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SUNTORY

November 10 2021

Bernadette Juarez
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Dear Dr. Juarez

RSR request 21-277-01rsr

Through this letter and document on subsequent pages, Suntory Flowers Limited are pleased to re-submit RSR dossier **21-277-01rsr (**blue-flower color transgenic cut-flower varieties of the plant species *Chrysanthemum morifolium* syn. *Dendranthema x grandiflora* Tzvelev (chrysanthemum), an ornamental plant used for cut-flower production).

The document has been revised as follows;
1. Figure 1 has been replaced with a version of the vector map in which nucleotide 1 is now the first nucleotide of the right border.
2. The FASTA sequence in Appendix 1 has been modified by removal of a TAG sequence at the start of the sequence and now begins at nucleotide 1 of the revised vector map.
3. Appendix 2 has been expanded to include description of all nucleotides in the inserted genetic material.

The document does not contain CBI. As before, the contact person for this request is Dr. Stephen Chandler (schandler@florigene.com.au; +61 409 387 386). We would appreciate it if you could please continue to address any queries and communication regarding this RSR request to Dr. Chandler.

Yours sincerely,

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1. REQUESTOR

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2. CONFIDENTIAL BUSINESS INFORMATION (CBI) STATEMENT

This request contains no confidential business information.

3. DESCRIPTION OF COMPARATOR PLANT

Scientific name
Chrysanthemum morifolium Ramat. syn. *Dendranthema x grandiflora* Tzvelev.

Common name
Chrysanthemum.

Cultivars
Table 1 lists the cultivars of the comparator plant included in this request.

Table 1. List of cultivars.

Cultivar
Candelatiera
T10
T37
Seisyore
T57
T44
Seialabera
S47
S25

4. GENOTYPE OF THE MODIFIED PLANT

4.1 Overview of modified genotype

The plant has been modified by the insertion of genetic material. Transgenic lines were generated after co-cultivation with disarmed *Agrobacterium tumefaciens* strain EHA105 carrying

the construct pB423 (Noda et al., 2017). The protocol used for chrysanthemum transformation is provided in Aida et al. (2004).
Three genes have been inserted:

- The nptII (neo) neomycin phosphotransferase selectable marker gene from *Escherichia coli*. This gene confers antibiotic resistance to transformed plant cells. Paromomycin was used as a selective agent during plant transformation.
- The flavonoid 3',5'-hydroxylase (F3'5'H) coding sequence (cDNA) from campanula (*Campanula medium*).
- The UDP-glucose: anthocyanin 3',5'-O-glucosyltransferase (UDPG) coding sequence (cDNA) from butterfly pea (*Clitoria ternatea*).

The result of expression of the F3'5'H and UDPG genes is a change in flower color to violet-purple-blue due to a re-direction of the anthocyanin biosynthesis pathway in flowers from the pink-red cyanidin-derived anthocyanins normally found in chrysanthemum to blue-purple delphinidin-derived anthocyanins.

4.2 Sequence of the insertion

Figure 1 provides a diagram of construct pB423.The inserted DNA is located at nucleotide positions 1 to 9007. The nucleotide sequence of the inserted DNA is provided at appendix 1.

