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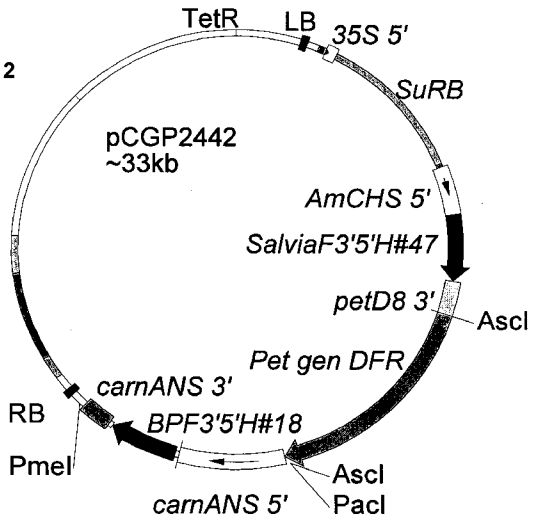
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(54) **Title:** GENETICALLY MODIFIED PLANTS WITH ALTERED INFLORESCENCE

Figure 2



(57) **Abstract:** The present invention relates to genetically modified plants expressing altered inflorescence. More particularly, expression of at least two flavonoid 3'5' hydroxylases and at least one dihydroflavonol-4-reductase is achieved, leading to the production of dephinidin pigments and altered colour phenotypes.

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GENETICALLY MODIFIED PLANTS WITH ALTERED INFLORESCENCE

FILING DATA

[0001] This application is associated with and claims priority from US Provisional Patent Application No. 60/988,293, filed on 15 November 2007, the entire contents of which, are incorporated herein by reference

FIELD

[0002] The present invention relates generally to the field of genetic modification of plants. More particularly, the present invention is directed to genetically modified plants expressing desired color phenotypes.

BACKGROUND

[0003] Bibliographic details of the publications referred to by the author in this specification are collected at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] The flower or ornamental or horticultural plant industry strives to develop new and different varieties of flowers and/or plants. An effective way to create such novel varieties is through the manipulation of flower color. Classical breeding techniques have been used with some success to produce a wide range of colors for almost all of the commercial varieties of flowers and/or plants available today. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for a single species to have the full spectrum of colored varieties. For example, the development of novel colored varieties of plants or plant parts such as flowers, foliage,

fruits and stems would offer a significant opportunity in both the cut flower, ornamental and horticultural markets. In the flower or ornamental or horticultural plant industry, the development of novel colored varieties of carnation is of particular interest. This includes not only different colored flowers but also anthers and styles.

[0006] Flower color is predominantly due to three types of pigment: flavonoids, carotenoids and betalains. Of the three, the flavonoids are the most common and contribute a range of colors from yellow to red to blue. The flavonoid molecules that make the major contribution to flower color are the anthocyanins, which are glycosylated derivatives of cyanidin and its methylated derivative peonidin, delphinidin and its methylated derivatives petunidin and malvidin and pelargonidin. Anthocyanins are localized in the vacuole of the epidermal cells of petals or the vacuole of the sub epidermal cells of leaves.

[0007] The flavonoid pigments are secondary metabolites of the phenylpropanoid pathway. The biosynthetic pathway for the flavonoid pigments (flavonoid pathway) is well established, (Holton and Cornish, *Plant Cell 7*:1071-1083, 1995; Mol *et al, Trends Plant Sci. 3*:212–217, 1998; Winkel-Shirley, *Plant Physiol. 126*:485-493, 2001a; and Winkel-Shirley, *Plant Physiol. 127*:1399-1404, 2001b, Tanaka and Mason, In *Plant Genetic Engineering*, Singh and Jaiwal (eds) SciTech Publishing Llc., USA, *1*:361-385, 2003, Tanaka *et al*, *Plant Cell, Tissue and Organ Culture 80*:1–24, 2005, Tanaka and Brugliera, *In Flowering and Its Manipulation, Annual Plant Reviews* Ainsworth (ed), Blackwell Publishing, UK, *20*:201-239, 2006) and is shown in Figure 1. Three reactions and enzymes are involved in the conversion of phenylalanine to *p*-coumaroyl-CoA, one of the first key substrates in the flavonoid pathway. The enzymes are phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA (provided by the action of acetyl CoA carboxylase (ACC) on acetyl CoA and CO_2) with one molecule of *p*-coumaroyl-CoA. This reaction is catalyzed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6', tetrahydroxy-chalcone, is normally rapidly isomerized by the enzyme chalcone flavanone isomerase (CHI) to produce naringenin. Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

[0008] The pattern of hydroxylation of the B-ring of DHK plays a key role in determining petal color. The B-ring can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) or dihydromyricetin (DHM), respectively. Two key enzymes involved in this part of the pathway are the flavonoid 3' hydroxylase (F3'H) and flavonoid 3', 5' hydroxylase (F3'5'H), both members of the cytochrome P450 class of enzymes.

