

SUNTORY

SUNTORY GLOBAL INNOVATION CENTER

Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
USA

Dear Dr. Firko


Suntory Global Innovation Center Limited has developed several transgenic lines of the important cut-flower crop rose. These lines (genetically modified for altered flower colour) are currently under trial in Colombia and through this letter and enclosed documents we would like to request an opinion on the regulatory status of imported cut flowers from these lines under 7 CFR Part 340.

In 2008, Florigene Pty. Ltd. received a letter from USDA-BRS indicating that import of genetically engineered cut roses that are not whole plants (cut roses) expressing a modified flower color to be imported into the U.S., did not fall under 7 CFR Part 340. At that time Florigene Pty. Ltd was a fully owned subsidiary of Suntory Ltd., as is Suntory Global Innovation Center Limited.

As the rose events we have developed are not identical to the transgenic rose events that USDA-BRS reviewed in 2008 we would like to confirm that the new events also do not meet the definition of a regulated article as described in 7 CFR part 340.

Thank you for your attention to this matter.

Yours sincerely,


Nobuyuki Fukui
Director, Member of the Board
Suntory Global Innovation Center Limited

Dated; NOV 5 2015

Email address for contact; Dr S.F. Chandler at schandler@florigene.com.au

Enclosures (1)

SUNTORY

SUNTORY GLOBAL INNOVATION CENTER

**Submission to the US Department of Agriculture Animal and
Plant Health Inspection Service Biotechnology Regulatory
Services enquiring whether imported transgenic rose flowers
are regulated articles under 7 CFR part 340**

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Contents

Abbreviations.....3

1. Company background.....4

2. Previous advice by USDA-APHIS-BRS relating to transgenic rose.....4

 2.1 Statement of grounds.....5

3. Scope of intended commercial activity in the USA.....6

4. Description of transgenic trait and nomenclature of events.....7

 4.1 Genetic modification strategy.....8

5. Transformation method.....11

6. Description of constructs.....11

7. Recipient organism.....11

8. Donor organisms for all DNA sequences in the construct that were actually inserted into the plant.....12

 Appendix 1. Construct elements.....13

 Appendix 2. Donor organisms.....21

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Abbreviations¹

ANS:	Anthocyanidin synthase
ADH-5'UTR:	5'-untranslated region of the tobacco alcohol dehydrogenase gene
AT:	anthocyanin 3-aromatic acyltransferase
CaMV35S or 35S:	Cauliflower mosaic virus 35S promoter fragment
CHS:	Chalcone synthase
D8:	Regulatory element from phospholipid transfer protein homologue
DFR:	Dihydroflavonol reductase
DHK:	Dihydrokaempferol
DHM:	Dihydromyricetin
DHQ:	Dihydroquercetin
DNA:	Deoxyribonucleic acid
F3'H:	Flavonoid 3' hydroxylase
F3'5'H:	Flavonoid 3' 5' hydroxylase
FW:	Fresh weight
g:	gram
GM:	Genetically Modified
IFD:	International Flower Developments
LB:	Left Border of T-DNA
Mas, mas:	Mannopine synthase
mg/g:	Milligrams per gram
Nos, nos:	Nopaline synthase
NPTII, nptII:	neomycin phosphotransferase
OS:	Ocean Song
p:	Promoter
RB:	Right Border of T-DNA
SIC:	Suntory Global Innovation Center Limited
t:	Terminator
T-DNA:	Transferred-deoxyribonucleic acid
Tet:	Tetracycline
US or USA:	United States of America
USDA – APHIS:	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-BRS:	United States Department of Agriculture – Animal and Plant Health Inspection Service- Biotechnology Regulatory Services
UTR	Untranslated region
VIT	Vacuolar iron transporter

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 document.

1. Company background

Suntory Global Innovation Center Limited (SIC; <http://www.suntory.com/sic/>) is a subsidiary company of Suntory Limited, Kyoto, Japan. SIC conducts research, including development of genetically modified ornamental plants. Since the early 1990s Suntory and Florigene Pty Ltd, Australia (Florigene) co-operated in research endeavors and in 2003 Suntory acquired Florigene. In 2010 all the research activities of Florigene were moved to Suntory in Japan. Suntory has continued all the marketing and commercial operations established by Florigene with transgenic carnation, rose and chrysanthemum, including imports of transgenic carnation flowers into the US.

2. Previous advice by USDA-APHIS-BRS relating to transgenic rose

Suntory (through its subsidiary Florigene) has previously contacted USDA-BRS regarding the regulatory status of two transgenic rose events; IFD-524Ø1 -4 and IFD-529Ø1-9¹. There is equivalence between these two events and the transgenic rose events described in this document, in these respects;

- The method of transformation (co-cultivation with disarmed *Agrobacterium tumefaciens*) is the same.
- The recipient organism (*Rosa hybrida*) is the same.
- The selectable marker (pnos: NPTII: tnos) is the same.
- The modified trait, altered flower colour, is the same. In IFD-524Ø1 -4 and IFD-529Ø1-9, altered flower colour is due to the accumulation of approximately 0.075 mg/g FW delphinidin-related anthocyanins in petals as a result of expression of an introduced *Viola* sp. flavonoid 3' 5' hydroxylase gene. Similar concentrations of delphinidin-related anthocyanins are measured in the flowers of the transgenic events described in this document, also as a result of expression of the *Viola* sp. flavonoid 3' 5' hydroxylase gene.