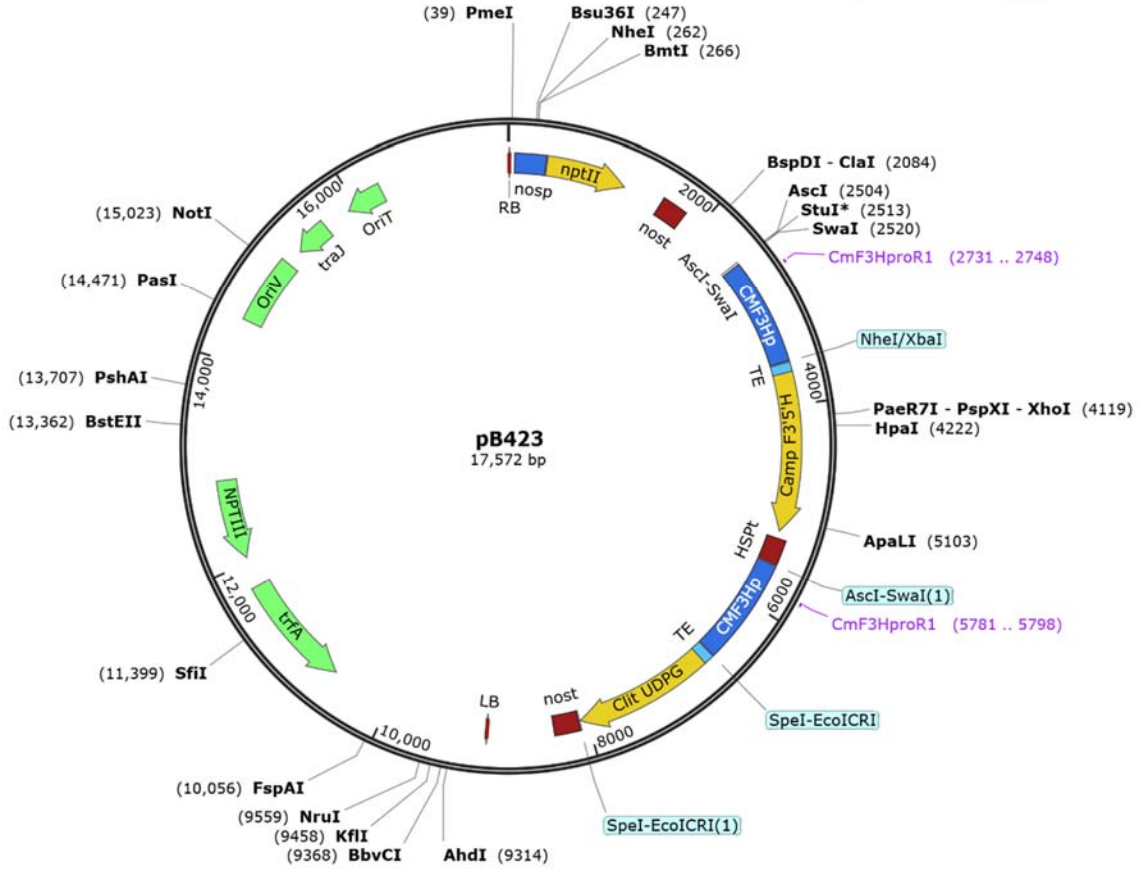


Figure 1. Map of construct pB423.

4.3 Reference numbers

Table 2 tabulates available reference numbers of the genetic components of the inserted DNA, in 5' to 3' direction.

Table 2. Available reference numbers of the genetic components of the inserted DNA.

Name of genetic component	Nucleotide sequence identification number	Protein accession number
RB	GenBank: AF485783.1[f]	-
nosp	AF485783.1	-
nptII	AF485783.1	GenBank: AAL92039.1[e]
nost	AF485783.1	-
CMF3Hp	GenBank accession number LC222467[a]	-
TE	LC222467	-
Camp F3'5'H	LC222467; GenBank accession number FW570860[b]	LC222467
HSPt	LC222467	-
CMF3Hp	LC222467; GenBank accession number FW570877.1[c]	-
TE	LC222467	-
Clit UDPG	LC222467; GenBank accession number AB115560.1[d]	LC222467; AB115560.1
nost	LC222467; AF485783.1	-
LB	AF485783.1	-

a. https://www.ncbi.nlm.nih.gov/nuccore/LC222467
b. https://www.ncbi.nlm.nih.gov/nuccore/FW570860
c. https://www.ncbi.nlm.nih.gov/nuccore/FW570877
d. https://www.ncbi.nlm.nih.gov/nuccore/AB115560
e. https://www.ncbi.nlm.nih.gov/protein/19569230
f. https://www.ncbi.nlm.nih.gov/nuccore/AF485783.1

4.4 Annotation of the inserted genetic material

Nucleotide position, name of inserted component, construct component, donor organism and construct component function are tabulated at appendix 2.