[0009] F3'H is a key enzyme in the flavonoid pathway leading to the cyanidin-based pigments which, in many plant species contribute to red and pink flower color.

[0010] The next step in the pathway, leading to the production of the colored anthocyanins from the dihydroflavonols (DHK, DHQ, DHM), involves dihydroflavonol-4-reductase (DFR) leading to the production of the leucoanthocyanidins. The leucoanthocyanidins are subsequently converted to the anthocyanidins, pelargonidin, cyanidin and delphinidin. These flavonoid molecules are unstable under normal physiological conditions and glycosylation at the 3-position, through the action of glycosyltransferases, stabilizes the anthocyanidin molecule thus allowing accumulation of the anthocyanins. In general, the glycosyltransferases transfer the sugar moieties from UDP sugars to the flavonoid molecules and show high specificities for the position of glycosylation and relatively low specificities for the acceptor substrates (Seitz and Hinderer, Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants.* Constabel and Vasil (eds.), Academic Press, New York, USA, *5:*49-76, 1988). Anthocyanins can occur as 3-monosides, 3-biosides and 3-triosides as well as 3, 5-diglycosides and 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose (Strack and Wray, In: *The Flavonoids - Advances in Research since 1986.* Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

[0011] Glycosyltransferases involved in the stabilization of the anthocyanidin molecule include UDP glucose: flavonoid 3-glucosyltransferase (3GT), which transfers a glucose moiety from UDP glucose to the 3-*O*-position of the anthocyanidin molecule to produce anthocyanidin 3-*O*-glucoside.

[0012] Many anthocyanidin glycosides exist in the form of acylated derivatives. The acyl groups that modify the anthocyanidin glycosides can be divided into two major classes based upon their structure. The aliphatic acyl groups include malonic acid or succinic acid and the aromatic class includes the hydroxy cinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid and the benzoic acids such as *p*-hydroxybenzoic acid.

[0013] Methylation at the 3' and 5' positions of the B-ring of anthocyanidin glycosides can also occur. Methylation of cyanidin-based pigments leads to the production of peonidin. Methylation of the 3' position of delphinidin-based pigments results in the production of petunidin, whilst methylation of the 3' and 5' positions results in malvidin production. Methylation of malvidin can also occur at the 5-O and 7-O positions to produce capensinin (5-O-methyl malvidin) and 5, 7-di-O-methyl malvidin.

[0014] In addition to the above modifications, pH of the vacuole or compartment where pigments are localized and co-pigmentation with other flavonoids such as flavonols and flavones can affect petal color. Flavonols and flavones can also be aromatically acylated (Brouillard and Dangles, In: *The Flavonoids -Advances in Research since 1986*. Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

[0015] Carnation flowers can produce two types of anthocyanidins, depending on their genotype - pelargonidin and cyanidin. In the absence of F3'H activity, pelargonidin is produced otherwise cyanidin is produced. Pelargonidin is usually accompanied by kaempferol, a colorless flavonol. Cyanidin pigments are usually accompanied by both kaempferol and quercetin. Both pelargonidin and kaempferol are derived from DHK; both cyanidin and quercetin are derived from DHQ (Figure 1).

[0016] The substrate specificity shown by DFR regulates the anthocyanins that a plant accumulates. Petunia and cymbidium DFRs do not reduce DHK and thus they do not accumulate pelargonidin-based pigments (Forkmann and Ruhnau, *Z Naturforsch C.* 42c, 1146-1148, 1987, Johnson *et al, Plant Journal, 19,* 81-85, 1999). Many important floricultural species including iris, delphinium, cyclamen, gentian, cymbidium are

presumed not to accumulate pelargonidin due to the substrate specificity of their endogenous DFRs (Tanaka and Brugliera, 2006, *supra*).

[0017] In carnation, the DFR enzyme is capable of metabolizing two dihydroflavonols to leucoanthocyanidins which are ultimately converted through to anthocyanins - pigments that are responsible for flower color. DHK is converted to leucopelargonidin, the precursor to pelargonidin-based pigments, giving rise to apricot to brick-red colored carnations. DHQ is converted to leucocyanidin, the precursor to cyanidin-based pigments, producing pink to red carnations. Carnation DFR is also capable of converting DHM to leucodelphinidin (Forkmann and Ruhnau, 1987 *supra*), the precursor to delphinidin-based pigments. However, wild-type or classically-derived carnation lines do not contain a F3'5'H enzyme and therefore do not synthesize DHM.