The main difference between IFD-524Ø1 -4 and IFD-529Ø1-9 and the transgenic rose events described in this document are;

- Additional genetic elements, from additional donor organisms, have been included in the constructs (see sections 4 and 6 of this document).
- Whereas IFD-524Ø1 -4 and IFD-529Ø1-9 are periclinal chimeras, none of the transgenic events described in this document are.

In 2008, Florigene enquired about the regulated status of IFD-524Ø1 -4 and IFD-529Ø1-9 when imported as cut flowers only into the United States. The advice received from USDA-BRS was, in part;

“Based on the information in your request we have determined that it is your intent to only import genetically engineered cut roses expressing a modified flower color into the U.S., and not grow or distribute intact plants, cuttings, or seeds. As such, APHIS has determined that

¹ Nakamura, N., Fukuchi-Mizutani, M., Katsumoto, Yukihiisa.,Togami, J.,Senior, M.,Matsuda, Y.,Furuichi, K.,Yoshimoto, M., Matsunaga, A.,Ishiguro, K.,Aida, M.,Tasaka, M.,Fukui, H.,Tsuda, S.,Chandler, S. and Tanaka, Y. (2011) Environmental risk assessment and field performance of rose (*Rosa*×*hybrida*) genetically modified for delphinidin production. *Plant Biotechnology* **28**; 251–261.

these cut roses do not fall under our regulations. This applies to both a need for a permit and for a non regulated determination.”

In 2009, International Flower Developments (then a joint venture between Florigene and Suntory and now a subsidiary company of Suntory) sought a determination of non-regulated status for IFD-52401-4 and IFD-52901-9, for cultivation in the USA. A determination on the dossier (09-315-01p) was made by USDA-APHIS--BRS in September 2011². This determination stated, in part;

“ In response to petition 08-315-01 p from International Flower Developments Pty, Ltd (hereafter referred to as IFD), the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has determined that IFD-524014 and IFD-52901-9 roses and progeny derived from them are unlikely to pose plant pest risks and are no longer to be considered regulated articles under APHIS' Biotechnology Regulations (Title 7 of Code of Federal Regulations (CFR), part 340). Since APHIS has determined that IFD-52401-4 and IFD-52901-9 roses are unlikely to pose plant pest risks, APHIS will approve the petition for nonregulated status of IFD-52401-4 and IFD52901-9 roses. Therefore, APHIS approved permits or acknowledged notifications that were previously required for environmental release, interstate movement, or importation of IFD-52401-4 and IFD-52901-9 roses and their progeny are no longer required.”

In the case of the decision made in 2008, for imported transgenic rose cut flowers (which is the intention for the events described in this document) the rationale presented by APHIS was;

“Your plans to import these cut flowers requires no further action on your part because APHIS interprets its regulations to apply to plant parts only if they are capable of self-propagation. APHIS considers cut roses as being incapable of self-propagation, gene flow from pollen produced from roses during transit is not reasonably foreseeable, and your indication that the pollen, although viable, is “non-transgenic”.

2.1 Statement of grounds

Though the events described in this document are not identical to the transgenic rose events that USDA-BRS reviewed in 2008 and 2009, our assumption is that the transgenic rose events described in this document also do not meet the definition of a regulated article as described in 7 CFR part 340. This is because;

- There is equivalence in trait, and overlap in genetic elements, between the transgenic rose events described in this document and IFD-524014 and IFD-52901-9. The submissions made in 2008 and 2009 included information to show that the primary modified trait (altered flower colour due to accumulation of delphinidin-related anthocyanins) does not present a risk to plant, animal or human health nor to the environment;
 - Delphinidin is a common anthocyanin, present in many other flower species, and at high concentrations in commonly consumed foods³.
 - The introduced flavonoid 3' 5' hydroxylase occurs in all plant foods containing delphinidin.
 - Genetically-modified carnation flowers containing the same pansy flavonoid 3' 5' hydroxylase that has been inserted in the transgenic rose events described

² https://www.aphis.usda.gov/brs/aphisdocs/08_31501p_det.pdf

³ Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E. and Prior, R.L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural Food Chemistry* **54**; 4069-4075.

in this document have been traded in the USA for nearly 15 years, with no reports of adverse effects⁴.

- APHIS has stated that transgenic cut flowers grown in other countries pose little if any risk and may not require permits⁵. This consideration was included in recent advice applied to import of cut flowers of transgenic gypsophila⁶. As section 3 below describes, the intended use of the transgenic rose events described in this document is for import of cut flowers only.
- Though additional genetic elements have been used in the transgenic events described in this document, these are not from donor organisms which pose a plant pest risk and in three cases are sense-antisense genes from the recipient organism, *Rosa hybrida* (please refer to sections 4, 6 and 8 in this document).
- Though the events described in this document are not periclinal chimeras (and so inserted genes are present in pollen) the biology of the rose precludes self-propagation from cut flowers after import into the USA and the imported cut flowers are therefore unlikely to pose a plant pest risk. The submissions made in 2008 and 2009 included information which concluded that cut flowers of transgenic rose were unlikely to establish in the environment by self-propagation or gene-flow;
 - Rose does not spread vegetatively. Suntory has experience of large scale production of rose in Colombia and Ecuador. Rose has never been found growing wild, even in the immediate vicinity of rose growing areas where waste material has been discarded or has been left for composting.
 - Formation of a seed in a cut flower of rose is not possible.
 - In major flower production areas throughout the world, including the USA, no rose population has ever been found in the wild. The parent variety, and other rose varieties, have been grown in the USA and imported into the USA, for decades. Cultivated rose is not a weed in the US. 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.