5. DESCRIPTION OF NEW TRAITS

5.1 Intended traits

The plant has two new traits:
a. Marker gene (antibiotic resistance).
b. Altered flower color.

5.2 Intended phenotypes

5.2.1 Marker gene (antibiotic resistance)

The modified phenotype is resistance to kanamycin, paromycin and neomycin during transformation. All transgenic events generated with construct pB423 contain a neomycin phosphotransferase gene from *Escherichia coli*. This gene confers antibiotic resistance. Paromomycin was used as a selective agent during plant transformation.

5.2.2 Altered flower color

The modified phenotype is purple, violet or blue flower color. Figure 2 illustrates typical flowers of three representative transgenic lines.

Figure 2. Typical flower color phenotypes of transgenic chrysanthemum lines 1B, 4A and 8D. For all three lines a flower from the transgenic line is on the left and a flower from the unmodified cultivar used for transformation is on the right.

Flower color in the modified plant is due to production of anthocyanins derived from the anthocyanidin delphinidin (Noda et al., 2017). Delphinidin-derived anthocyanins are not accumulated in chrysanthemum flowers naturally. The color of the flowers from the transgenic chrysanthemum cultivars are in the range of Royal Horticultural Society color groups violet (RHS code 85D), violet-blue (RHS code 94B) and blue (RHS code 100C) (Noda et al., 2017). Depending on the transgenic line, the amount of delphinidin-derived anthocyanin in the petals of the transgenic flowers ranges from 0.2 to 5.8 nmol mg^{-1} and represents 40 - 100% of total anthocyanins (Noda et al., 2017). Delphinidin accumulation is confined to the flowers of the modified plant, due to endogenous enhancement of the downstream anthocyanin pathway in flowers and the use of petal-specific promoters with the introduced flavonoid 3' 5' hydroxylase and UDP-glucose:anthocyanin 3',5'-O-glucosyltransferase genes.

5.3 Description of the mechanism of action (MOA)

5.3.1 Marker gene (antibiotic resistance)

Expression of neomycin phosphotransferase (NPTII) confers resistance to certain antibiotics including kanamycin, paromycin and neomycin.

5.3.2 Altered flower color

F3'5'H (flavonoid 3' 5' hydroxylase) catalyses production of dihydromyricetin, a precursor to delphinidin derived anthocyanins; UDPG (UDP-glucose:anthocyanin 3',5'-O-glucosyltransferase) glucosylates delphinidin-derived anthocyanin to form 3′-glucosylated cyanidin-derived anthocyanin, 3′,5′-glucosylated delphinidin-derived anthocyanins and/or related ternatin-type anthocyanins, resulting in violet, mauve or blue flower color.

5.4 Other information on the MOA (altered flower color)

The flower color change in the chrysanthemum events is due to expression of two plant genes; a cDNA clone of flavonoid 3', 5' -hydroxylase (F3'5'H) from campanula and a cDNA clone of UDP-glucose: anthocyanin 3',5'-O-glucosyltransferase (UDPG) gene from butterfly pea.

<u>F3'5'H gene</u>

Flower color is generally the result of the relative concentration of two pigment types - carotenoids and flavonoids. Carotenoids are responsible for yellow through orange colors. Anthocyanins are flavonoid derived colored pigments. There are three groups of anthocyanins, the delphinidin-derived anthocyanins that generally produce purple-blue flower color, cyanidin-derived anthocyanins that produce red or pink flower color and pelargonidin-derived anthocyanins that produce orange or brick red flower color (Yoshida et al., 2009; Ohmiya, 2018). Figure 3 shows a simplified anthocyanin pathway.