[0018] The petunia DFR enzyme has a different specificity to that of the carnation DFR. It is able to convert DHQ through to leucocyanidin, but it is not able to convert DHK to leucopelargonidin (Forkmann and Ruhnau, 1987 *supra*). It is also known that in petunia lines containing the F3'5'H enzyme, the petunia DFR enzyme can convert the DHM produced by this enzyme to leucodelphinidin which is further modified giving rise to delphinidin-based pigments which are predominantly responsible for blue colored flowers (see Figure 1). Even though the petunia DFR is capable of converting both DHQ and DHM, it is able to convert DHM far more efficiently, thus favoring the production of delphinidin (Forkmann and Ruhnau 1987 *supra*).

[0019] The anthocyanins found in chrysanthemum are generally based on cyanidin. Delphinidin-based pigments are not present due to the lack of a F3'5'H activity and pelargonidin-based pigments are rarely found. It has been suggested that the absence of pelargonidin-based pigments in chrysanthemum is due to the presence of a F3'H activity rather than the DFR specificity. For example, when chrysanthemum petals were fed with a cytochrome P450 inhibitor, pelargonidin-based pigments were detected (Schwinn *et al, Phtochemistry*, *35*:145-150, 1993).

[0020] Roses and gerberas generally accumulate anthocyanins based on cyanidin and/or pelargonidin. Delphinidin-based anthocyanins are generally not found in wild-type or classically derived rose or gerbera flowers primarily due to the absence of F3'5'H activity.

[0021] Nucleotide sequences encoding F3'5'Hs have been cloned (see International Patent Application No. PCT/AU92/00334 incorporated herein by reference and Holton *et al, Nature*, *366*:276-279, 1993 and International Patent Application No. PCT/AU03/01111 incorporated herein by reference). These sequences were efficient in modulating 3', 5' hydroxylation of flavonoids in petunia (see International Patent Application No. PCT/AU92/00334 and Holton *et al,* 1993 *supra*), tobacco (see International Patent Application No. PCT/AU92/00334), carnations (see International Patent Application No. PCT/AU96/00296 incorporated herein by reference) and roses (see International Patent Application No. PCT/AU03/01111).

[0022] Carnations are one of the most extensively grown cut flowers in the world.

[0023] There are thousands of current and past cut-flower varieties of cultivated carnation. These are divided into three general groups based on plant form, flower size and flower type. The three flower types are standards, sprays and midis. Most of the carnations sold fall into two main groups –the standards and the sprays. Standard carnations are intended for cultivation under conditions in which a single large flower is required per stem. Side shoots and buds are removed (a process called disbudding) to increase the size of the terminal flower. Sprays and/or miniatures are intended for cultivation to give a large number of smaller flowers per stem. Only the central flower is removed, allowing the laterals to form a 'fan' of stems.

[0024] Spray carnation varieties are popular in the floral trade, as the multiple flower buds on a single stem are well suited to various types of flower arrangements and provide bulk to bouquets used in the mass market segment of the industry.

[0025] Standard and spray cultivars dominate the carnation cut-flower industry, with approximately equal numbers sold of each type in the USA. In Japan, Spray-type varieties

account for 70% of carnation flowers sold by volume, whilst in Europe spray-type carnations account for approximately 50% of carnation flowers traded through out the Dutch auctions. The Dutch auction trade is a good indication of consumption across Europe.

[0026] Whilst standard and midi –type carnations have been successfully manipulated genetically to introduce new colors (Tanaka and Brugliera, 2006, *supra*; see International Patent Application No. PCT/AU96/00296) , this has not been applied to spray carnations. There is an absence of blue color in color-assortment in carnation, only recently filled through the introduction of genetically-modified standard-type carnation varieties. However, standard-type varieties can not be used for certain purposes, such as bouquets and flower arrangements where a large number of smaller carnation flowers are needed, such as hand-held arrangements, and small table settings.

[0027] One particular spray carnation which is particularly commercially popular is the Kortina Chanel line of carnations (*Dianthus caryophyllus* cv. Kortina Chanel). The variety has excellent growing characteristics and a moderate to good resistance to fungal pathogens such as *Fusarium*. There are a number of varieties which have been released as "sports" of Kortina Chanel. These include Kortina, Royal Red Kortina, Cerise Kortina and Dusty Kortina. However, before the advent of the present invention, purple/blue spray carnations were not available.