3. Scope of intended commercial activity in the USA

The scope of the intended commercial activity associated with the rose events described in this document is import into the USA of cut-flowers of the transgenic rose events only. There is no intention to grow these events in the USA. Flowers are being trialled at present in Colombia, which is the intended site of production of the flowers from the transgenic rose events described in this document. Cut flowers of rose are exported on a daily basis from Colombia to the USA and it is intended that the flowers of the transgenic events will be harvested, treated, packed and exported in the same way as flowers from non-transgenic rose plants. It is intended that once in the USA the transgenic flowers will be imported, distributed, retailed and used in the same way as non-transgenic rose flowers.

⁴ Chandler, S. F., Senior, M., Nakamura, N., Tsuda, S. and Tanaka, Y. (2013). Expression of flavonoid 3',5'-hydroxylase and acetolactate synthase genes in transgenic carnation: assessing the safety of a non-food plant. *Journal of agricultural and food chemistry* 61;11711-11720

⁵ Federal Register /Vol. 73, No. 197 /Thursday, October 9, 2008 / Proposed Rules; Department Of Agriculture, Animal and Plant Health Inspection Service 7 CFR Part 340 [Docket No. APHIS-2008-0023]

⁶ https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/Response_Babysbreath_122011.pdf

4. Description of transgenic trait and nomenclature of events

Seven events are described in this document, generated after transformation with 6 different constructs. Table 1 lists the events.

Table 1. List of transgenic rose events included in this document

Lab code	Unique identifier	Construct
OS/1003-37-01	SHD-03701-6	pSFL1003
OS/1003-61-01	SHD-06101-3	pSFL1003
OS/4538-20-02	SHD-02002-8	pSPB4538
OS/4569-31-01	SHD-03101-9	pSPB4569
OS/4715-01-02	SHD-00102-7	pSPB4715
OS/903-13-01	SHD-01301-9	pSFL903
OS/910-22-02	SHD-02202-1	pSFL910

The transgenic trait expressed in all seven events is the same;

1. The transgenic rose events have been modified to produce an **altered flower colour**. This has been achieved through the introduction of a flavonoid 3', 5'-hydroxylase (known also as "Blue Gene") from *Viola sp*⁷. Expression of this gene results in the production of delphinidin, a common and naturally occurring secondary metabolite, which confers a mauve-violet hue to the flowers. Though there are six constructs described in this document, the phenotype change in the transgenic lines is the same—production of the anthocyanidin delphinidin in the flowers, then leading to an altered flower colour. All constructs confer the same class of modified trait; altered flower colour. Table 2 provides quantitative data for the modified trait.

Table 2. Anthocyanin profiles of the seven transgenic events included in this document

Lab code	% delphinidin	delphinidin (mg/g fresh weight petal)
Parental variety	0	0
OS/1003-37-01	87	0.13
OS/1003-61-01	89	0.13
OS/4538-20-02	86	0.03
OS/4569-31-01	73	0.12
OS/4715-01-02	96	0.09
OS/903-13-01	92	0.09
OS/910-22-02	88	0.08

2. All events contain a neomycin phosphotransferase from *Escherichia coli*. This gene confers **antibiotic resistance**⁸ (kanamycin was used as a selectable agent during transformation) and is present in all constructs.

⁷ Katsumoto Y, Mizutani M, Fukui Y, Brugliera F, Holton T, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, et al. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. *Plant Cell Physiol* **48**: 1589–1600

⁸ Goldstein, D. A., Tinland, B., Gilbertson, L. A., Staub, J. M., Bannon, G. A., Goodman, R. E., McCoy, R. L. and Silvanovich, A. (2005). Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection technologies. *Journal of Applied Microbiology* **99**; 7–23

Qualitatively, the flower colour change is measured using the colour codes from the Royal Horticultural Society. The code for the flowers from the non-transgenic parental variety is 69C/76D and the codes for the flowers from the transgenic lines is 76A or 76B. A representative example of colour change is shown in Figure 1.