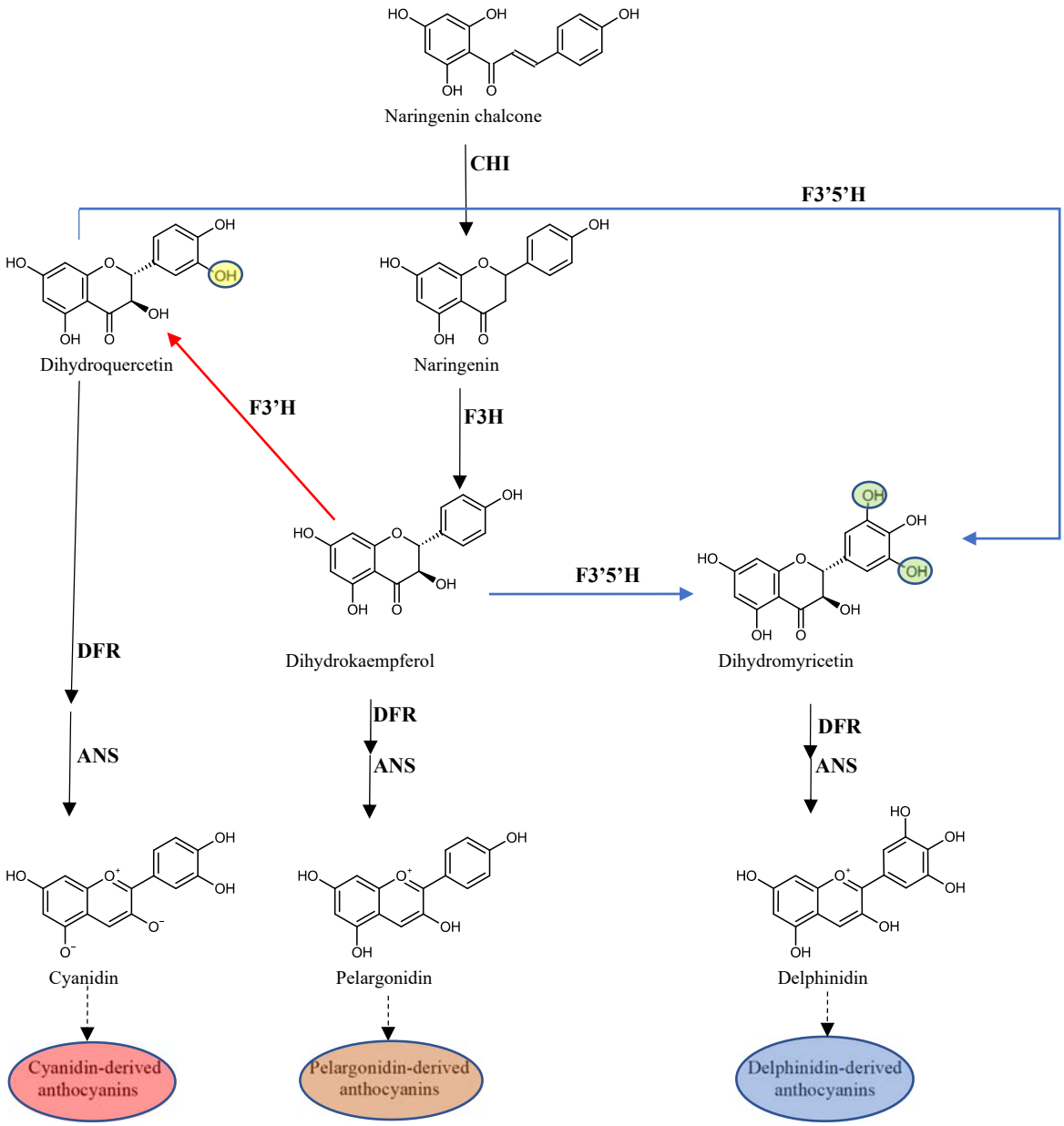


Figure 3. Simplified anthocyanin pathway. CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase. The dihydroflavonols are substrates for the biosynthesis of the non-colored anthocyanidins. Anthocyanidins are produced in two steps; firstly, a reduction, catalysed by the enzyme dihydroflavonol 4-reductase (DFR) and secondly the production of colorless anthocyanidin through the action of anthocyanidin synthase (ANS). Addition of glycosyl, acyl and/or methyl molecules to the anthocyanidins produces colored anthocyanins.

