O. Box 9514, 2300 RA Leiden, THE NETHERLANDS
41	Norway	Botanical Garden	Professor Victor A. Albert, The Botanical Garden, PO Box 1172 Blindern, N-0318, Oslo NORWAY
42	Poland	W. Szafer Institute of Botany	W. Szafer Institute of Botany of the Polish Academy of Sciences in Cracow. 31-512 KRAKÓW, ul. Lubicz 46 POLAND
43	Portugal	Botanic Garden, University of Coimbra	António Xavier Coutinho, Curator, Botanic Garden, University of Coimbra, Calçada Martim de Freitas, 3000 Coimbra PORTUGAL
44	Portugal	Botanical Garden and Museum	The Curator, Jardim Botanico e Museu, Univeristy of Porto, Rua do Campo Alegre, 1191, 4150-181 Porto, PORTUGAL
45	Russia	Botanic Garden, Irkutsk State University	Dr. Victor Kuzevanov, Director, Botanic Garden, Irkutsk State University, 93 Koltsov Street, P.O. Box 1457, Irkutsk, 664039 RUSSIA
46	Russia	The Botanical Gardens of Moscow State University	Prof. M. V. Gusev, Dean - Department of Biology, Moscow State University, Moscow, 119899 RUSSIA

47	Slovakia	Department of Botany	Department of Botany, Faculty of Natural Science, Comenius University, Révova ulica 39, 811 02 Bratislava. SLOVAKIA
48	Slovenia	Herbarij LJM, Univeristy of Ljubljana	Dr. Nejc Jogan, Curator, Herbarij LJM, Univeristy of Ljubljana, Odd. za biologijo, Biotehniske fakultete UL, Vecna pot 111, SI-1000 Ljubljana SLOVENIA
49	Spain	Herbarium, University of Granada	Manuel Casares Porcel, Director - Departamento de Botánica, Facultad de Ciencias, Universidad DE Granada, Planta 6ª SPAIN
50	Spain	Herbario, University of Navarra	De Miguel Velasco, Ana Mª, Directora, Herbario, University of Navarra, Edificio de Ciencias, C/ Irunlarrea, s/n, Apartado 177, E- 31080 PAMPLONA SPAIN
51	Spain	Herbarium, University Complutense of Madrid	Prof. Dr. Maria Andrea Carrasco de Salazar, Curator, Dpto. de Biología Vegetal I. Facultad de CC. Biológicas , Universidad Complutense de Madrid , Ciudad Universitaria s/n. 28040 – Madrid SPAIN
52	Sweden	Herbarium SUNIV, Department of Botany, Stockholm University	Jan Thomas Johansson, Botaniska institutionen, Stockholms universitet, S-106 91 Stockholm SWEDEN
53	Sweden	Swedish Museum of Natural History - Botany	Professor Arne A. Anderberg, Swedish Museum of Natural History - Botany, Box 50007, SE-104 05, Stockholm, SWEDEN
54	Switzerland	Geobotanical Institute, ETH Zurich	Prof. E. Horak, Curator, Geobotanisches Institut, Zollikerstrasse 107 ZOC 7, 8044 Zürich SWITZERLAND
55	UK	Herbarium, University of Cambridge	Professor John Parker, Curator, Department of Plant Science, University of Cambridge, Dowing Street, Cambridge BD@ 3EA UNITED KINGDOM
56	UK	Royal Botanic Garden, Edinburgh	Professor Stephen Blackmore FRSE, Regius Keeper, Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh, EH3 5LR UNITED KINGDOM
57	UK	Royal Botanic Gardens, Kew	Professor Simon Owens, Keeper of the Herbarium, Royal Botanic Gardens, Kew, Richmond, Surry TW9 3AB UNITED KINGDOM
58	UK	Oxford University Herbaria	Stephen A. Harris, Curator, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB UNITED KINGDOM
59	UK	Herbarium, University of Aberdeen	Dr Chris Wilcock, Keeper, Department of Plant & Soil Science, Cruickshank Building, St Machar Drive, Aberdeen, AB24 3UU UNITED KINGDOM
60	UK	Herbarium, Department of Geography, University of Hull	Professor Graham Haughton, Head - Department of Geography, University of Hull, Cottingham Road, Hull, HU6 7RX UNITED KINGDOM
61	UK	Herbarium, Department of Geography & Environmental Science	Prof. Mark Seaward, Department of Geography & Environmental Science, University of Bradford, Bradford, West Yorkshire, BD7 1DP UNITED KINGDOM
62	UK	Ida Roper Herbarium, University of Leeds	Mary Beckett, Documentation Officer (Herbarium), School of Biology, The Louis Compton Miall Building, University of Leeds, LS2 9JT UNITED KINGDOM