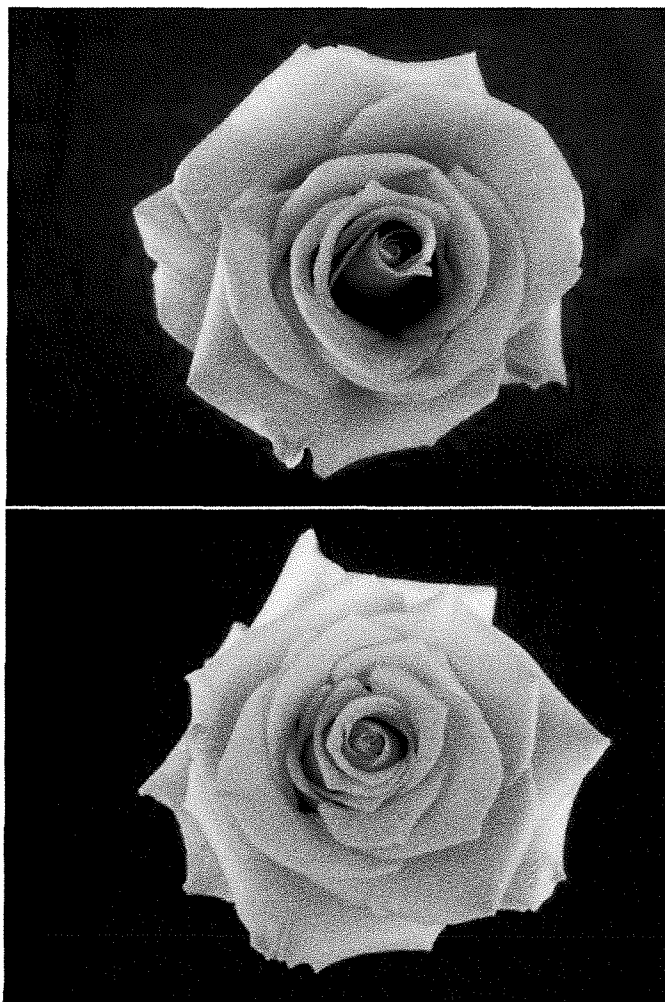


Figure 1. Appearance of OS/4569-31-01 compared to the parental control, in lower panel. The RHS (Royal Horticulture Society) code of the transgenic line is 76B.

4.1 Genetic modification strategy

There are three groups of anthocyanins, the delphinidin-based anthocyanins that generally produce purple-blue flower colour, cyanidin-based anthocyanins that produce red or pink flower colour and pelargonidin-based anthocyanins that produce orange or brick red flower colour⁹. Non-genetically modified rose lacks 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). This enzyme converts dihydrokaempferol (DHK) and dihydroquercetin (DHQ) to dihydromyricetin (DHM). In the genetically modified rose lines described in this document a *Viola* sp. gene encoding F3'5'H has been introduced and delphinidin is produced as a result of the expression of this gene¹⁰ (figure 2).

⁹ Yoshida K, Mori M, Kondo T (2009). Blue flower color development by anthocyanins: From chemical structure to cell physiology. *Nat Prod Rep* 26: 857–974

¹⁰ Tanaka Y, Brugliera F (2014). Metabolic engineering of flower color pathway using cytochromes P450. In: Yamazaki M (ed) *Fifty Years of Cytochrome P450 Research*. Springer, Tokyo, Japan, pp 207–229

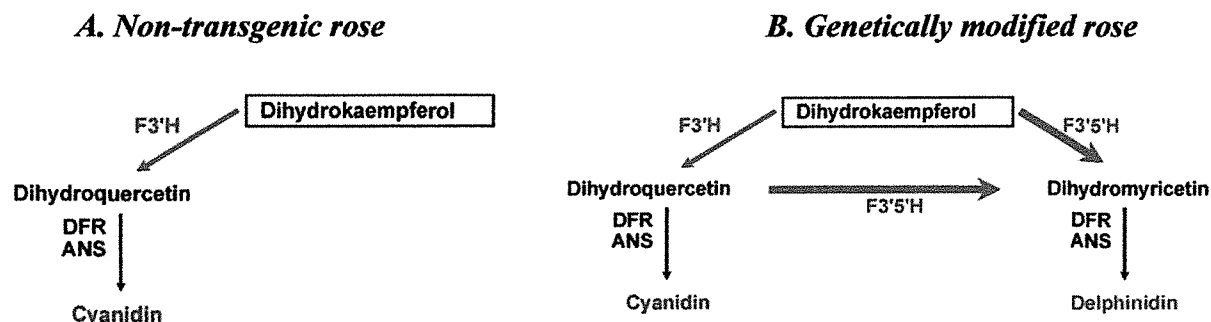


Figure 2. A. The anthocyanin pathway present in untransformed rose plants. B. The anthocyanin pathway in rose genetically modified through addition of a *Viola sp.* gene for flavonoid 3'5' hydroxylase. Dihydroflavonols are substrates for the biosynthesis of the anthocyaninidin pigments, which are produced firstly by a reduction catalysed by the enzyme dihydroflavonol 4-reductase (DFR) and secondly by the production of colourless anthocyanidin through the action of the enzyme anthocyanidin synthase (ANS).

The lines included in this document have been generated using six constructs. In combination with the introduction of flavonoid 3'5' hydroxylase, four other strategies have been employed;

1. In construct pSPB4715 a 5'-untranslated region from the tobacco alcohol dehydrogenase gene¹¹ has been fused to flavonoid 3'5' hydroxylase to enhance translational efficiency.
2. In pSPB4538 and pSPB4569 the concentration of ferrous ions in the vacuole was increased by a) down-regulation of endogenous ferritin¹², increasing the pool of available iron ions b) introduction of an iron transporter gene from either tulip¹³ (pSFL4538) or arabidopsis¹⁴ (pSFL4569), increasing the concentration of iron ions in the vacuole. An interaction between ferrous ions increased the blueness of the delphinidin-based anthocyanins. This strategy is illustrated in figure 3.

¹¹ Satoh, J., Kato, K. and Shinmyo, A. (2004). The 5'-untranslated region of the tobacco alcohol dehydrogenase gene functions as an effective translational enhancer in plant. *Journal of bioscience and bioengineering* **98**; 1-8.

¹² Shoji, K., Momonoi, K., and Tsuji, T. (2010). Alternative expression of vacuolar iron transporter and ferritin genes leads to blue/purple coloration of flowers in tulip cv. 'Murasakizuisho'. *Plant and cell physiology* **51**::215-224

¹³ Momonoi, K., Yoshida, K., Mano, S., Takahashi, H., Nakamori, C., Shoji, K. and Nishimura, M. (2009). A vacuolar iron transporter in tulip, TgVit1, is responsible for blue coloration in petal cells through iron accumulation. *The Plant Journal*, **59**; 437-447

¹⁴ Kim, S. A., Punshon, T., Lanzirrotti, A., Li, L., Alonso, J. M., Ecker, J. R. and Guerinot, M. L. (2006). Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1. *Science* **314**; 1295-1298.