As figure 3 shows, the key intermediate in the anthocyanin pathway is the dihydroflavonol dihydrokaempferol, which is produced after enzymatic reactions catalysed by chalcone isomerase and flavanone 3β-hydroxylase. The enzyme flavonoid 3'-hydroxylase

converts dihydrokaempferol to dihydroquercetin through addition of a hydroxyl group (highlighted in yellow in figure 3).The enzyme flavonoid 3'5'-hydroxylase converts dihydrokaempferol and dihydroquercetin to dihydromyricetin by addition of one or two hydroxyl groups (highlighted in green in figure 3).The number of hydroxyl groups on the B-ring of the anthocyanidin is the determinant of anthocyanin colour and only anthocyanins derived from dihydromyricetin are blue, violet or mauve. F3'5'H expression is therefore a pre-requisite for the biosynthesis of delphinidin. Non-transgenic chrysanthemums lack the gene for the enzyme flavonoid 3'5'- hydroxylase and only accumulate cyanidin-derived anthocyanins (Noda et al.,2013, 2017; Ohmiya, 2018). In the genetically modified chrysanthemum, a campanula gene encoding F3'5'H has been introduced (Noda et al., 2013, 2017) and delphinidin-derived anthocyanins are produced because of the expression of this gene (figure 4). The change in anthocyanin type from pink-red cyanidin-derived anthocyanins to blue-purple delphinidin-derived anthocyanins results in an alteration in flower color.