SUMMARY

[0028] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0029] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc.

[0030] A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

[0031] The present invention provides genetically modified plants exhibiting altered inflorescence. More particularly, the present invention provides genetically modified carnations and even more particularly genetically modified carnation sprays exhibiting altered inflorescence. The altered inflorescence is a color in the range of red-purple to blue such as purple to blue color in the tissue or organelles including flowers, petals, anthers and styles. In one embodiment, the color is determined using the Royal Horticultural Society (RHS) color chart where colors are arranged in order of the fully saturated colors with the less saturated and less bright colors alongside. The color groups proceed through the observable spectrum and the colors referred to in this application are generally in the red-purple (RHSCC 58-74), purple (RHSCC 75-79), purple-violet (RHSCC 81-82), violet (RHSCC 83-88), violet-blue (89-98), blue (RHSCC 99-110) groups contained in Fan 2. Colors are selected from the range including 61A, 64A, 71A, 71C, 72A, 81A, 86A and 87A and colors in between or proximal thereto.

TABLE 1

Summary of sequence identifiers

SEQ ID NO:	NAME	SPECIES	TYPE OF SEQ	DESCRIPTION
1	*BPF3'5'H#18*.nt	*Viola* sp	nucleotide	*F3'5'H cDNA*
2	*BPF3'5'H#18*.aa	*Viola* sp	amino acid	*deduced F3'5'H amino acid sequence*
3	*SalF3'5'H#47*.nt	*Salvia* sp.	nucleotide	*F3'5'H cDNA*
4	*SalF3'5'H#47*.aa	*Salvia* sp	amino acid	*deduced F3'5'H amino acid sequence*
5	*Pet gen DFR.nt*	*Petunia* sp	nucleotide	*DFR genomic clone*
6	*Pet gen DFR.aa*	*Petunia* sp	amino acid	*deduced DFR amino acid sequence*
7	*CarnANS 5'*	*Dianthus caryophyllus*	nucleotide	*Carnation ANS promoter fragment*
8	*CarnANS 3'*	*Dianthus caryophyllus*	nucleotide	*Carnation ANS terminator fragment*
9	*Cin gF3'5'H.nt*	*Cineraria* sp	nucleotide	*F3'5'H genomic clone*
10	*Cin gF3'5'H.aa*	*Cineraria* sp	amino acid	*deduced F3'5'H amino acid sequence*

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[0032] The modified plants and in particular genetically modified spray carnations comprise genetic sequences encoding at least two F3'5'H molecules and at least one DFR. Insofar as the present invention relates to carnations, the carnation sprays are conveniently in a Kortina Chanel genetic background including sports of Kortina Chanel such as Kortina, Royal Red Kortina, Cerise Kortina and Dusty Kortina. Other carnation cultivars included within the present invention are Cream Cinderella, Cinderella, Cerise Westpearl, Vega, Artisan and Rendezvous.

[0033] Hence, one aspect of the present invention is directed to a genetically modified plant exhibiting altered inflorescence in selected tissue, the plant comprising expressed genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme. The term "altered inflorescence" in this context means compared to the inflorescence of a plant (e.g. parent plant or plant of the same species) prior to genetic manipulation. The term "encoding" includes the expression of the genetic material to produce functional F3'5'H and DFR enzymes.

[0034] In a particular embodiment, the plant is a spray carnation.

[0035] Accordingly, another aspect of the present invention is directed to a spray carnation exhibiting altered inflorescence in selected tissue, the spray carnation comprising expressed genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0036] Yet another, aspect of the present invention is directed to a genetically modified Kortina Chanel spray carnation line or sport thereof exhibiting tissues of a purple to blue color, the carnation comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0037] Another aspect of the present invention is directed to a genetically modified chrysanthemum exhibiting tissues of a purple to blue color, the chrysanthemum comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0038] Still another aspect of the present invention is directed to a genetically modified rose exhibiting tissues of a purple to blue color, the rose comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0039] Even yet another aspect of the present invention is directed to a genetically modified gerbera exhibiting tissues of a purple to blue color, the gerbera comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0040] Yet another aspect of the present invention is directed to a genetically modified ornamental or horticultural plant exhibiting tissues of a purple to blue color, the ornamental or horticultural plant comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0041] In a particular embodiment, the present invention provides a genetically modified spray carnation identified herein as Kortina Chanel (KC)/pCGP2442 and its progeny and sports.