63	UK	Herbarium RNG & Plant Identification Service	Stephen L. Jury, Curator-Herbarium, School of Plant Sciences, The University of Reading, Whiteknights Reading, RG6 6AS UNITED KINGDOM

National Botanical survey Networks/ societies

	Country	Organization Name	Address
1	Austria	Faculty center Botany	FACULTY CENTER BOTANY, Rennweg 14, A-1030, Vienna AUSTRIA
2	Belgium	Société Royale de Botanique de Belgique	Société Royale de Botanique de Belgique c/o Pierre Meerts, 1850 chaussée de Wavre, 1160 Bruxelles BELGIUM
3	Croatia	Croatian Botanical Society	Croatian Botanical Society, Marulićev Trg 20/II, HR-10000 Zagreb CROATIA
4	Cyprus	Cyprus Conservation Foundation	Cyprus Conservation Foundation, P.O. Box 50257, CY-3602 Limassol CYPRUS
5	Czech republic	The Czech Botanical Society	Ceska botanicka spolecnost, Benatska 2, Praha 2, CZ-128 01 CZECH REPUBLIC
6	Denmark	Dansk Botanisk Forening	Dansk Botanisk Forening Sølvgade 83, DK-1307 Copenhagen K. DENMARK
7	Estonia	Estonian Naturalists' Society	Estonian Naturalists' Society, Struve Street 2, Tartu ESTONIA
8	Finland	Societas pro fauna et flora fennica	Secretary Roland Skytén, Finnish Museum of Natural History, Mycology Division P.O. Box 7 (Unionsgatan 44) FI-00014 HELSINKI UNIVERSITY FINLAND
9	France	Société Botanique de France	Secrétaire général Elisabeth DODINET 20, avenue de Longchamp 92210 SAINT-CLOUD FRANCE
10	Germany	Deutsche Botanische Gesellschaft e.V, DBG	Prof. Dr. Rudolf Ehwald, Institut für Biologie Humboldt-Universität zu Berlin Invalidenstr. 42 D-10115 Berlin GERMANY
11	Greece	Hellenic Society for the Protection of Nature	Hellenic Society for the Protection of Nature, Nikis 24, 105 57 Athens GREECE
12	Hungary	Institute of Ecology and Botany of the Hungarian Academy of Sciences	Ökológiai és Botanikai Kutatóintézete, Magyar Tudományos Akadémia, Alkotmány u. 2-4, 2163 Vácrátót HUNGARY
13	Ireland	Botanical Society of the British Isles	Botanical Society of the British Isles, Co-ordinator's Office 66 North Street, Shrewsbury, Shropshire SY1 2JL UNITED KINGDOM
14	Italy	Italian Botanical Society	The secretariat, Italian Botanical Society, Via G. La Pira 4, I-50121 Florence, ITALY
15	Latvia	Latvijas Botaniku Biedriba	Kronvalda bulv. 4, Rīga, LV-1586 LATVIA
16	Lithuania	Lithuanian Botanical Society	Lithuanian Botanical Society, Institute of Botany. Žaliųjų ežerų 49. LT-2021 Vilnius LITHUANIA
17	Luxembourg	Musée national d'histoire naturelle	Musée national d'histoire naturelle, Section botanique, 24, rue Münster, L-2160