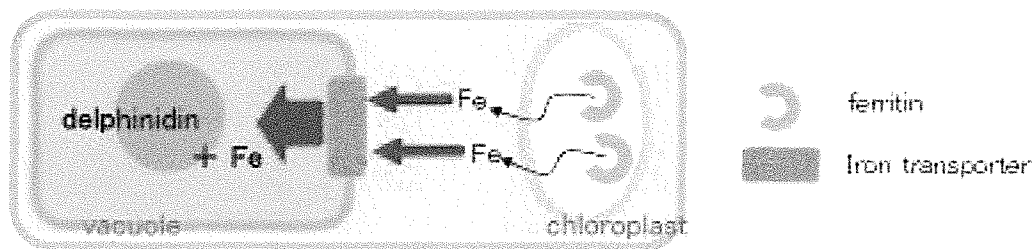


Figure 3. Pathway modification using constructs pSFL4538 and pSFL4569. The flavonoid biosynthesis pathway was directed towards accumulation of delphinidin as a result of introduction of F3'5'H. In addition, the concentration of ferrous ions in the vacuole was increased by a) down-regulation of endogenous ferritin, increasing the pool of available iron ions b) introduction of an iron transporter gene from either tulip (pSFL4538) or arabidopsis (pSFL4569), increasing the pool of iron ions in the vacuole. There is a colour shift in the delphinidin molecule as a result of interaction with the iron ions.

3. In pSFL903 and pSFL1003 the endogenous activity of the rose DFR gene has been suppressed and an external DFR gene from iris added¹⁵. The iris DFR has affinity to dihydromyricetin and is therefore more effective in biosynthesis of delphinidin. This strategy is illustrated in figure 4. In constructs pSFL903 and pSFL1003 different regions of the rose DFR gene have been selected for inclusion in the sense-antisense part of the construct.

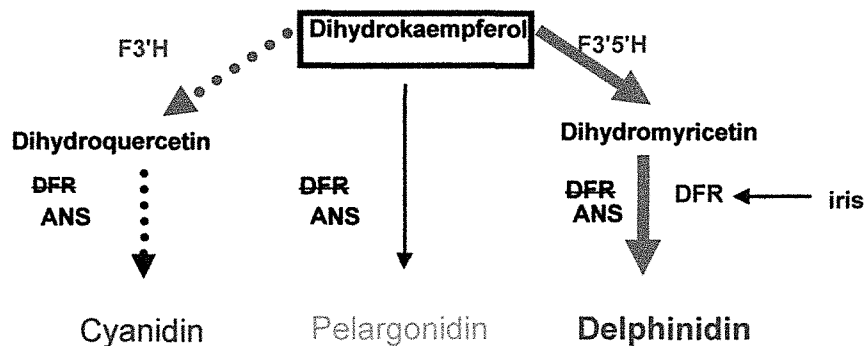


Figure 4. Pathway modification using constructs pSFL903 and pSFL1003. The flavonoid biosynthesis pathway was directed towards accumulation of delphinidin as a result of; a) Introduction of F3'5'H b) suppression of endogenous DFR activity c) introduction of an iris DFR, which has affinity for dihydromyricetin.

4. In pSFL910 the endogenous activity of the rose flavonoid 3'-hydroxylase gene has been suppressed and the external DFR gene from iris added. Suppression of the endogenous flavonoid 3'-hydroxylase increases the pool of dihydrokaempferol, the substrate for flavonoid 3'5' hydroxylase. The iris DFR has affinity to dihydromyricetin and is therefore more effective in biosynthesis of delphinidin. This

¹⁵ Katsumoto, Y., Fukuchi-Mizutani, M., Fukui, Y., Brugliera, F., Holton, T. A., Karan, M. and Tanaka, Y. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. *Plant and Cell Physiology* 48; 1589-1600.

strategy is illustrated in figure 5.

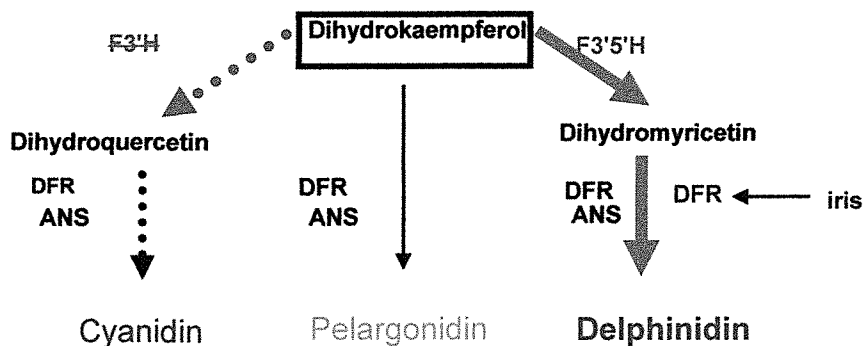


Figure 5. Pathway modification using construct pSFL910. The flavonoid biosynthesis pathway was directed towards accumulation of delphinidin as a result of; a) Introduction of F3'5'H b) suppression of endogenous Flavonoid 3'-hydroxylase activity c) introduction of an iris DFR, which has affinity for dihydromyricetin.