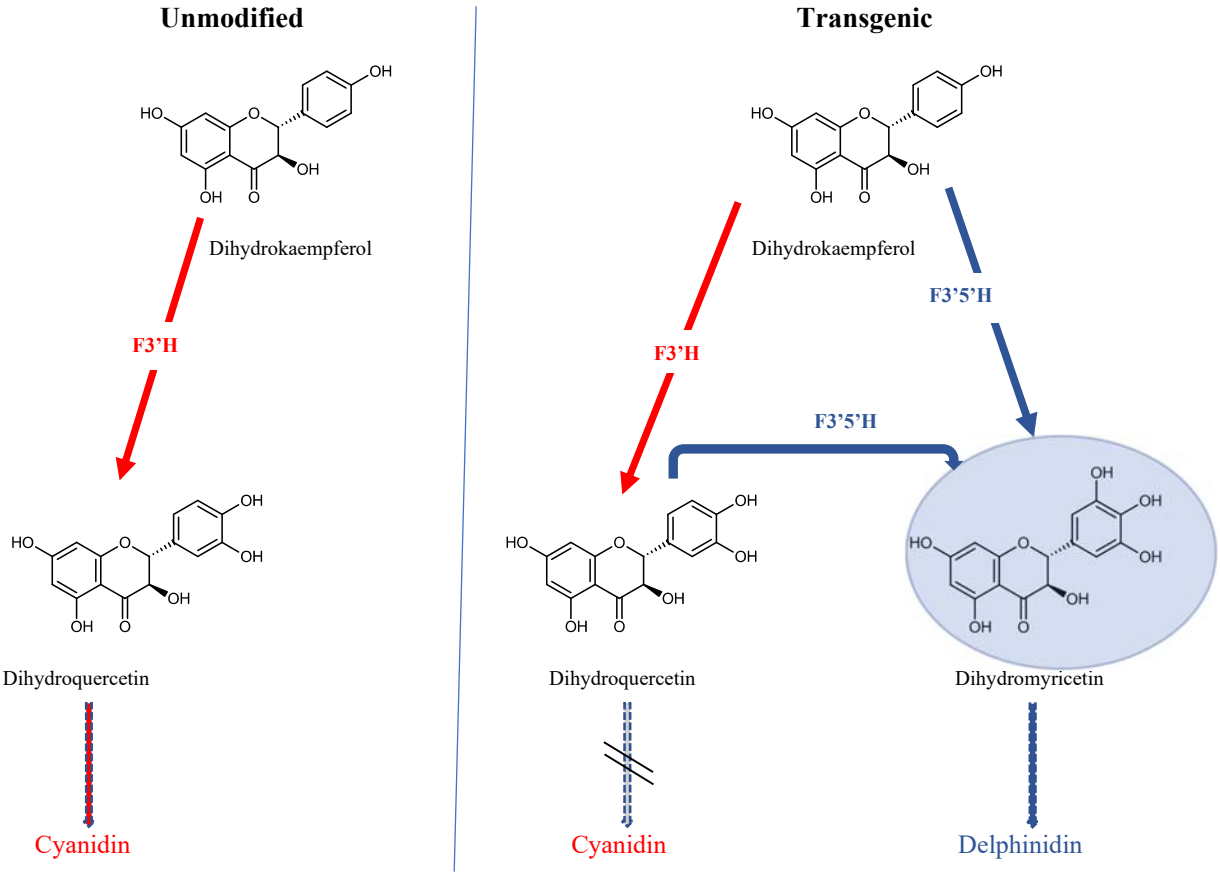
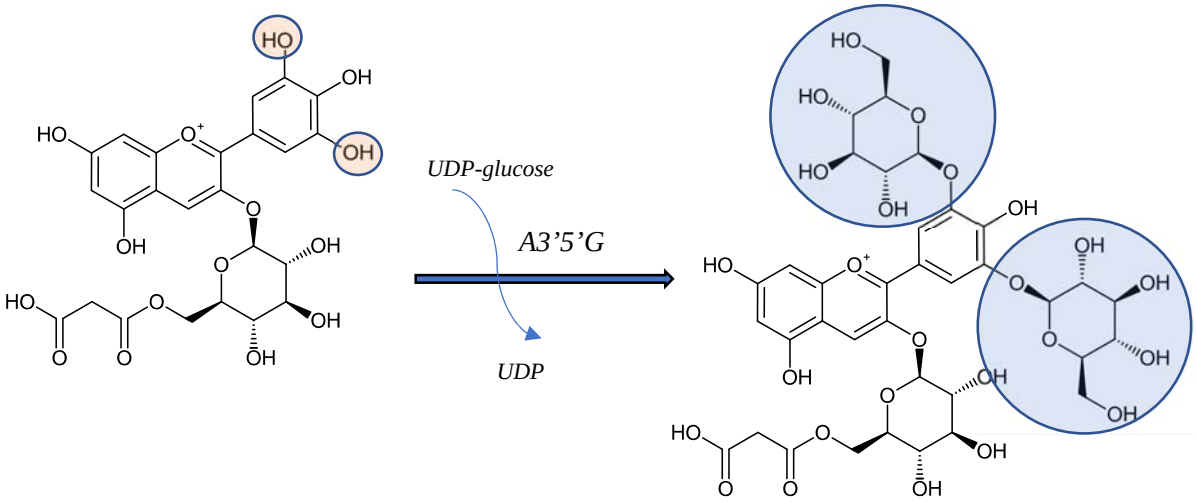


Figure 4. Modification of the anthocyanin pathway in chrysanthemum. Left hand side. Untransformed chrysanthemum plants, expressing endogenous flavonoid 3' hydroxylase (F3'H). Right hand side; The anthocyanin pathway in transgenic chrysanthemum after addition of a gene for flavonoid 3'5' hydroxylase (F3'5'H). Expression of this gene out competes the endogenous F3'H, resulting in biosynthesis of delphinidin, from which delphinidin-derived anthocyanins are produced.

UDPG gene

Expression of F3'5'H results in accumulation of the delphinidin-derived anthocyanins delphinidin-3-O-(6"-O-malonyl)glucoside and delphinidin-3-O-(3",6"-O-dimalonyl)glucoside in flowers of the transgenic chrysanthemum plant (Noda et al.,2013). The second introduced gene, UDP-glucose: anthocyanin 3',5'-O-glucosyltransferase (UDPG or A3′5′GT) functions to add two glucosyl groups to these anthocyanins (Kogawa et al., 2007) as illustrated in figure 5. The addition of these side groups confers a bluer hue to the anthocyanin (Noda et al., 2017).