			LUXEMBOURG
18	Malta	Argotti Herbarium and University Botanic Gardens	Argotti Herbarium and University Botanic Gardens, University of Malta, Msida MALTA
19	Netherlands	Royal Botanical Society of the Netherlands (KNBV)	Dr. Janny L. Peters , Second Secretary, Royal Botanical Society of the Netherlands (KNBV) Radboud Universiteit Nijmegen NETHERLANDS
20	Poland	POLSKIE TOWARZYSTWO BOTANICZNE	dr ANNA MIKUŁA, Sekretarz generalny Ogród Botaniczny Centrum Zachowania Różnorodności Biologicznej Polskiej Akademii Nauk, ul. Prawdziwka 2, 02-973 WarszawaPOLAND
21	Portugal	Museu, Laboratório e Jardim Botânico	Museu, Laboratório e Jardim Botânico, Rua da Escola Politécnica, 1294 Lisboa Codex PORTUGAL
22	Serbia and Montenegro	Serbian Ecological Society	Serbian Ecological Society University of Belgrade, Fac. Biol. Inst.of Bot. and Bot. Garden "Jevremovac" Takovska 43, 11000, Belgrade REPUBLIC OF SERBIA
23	Slovakia	Slovak Botanical Society	Slovenská botanická spoločnosť pri SAV Dúbravská cesta 14, 845 23 Bratislava SLOVAKIA
24	Slovenia	Botanical Society of Slovenia	Botanicno drustvo Slovenije Izanska 15, SI-1000 Ljubljana SLOVENIA
25	Spain	Sociedad Española de Biología de la Conservación de Plantas	Jaime Güemes Sociedad Española de Biología de la Conservación de Plantas Jardí Botànic de la Universitat de València, Quart, 80, E-46008 Valencia, SPAIN
26	Spain	Organisation for the Phyto-Taxonomic Investigation of the Mediterranean Area	The secretary Organisation for the Phyto-Taxonomic Investigation of the Mediterranean Area Dpto. Biologia Vegetal E.U.I.T Agricola Universidad Politecnica Ciudad Universitaria Madrid SPAIN
27	Sweden	Svenska Botaniska Föreningen	Evastina Blomgren Sekreterare Dalgatan 7-9, 456 32 KUNGSHAMN SWEDEN
28	United Kingdom	Botanical Society of the British Isles	Botanical Society of the British Isles, Co-ordinator's Office 66 North Street, Shrewsbury, Shropshire SY1 2JL UNITED KINGDOM

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2	Belgium	Federal Public Service of Public Health, Food Chain Security and	Mr Lieven VAN HERZELE Federal Public Service of Public Health, Food Chain Security

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3	Croatia	Ministry of Agriculture and Forestry	Ministry of Agriculture and Forestry Ul. grada Vukovara 78 10000 ZAGREB CROATIA
4	Cyprus	Ministry of Agriculture, Natural Resources and Environment	Mr A. CONSTANTINOU Department of Agriculture Ministry of Agriculture, Natural Resources and Environment 1412 NICOSIA CYPRUS
5	Czech republic	State Phytosanitary Administration	Mr M. HUSAK State Phytosanitary Administration Tesnov 17 11705 PRAHA 1 CZECH REPUBLIC
6	Denmark	Ministry of Food, Agriculture and Fisheries	Mr J. SØGAARD HANSEN Dept. Plants and Plant Health Ministry of Food, Agriculture and Fisheries Danish Plant Directorate Skovbrynet 20 2800 LYNGBY DENMARK
7	Estonia	Estonian Plant Production Inspectorate	Ms R. KOIDUMAA Estonian Plant Production Inspectorate Plant Health Department Teaduse 2 Saku 75501 HARJUMAA ESTONIA
8	Finland	Ministry of Agriculture and Forestry	Mr R. LOPIAN Ministry of Agriculture and Forestry Quality Policy Unit Hallituskatu 5, P.O. Box 30 FIN-00023 Government FINLAND
9	France	Direction de la Qualité et de la Protection des Végétaux	Monsieur J. MATHURIN Direction Générale de l'Alimentation Sous Direction de la Qualité et de la Protection des Végétaux 251 rue de Vaugirard 75732 PARIS CEDEX 15 FRANCE
10	Germany	Federal Ministry for Consumer Protection	Ms Karola SCHORN Federal Ministry for Consumer Protection, Food and Agriculture Rochusstrasse 153123 BONN GERMANY
11	Greece	Ministry of Rural Development and Food	Mr Theofanis KAFRITSAS Director of Plant Produce Protection Ministry of Rural Development and Food 150 Leoforos Syggrou Avenue 17671 ATHENS GREECE
12	Hungary	Ministry of Agriculture and Rural	Mr Lajos SZABÓ