5. Transformation method

All seven events included in this document were generated after co-cultivation with disarmed *Agrobacterium tumefaciens*.

6. Description of constructs

As outlined in section 4, in addition to flavonoid 3', 5'-hydroxylase, which is present in all constructs, constructs also contain;

- A sense-antisense construct for suppression of expression of the rose endogenous ferritin biosynthesis gene.
- An iron transporter gene from either tulip or arabidopsis.
- A sense-antisense construct for suppression of expression of the rose endogenous dihydroflavonol reductase (DFR) gene.
- A sense-antisense construct for suppression of expression of the rose endogenous flavonoid 3'-hydroxylase gene.
- A DFR gene from iris.

Each of the additional genetic elements is designed to optimize the accumulation of delphinidin or to enhance the "blueness" of the accumulated delphinidin.

Appendix 1 of this document lists the elements of each of the six constructs used.

7. recipient organism

The common name of the recipient organism is the cultivated rose, variety OS. The scientific name is *Rosa hybrida*, or *Rosa X hybrida*.

8. Donor organism for all DNA sequences in the construct that were actually inserted into the plants

Southern analysis has been carried out on all seven events, using probes for left border, right border and inserted genes. The analysis indicates the T-DNA elements of the constructs has been inserted in the transgenic plants. The donor organisms are described in Appendix 2 of this document.

Appendix 1. Description of constructs

Tables 1 - 6 below list the elements of each construct in the order in which they occur in the construct, with the tabulation in all constructs starting from the left border of the T-DNA in the construct.

Table 1	Construct pSFL1003
Table 2	Construct pSPB4538
Table 3	Construct pSPB4569
Table 4	Construct pSPB4715
Table 5	Construct pSFL903
Table 6	Construct pSFL910

Table 7 provides a summary of inserted genes by construct as there are some elements which are found in all, or more than one, construct.

Table 1. Construct pSFL1003 (pnos: NPTII: tnos + p35S: iris DFR: tD8+ p35S: ds roseDFR(3'UTR): tmas + p35S: pansyF 3',5'-H : tnos)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	Nos (nopaline synthase)	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Gene	Dihydroflavonol reductase(DFR)	<i>Iris sp.</i> [iris]	DFR coding sequence (cDNA)
Terminator	D8	<i>Petunia hybrida</i> [petunia]	Regulatory element from phospholipid transfer protein homologue
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	DFR genomic clone (3'UTR)-sense	<i>Rosa hybrida</i> [rose]	Sense strand of co-suppression construct. Suppression of endogenous DFR activity.
Gene	DFR genomic clone (3'UTR)-antisense	<i>Rosa hybrida</i>	Anti-sense strand of co-suppression construct. Suppression of endogenous DFR activity.
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Flavonoid 3',5'-hydroxylase (F 3',5'-H)	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 2. Construct pSPB4538 (pnos: NPTII: tnos + p35S: dsroseFER1: tmas + p35S: dsroseFER2: tmas + p35S: tulipVIT1: tmas + p35S: pansyF 3', 5'-H : tmas)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	nos	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Gene	Ferritin1 cDNA-sense	<i>Rosa hybrida</i> [rose]	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Gene	Ferritin1 cDNA-antisense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Terminator	mas (mannopine synthase)	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Ferritin2 cDNA-sense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Gene	Ferritin2 cDNA-antisense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Terminator	mas (mannopine synthase)	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	VIT1 cDNA	<i>Tulipa gesneriana</i> [tulip]	Expression of a vacuole membrane based iron transporter gene promoting transport of ferrous ions into the vacuole
Terminator	mas (mannopine synthase)	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Flavonoid 3',5'-hydroxylase	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 3. Construct pSPB4569 (pnos: NPTII: tnos + p35S: pansyF 3', 5'-H : tmas + pAT: dsroseFER1: tmas + pAT: dsroseFER2: tmas + pCHS: arabidopsisVIT1: tmas)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	nos	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Gene	Flavonoid 3',5'-hydroxylase	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	AT promoter	<i>Perilla frutescens</i> [Perilla, shiso, wild coleus]	Promoter element from anthocyanin 3-aromatic acyltransferase gene driving gene expression
Gene	Ferritin1 cDNA-sense	<i>Rosa hybrida</i> [rose]	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Gene	Ferritin1 cDNA-antisense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	AT promoter	<i>Perilla frutescens</i>	Promoter element from anthocyanin 3-aromatic acyltransferase gene driving gene expression
Gene	Ferritin2 cDNA-sense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Gene	Ferritin2 cDNA-antisense	<i>Rosa hybrida</i>	Suppression of endogenous ferritin activity, reducing ferritin pool in chloroplasts, increasing the pool of free ferrous ions in vacuoles.
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	CHS (chalcone synthase)	<i>Rosa hybrida</i>	Promoter region of chalcone synthase gene driving constitutive gene expression
Gene	VIT1 cDNA	<i>Arabidopsis thaliana</i> [arabidopsis]	Expression of a vacuole membrane based iron transporter gene promoting transport of ferrous ions into the vacuole
Terminator	mas	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 4. Construct pSPB4715 (pnos: NPTII: tnos + p35S-ADH: pansyF 3', 5'-H: tAT)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	nos	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Translational enhancer	ADH-5'UTR	<i>Nicotiana tabacum</i> [tobacco]	5'-untranslated region of the tobacco alcohol dehydrogenase gene. Enhances gene expression at the translational level.
Gene	Flavonoid 3',5'-hydroxylase	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	AT terminator	<i>Perilla frutescens</i> [Perilla, shiso, wild coleus]	Terminator element from anthocyanin 3-aromatic acyltransferase gene driving gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 5. Construct pSFL903 (pnos: NPTII: tnos + p35S: iris DFR: tD8+ p35S: ds roseDFR(exon 3): tnos + p35S: pansyF 3',5'-H : tnos)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	Nos (nopaline synthase)	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Gene	Dihydroflavonol reductase(DFR)	<i>Iris sp.</i> [iris]	DFR coding sequence (cDNA)
Terminator	D8	<i>Petunia hybrida</i> [petunia]	Regulatory element from phospholipid transfer protein homologue
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	DFR genomic clone (exon3, intron3)-sense	<i>Rosa hybrida</i> [rose]	Sense strand of co-suppression construct. Suppression of endogenous DFR activity.
Gene	DFR genomic clone (exon3, intron3)-antisense	<i>Rosa hybrida</i>	Anti-sense strand of co-suppression construct. Suppression of endogenous DFR activity.
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Flavonoid 3',5'-hydroxylase	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 6. Construct pSFL910 (pnos: NPTII: tnos + p35S: iris DFR: tD8+ p35S: ds roseF3'H: tnos + p35S: pansyF 3', 5'-H : tnos)