Delphinidin-3-O-(6"-O-malonyl) -ß-glucoside

Delphinidin-3-*O*-(6"-*O*-malonyl)-ß-glucoside - 3',5'-di-*O*-ß-glucoside (ternatin C5)

Figure 5. Schematic representation of the mechanism of action of A3'5'GT (UDP-glucose: anthocyanin 3',5'-O-glucosyltransferase) in transgenic chrysanthemum. Two glucosides (shown in the blue shade on the right-hand side of the figure) are added at the 3' and 5' hydroxyl positions (shown shaded in orange on the left-hand side of the figure) of the B-ring of the anthocyanin delphinidin-3-O-(6"-O-ß-malonyl)glucoside.

The UDPG introduced into the modified chrysanthemum described in this request is from *Clitoria ternatea*, a blue flowered plant which contains a range of delphinidin-derived anthocyanins, including polyacylated anthocyanins called ternatins (Zhang et al., 2021). Transgenic chrysanthemum generated by co -expression of either the campanula F3'5'H gene and *Clitoria ternatea* UDPG gene (Noda et al., 2017) or an *Osteospermum hybrid* F3'5'H gene and UDPG *Clitoria ternatea* gene (Han et al., 2021) also contain ternatins.

6. REFERENCES

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APPENDIX 1. SEQUENCE OF THE INSERTED DNA IN FASTA FORMAT

Nucleotide positions 1 to 9007 of construct pB423.

>pB423 T-DNA
GTTTACCCGCCAATATATCCTGTCAAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGAT
CATGAGCGGAGAATTAAGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGT
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CGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCAC
TGGTGAAAAGAAAAACCACCCCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGCGT
CAATTTGTTTACACCACAATATATCCTGCCA

APPENDIX 2. ANNOTATION OF THE INSERTED GENETIC MATERIAL

Nucleotide positions 1 to 9007 of construct pB423.

Nucleotide position	Name of inserted component	Construct component donor species name	Function
1 - 25	RB (right border)	*Agrobacterium tumefaciens*	A 25bpT-DNA right border sequence from plasmid pTiT37 (Bevan, 1984) The sequence is utilized in transfer and integration of the binary vector genes into the plant genome.
26 - 65	Intervening non-coding sequence	*E.coli* sequence modified with synthetic sequence for use in DNA cloning and vector construction (Bevan, 1984; Jefferson et al., 1987)	Sequence introduced during cloning
66 - 372	nosp (nos promoter)	*Agrobacterium tumefaciens*	The nopaline synthase gene promoter (Bevan, 1984).
373 - 384	Intervening non-coding sequence	*E.coli* sequence modified with synthetic sequence for use in DNA cloning and vector construction (Bevan, 1984; Jefferson et al., 1987)	Sequence introduced during cloning
385 – 1179	nptII (neomycin phosphotransferase)	*Escherichia coli*	Tn5 derived neomycin phosphotransferase gene (Bevan, 1984). A selectable marker gene used for selection of transgenic plant cells resistant to antibiotics such as paromomycin and kanamycin.
1180 - 1568	Intervening non-coding sequence	*E.coli* sequence modified with synthetic sequence for use in DNA cloning and vector construction (Bevan, 1984; Jefferson et al., 1987)	Sequence introduced during cloning
1569 – 1824	nost (nos terminator)	*Agrobacterium tumefaciens*	Terminator (polyadenylation site) region of the nopaline synthase gene (Jefferson et al., 1987).
1825 - 2524	Intervening non-coding sequence	Synthetic (Chen et al., 2003)	Sequence introduced during cloning