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13	Ireland	Department of Agriculture and Food	Mr M. HICKEY Department of Agriculture and Food Horticulture and Plant Health Division Maynooth Business Campus MAYNOOTH Co. Kildare IRELAND
14	Italy	Ministero per le Politiche Agricole	Monsieur P. MAINOLFI Dir. Gen. delle Politiche Agricole ed Agroindustriali Nazionali, Ministero per le Politiche Agricole Via XX Settembre 20 00187 ROME ITALY
15	Latvia	Ministry of Agriculture	Mr R. ARNITIS Director State Plant Protection Service Ministry of Agriculture Republikas Lauk. 2 RIGA, LV – 1981 LATVIA
16	Lithuania	State Plant Protection Service	Dr E. MORKEVICIUS State Plant Protection Service Kalvariju str. 62 2005 VILNIUS LITHUANIA
17	Luxembourg	Administration des Services techniques de l'Agriculture	Monsieur A. ASCHMAN Service Protection des Végétaux Administration des Services techniques de l'Agriculture Case postale 1904 Route d'Esch 16 LUXEMBOURG
18	Malta	Plant Health Department	Dr David MIFSUD Director, Plant Health Department Plant Biotechnology Centre Preca Street LIJA BZN 04 MALTA
19	Netherlands	Plant Protection Service	Dr R.J.T. VAN LINT Director Plant Protection Service Geertjesweg 15 P.O. Box 9102 6700 HC WAGENINGEN NETHERLANDS
20	Poland	The State Plant Health and Seed Inspection Service	Mrs Miroslawa KONICKA Main Inspector of Plant Health and Seed Inspection The State Plant Health and Seed Inspection Service Ul. Wspolna 30 00 930 WARSAW POLAND
21	Portugal	Protecçao das Culturas (DGPC)	Mr C. Sao Simao de CARVALHO Direcçao Geral de Protecçao

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22	Serbia and Montenegro	Plant Protection Directorate	Mr Miroslav VUJOVIC Head of the Department for Pesticides Plant Protection Directorate Omladinskih brigada 1 11 000 BELGRADE SERBIA
23	Slovakia	Ministry of Agriculture	Mr Ing. J. KOTLEBA Head Officer, Plant Protection Service Ministry of Agriculture Dobrovicova 12 812 66 BRATISLAVA SLOVAKIA
24	Slovenia	Ministry of Agriculture, Forestry and Food	Mrs K. GROZNIK Phytosanitary Administration of Slovenia Ministry of Agriculture, Forestry and Food Plant Health Division Einspierlerjeva 6 1000 LJUBLJANA SLOVENIA
25	Spain	Ministerio de Agricultura, Pesca y Alimentacion	Subdireccion General de Agricultura Integrada y Sanidad Vegetal Ministerio de Agricultura, Pesca y Alimentacion c/Alfonso XII, 62, 2a Planta 28014 MADRID SPAIN
26	Sweden	Swedish Board of Agriculture	Mr G. KROEKER Swedish Board of Agriculture Dragarbrunnsgatan 35 753 20 Uppsala SWEDEN
27	United Kingdom	Department for Environment, Food and Rural Affairs	Mr S. HUNTER Head, Plant Health Quarantine Branch Department for Environment, Food and Rural Affairs Foss House, Kings Pool 1-2 Peasholme Green YORK Y01 7PX UNITED KINGDOM