Element type	Element name	Organism from which element derived	Description of elements function
T-DNA border	LB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Promoter	Nos (nopaline synthase)	<i>Agrobacterium. tumefaciens</i>	Constitutive promoter for gene expression
Gene	nptII	<i>Escherichia coli</i>	Selectable marker conferring resistance to the antibiotic kanamycin, which is used for selection of transgenic plants <i>in vitro</i> .
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus (CaMV)	Constitutive promoter for gene expression
Gene	Dihydroflavonol reductase(DFR)	<i>Iris sp.</i> [iris]	DFR coding sequence (cDNA)
Terminator	D8	<i>Petunia hybrida</i> [petunia]	Regulatory element from phospholipid transfer protein homologue
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Flavonoid 3'- hydroxylase genomic clone-sense	<i>Rosa hybrida</i> [rose]	Sense strand of co-suppression construct. Suppression of endogenous Flavonoid 3'- hydroxylase activity.
Gene	Flavonoid 3'- hydroxylase genomic clone -antisense	<i>Rosa hybrida</i>	Sense strand of co-suppression construct. Suppression of endogenous Flavonoid 3'- hydroxylase activity.
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
Promoter	35S	Cauliflower mosaic virus	Constitutive promoter for gene expression
Gene	Flavonoid 3',5'-hydroxylase	<i>Viola sp.</i> [pansy]	Flavonoid 3',5'-hydroxylase coding sequence (cDNA)
Terminator	nos	<i>Agrobacterium. tumefaciens</i>	Regulation of gene expression
T-DNA border	RB	<i>Agrobacterium. tumefaciens</i>	Defines junction between T-DNA and plant genomic or vector DNA. Utilized in transfer of insert to plant cell and integration into genome.
Construct backbone	pVS1 replicon & pPZP100 seq	<i>Pseudomonas aeruginosa</i>	For replication of transformation vector in <i>A. tumefaciens</i> .
Construct backbone	Tet resistance gene complex	<i>Escherichia coli</i>	For selection of bacteria carrying the transformation vector.
Construct backbone	Modified pACYC184 replicon	<i>Escherichia coli</i>	For replication of transformation vector in <i>E. coli</i> only.

Table 7. Summary table. Green shading indicates the inserted gene is present in the construct.

All constructs contain the same borders and the same non T-DNA elements (LB, RB, backbone)

Inserted gene			Construct					
Promoter	Gene	Terminator	pSFL 1003	pSPB 4538	pSPB 4569	pSPB 4715	pSFL 903	pSFL 910
Nos (nopaline synthase)	nptII	nos						
35S	Iris dihydroflavonol reductase(DFR)	D8						
35S	Rose DFR sense-antisense	mas						
35S	Rose DFR sense-antisense	nos						
35S	Viola Flavonoid 3',5'-hydroxylase	nos						
35S	Viola Flavonoid 3',5'-hydroxylase	mas						
35S	ADH-5'UTR ;Viola Flavonoid 3',5'-hydroxylase	AT						
35S	Rose ferritin 1 sense-anti-sense	mas						
35S	Rose ferritin 2 sense-anti-sense	mas						
35S	Tulip vacuole membrane based iron transporter gene	mas						
AT	Rose ferritin 1 sense-anti-sense	mas						
AT	Rose ferritin 2 sense-anti-sense	mas						
CHS	Arabidopsis vacuole membrane based iron transporter gene	mas						
35S	Rose Flavonoid 3'-hydroxylase sense-antisense	nos						

Appendix 2. Donor organisms

Table 1 summarizes, by construct, the donor organisms from which DNA sequences actually inserted into the transgenic rose events described in this document were derived.