2525 – 3571	CMF3Hp (flavanone 3-hydroxylase promoter)	*Chrysanthemum morifolium*	Promoter region of the flavanone 3-hydroxylase gene. A petal-specific chrysanthemum promoter which has maximum expression efficiency in chrysanthemum (Noda et al., 2010, 2013).
3572 - 3583	Intervening non-coding sequence	Synthetic (Noda et al., 2017)	Sequence introduced during cloning
3584 - 3677	TE (translational enhancer)	*Nicotiana tabacum*	5'-untranslated region of the alcohol dehydrogenase gene (Satoh et al., 2004) .A translational enhancer which enhances the translation of introduced genes in chrysanthemum (Aida et al., 2008; Noda et al., 2013).
3678 – 5243	Camp F3'5'H (flavonoid 3',5'-hydroxylase gene)	*Campanula medium*	Coding sequence from the flavonoid 3',5'-hydroxylase gene (Noda et al., 2013). Expression of this gene is required for the biosynthesis of delphinidin-derived anthocyanins, which confer blue flower color. Campanula has been identified as the source organism for optimal expression of the F3'5'H gene in chrysanthemum (Noda et al., 2013)
5244 - 5316	Intervening non-coding sequence	Synthetic (Noda et al., 2017)	Sequence introduced during cloning
5317 - 5566	HSPt (heat shock protein terminator)	*Arabidopsis thaliana*	Terminator region from heat shock protein 18.2 (Nagaya et al., 2010). In combination with translational enhancers, gene expression levels are enhanced with this terminator.
5567 - 5574	Intervening non-coding sequence	Synthetic (Noda et al., 2017)	Sequence introduced during cloning
5575 - 6621	CMF3Hp (flavanone 3-hydroxylase promoter)	*Chrysanthemum morifolium*	Promoter region of the flavanone 3-hydroxylase gene. A petal-specific chrysanthemum promoter which has maximum expression efficiency in chrysanthemum (Noda et al., 2010, 2013).

6622 - 6628	Intervening non-coding sequence	Synthetic (Noda et al., 2017)	Sequence introduced during cloning
6629 - 6727	TE (translational enhancer)	*Nicotiana tabacum*	5'-untranslated region of the alcohol dehydrogenase gene (Satoh et al., 2004) .This a translational enhancer which enhances the translation of introduced genes in chrysanthemum (Aida et al., 2008; Noda et al., 2013).
6728 - 8071	Clit UDPG (UDP-glucose:anthocyanin 3',5'-O-glucosyltransferase gene)	*Clitoria ternatea*	UDP-glucose:anthocyanin 3',5'-O-glucosyltransferase gene. Expression functions to add two glucosyl side groups to delphinidin derived anthocyanin (Kowaga et al., 2007) conferring a bluer hue to the pigment (Noda et al., 2017).
8072 - 8087	Intervening non-coding sequence	Synthetic (Noda et al., 2017)	Sequence introduced during cloning
8088 – 8340	nost (nos terminator)	*Agrobacterium tumefaciens*	Terminator (polyadenylation site) region of the nopaline synthase gene (Jefferson et al., 1987).
8341 - 8981	Intervening non-coding sequence	Synthetic (Chen et al., 2003)	Sequence introduced during cloning
8982 - 9007	LB (left border)	*Agrobacterium tumefaciens*	A 26bpT-DNA left border sequence from plasmid pTiT37 (Bevan, 1984) The sequence is utilized in transfer and integration of the binary vector genes into the plant genome.

APPENDIX 1. SEQUENCE OF THE INSERTED DNA IN FASTA FORMAT

Nucleotide positions 1 to 9007 of construct pB423.

>pB423 T-DNA
GTTTACCCGCCAATATATCCTGTCAAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGAT
CATGAGCGGAGAATTAAGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGT
TTGGAACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCGCGGGTTTCTGGAGTTTAATGAGCTA
AGCACATACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCCTAAGGTCACTATCAGCTAGCAAATA
TTTCTTGTCAAAAATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAATTAGAGTCTCATATT
CACTCTCAATCCAAATAATCTGCACCGGATCTGGATCGTTTCGCATGATTGAACAAGATGGATTGCAC
GCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG
CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGT
CCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCT
TGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGG
GCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGC
GGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCA
CGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCC
AGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATCTCGTCGTGACCCATGGCG
ATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTG
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