Table 1. Summary of donor organisms by construct

Donor organism	Construct					
	pSFL 1003	pSPB 4538	pSPB 4569	pSPB 4715	pSFL 903	pSFL 910
<i>Agrobacterium tumefaciens</i>	√	√	√	√	√	√
<i>Escherichia coli</i>	√	√	√	√	√	√
<i>Viola hortensis</i>	√	√	√	√	√	√
Cauliflower mosaic virus	√	√	√	√	√	√
<i>Perilla frutescens</i>			√	√		
<i>Nicotiana tabacum</i>				√		
<i>Iris sp.</i>	√				√	√
<i>Petunia hybrida</i>	√				√	√
<i>Rosa hybrida</i>	√	√	√		√	√
<i>Tulipa gesneriana</i>		√				
<i>Arabidopsis thaliana</i>			√			

Donor organisms are described in table 2.

Table 2. Description of donor organism

Donor organism	Description
<i>Agrobacterium tumefaciens</i>	<i>Agrobacterium tumefaciens</i> is a plant bacterial pathogen. In the armed form the bacterium causes crown gall disease in many dicotyledonous plant species (Mansfield et al., 2012 ¹⁶). <i>Agrobacterium tumefaciens</i> is ubiquitous in nature and has a worldwide distribution in soil and in association with plants.

¹⁶ Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P. and Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular plant pathology*, 13; 614-629

<i>Escherichia coli</i>	<i>Escherichia coli</i> has no plant pathogenicity, though certain strains may cause disease in humans. <i>E. coli</i> is an Enterobacterium, and some bacterium in the group, such as <i>Erwinia</i> do exploit plants as hosts, <i>E. coli</i> is found in the gastrointestinal tract of animals (Todar, 2004 ¹⁷). Certain strains of <i>Escherichia coli</i> may cause disease in humans, with toxic effects in some cases, associated with infection. Ubiquitous in nature. Worldwide distribution in numerous aquatic habitats and in association with animals and humans.
<i>Viola hortensis</i>	Pansy (<i>Viola hortensis</i>) is a non-pathogenic plant species. The species is a cultivated annual and is non-epiphyte i.e. it does not colonise other plant species. Pansy is a cultivated plant, developed for both bedding situations and pot plant use. Commercial varieties are now propagated throughout the world.
Cauliflower mosaic virus	Cauliflower mosaic virus (CaMV) is a plant virus pathogen, infecting many cruciferous plants. The virus has a global distribution (Yasaka et al., 2014 ¹⁸) and is ubiquitous in nature.
<i>Perilla frutescens</i>	Perilla, or wild coleus (<i>Perilla frutescens</i>) is a non-pathogenic plant species. The species is a cultivated and wild plant harvested as a food and is non-epiphyte i.e. it does not colonise other plant species. <i>Perilla frutescens</i> is widely used as a colorant and traditional medicine in Asia (Saito and Yamazaki, 2002 ¹⁹). <i>Perilla frutescens</i> is cultivated in Asia; primarily Korea and Japan. The plant is found in the wild in China, India and other parts of South-East Asia.
<i>Nicotiana tabacum</i>	Tobacco (<i>Nicotiana tabacum</i>) is a non-pathogenic plant species. The species is an annual only found in cultivation (for the tobacco industry) and is non-epiphyte i.e. it does not colonise other plant species.
<i>Iris</i> sp.	Iris (<i>Iris</i> sp.) comprises a group of plant species cultivated for the ornamental industry and none of the species are pathogenic. Iris is a perennial and is propagated by rhizomes or bulbs. Iris species are non-epiphyte i.e. they do not colonise other plant species. Iris is cultivated in temperate regions of the world but cut flowers may be distributed globally.
<i>Petunia hybrida</i>	<i>Petunia X hybrida</i> represents a hybrid of uncertain parentage and the common name of such hybrids are petunia. Petunia species are annual ornamentals found in cultivation and are non-pathogenic and non-epiphytic. <i>Petunia hybrida</i> is a cultivated plant which is grown on a worldwide basis.

¹⁷ Todar, K (2004). The enteric bacteria. In; Todars, online textbook of Bacteriology. <http://textbookofbacteriology.net>.

¹⁸ Yasaka, R., Nguyen, H. D., Ho, S. Y., Duchêne, S., Korkmaz, S., Katis, N. and Ohshima, K. (2014). The Temporal Evolution and Global Spread of Cauliflower mosaic virus, a Plant Pararetrovirus. PloS one, 9;e85641

¹⁹ Saito, K., and Yamazaki, M. (2002). Biochemistry and molecular biology of the late-stage of biosynthesis of anthocyanin: lessons from *Perillafrutescens* as a model plant. New Phytologist, 155; 9-23

<i>Rosa hybrida</i>	Cultivated rose varieties are designated <i>Rosa hybrida</i> or <i>Rosa X hybrida</i> due the complex and sometimes uncertain heritage of modern cut-flower varieties. Cultivated rose is perennial and is non-pathogenic and non-epiphytic.
<i>Tulipa gesneriana</i>	<i>Tulipa gesneriana</i> is commonly known as tulip or gardener's tulip. Cultivated via bulbs, this plant species is non-pathogenic and non-epiphytic. <i>Tulipa gesneriana</i> contains an allergen, tuliposide A, which causes dermatitis in sensitive individuals. Ornamental varieties of <i>Tulipa gesneriana</i> are found in cultivation only. These varieties developed primarily for cut flowers but also for pot situations.
<i>Arabidopsis thaliana</i>	Thale or mouse-ear cress (<i>Arabidopsis thaliana</i>) is an annual plant found across much of Eurasia. This species is not cultivated but is commonly grown for experimental research in plants. <i>Arabidopsis thaliana</i> is non-pathogenic and non-epiphytic.