[0042] Progeny, reproductive material, cut flowers, tissue culturable cells and regenerable cells from the genetically plants also form part of the present invention.

[0043] The present invention is also directed to the use of genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme in the manufacture of a genetically modified plant such as a spray carnation including a Kortina Chanel carnation or sports thereof exhibiting altered inflorescence including tissue having a purple to blue color.

[0044] The F3'5'H enzymes may be from any source and the two may come from the same or different plant species. Nucleotide sequences encoding F3'5'H enzymes from *Viola* sp and *Salvia* sp are particularly useful (see Table 1). Similarly, the nucleotide sequence encoding the DFR enzyme may come from any species such as but not limited to *Petunia* sp (e.g. see Table 1), iris, cyclamen, delphinium, gentian, *Cymbidium*. Suitable nucleotide

sequences for F3'5'H from *Viola* sp and *Salvia* sp and a DFR from *Petunia* sp are set forth in Table 1.

BRIEF DESCRIPTION OF THE FIGURES

[0045] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0046] Figure 1 is a schematic representation of the biosynthesis pathway for the flavonoid pigments showing production of the anthocyanidin 3-glucosides that occur in most plants that produce anthocyanins. Enzymes involved in the pathway have been indicated as follows: PAL = Phenylalanine ammonia-lyase; C4H = Cinnamate 4-hydroxylase; 4CL = 4-coumarate:CoA ligase; CHS = Chalcone synthase; CHI = Chalcone flavanone isomerase; F3H = Flavanone 3-hydroxylase; DFR = Dihydroflavonol-4-reductase; ANS = Anthocyanidin synthase, 3GT = UDP-glucose: flavonoid 3-*O*-glucosyltransferase; Other abbreviations include: DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin.

[0047] Figure 2 is a diagrammatic representation of the binary plasmid pCGP2442. chimeric. The construction of pCGP2442 is described in Example 1. Selected restriction endonuclease sites (AscI, PacI, PmeI) are marked. Abbreviations include LB = Left Border from *A. tumefaciens* Ti plasmid, RB = Right border region from *A. tumefaciens* Ti plasmid, TetR = antibiotic, tetracycline resistance gene complex Refer to Table 2 for a description of gene elements.

[0048] Figure 3 is a photographic representation showing flowers from transgenic Kortina Chanel/pCGP2442 spray carnation lines. A: flowers from transgenic line 19890 (left hand side) and Kortina Chanel non-transgenic control (right-hand side); B: flower from transgenic line 19907.

[0049] Figure 4 is a photographic representation showing petal size and shape of Kortina Chanel/pCGP2442 spray carnation lines. A: petals from Kortina Chanel non-transgenic control flower; B: petals from flowers of the transgenic Kortina Chanel/pCGP2442 line 19907; C: petals from flowers of the transgenic Kortina Chanel/pCGP2442 line 19890.

[0050] Figure 5 is a photographic representation showing flower parts of Kortina Chanel/pCGP2442 spray carnation lines A: non-transgenic Kortina Chanel control; B: transgenic line 19907; C: transgenic line 19890. Pollen is visible (arrow).

DETAILED DESCRIPTION

[0051] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes a single plant, as well as two or more plants; reference to "an anther" includes a single anther as well as two or more anthers; reference to "the invention" includes a single aspect or multiple aspects of an invention; and so on.

[0052] The present invention contemplates genetically modified plants such as carnation plants and in particular spray carnations exhibiting altered inflorescence. The altered inflorescence may be in any tissue or organelle including flowers, petals, anthers and styles. Particular inflorescence contemplated herein includes a color in the range of red-purple to blue color such as a purple to blue color. The color determination is conveniently measured against the Royal Horticultural Society (RHS) color chart (RHSCC) and includes colors 81A, 86A, 87A and colors in between or proximal to either end of the above range. The term "inflorescence" is not to be narrowly construed and relates to any colored cells, tissues organelles or parts thereof, as well as flowers and petals.

[0053] Hence, one aspect of the present invention is directed to a genetically modified plant exhibiting altered inflorescence in selected tissue, the plant comprising expressed genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0054] In a particular embodiment, the plant is a carnation. Accordingly, another aspect of the present invention is directed to a spray carnation exhibiting altered inflorescence in selected tissue, the spray carnation comprising expressed genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0055] Whilst the present invention encompasses any spray carnation, a carnation of the Kortina Chanel line is particularly useful including sports thereof. Useful sports of Kortina Chanel include Kortina, Royal Red Kortina, Cerise Kortina and Dusty Kortina.

[0056] Accordingly, another aspect of the present invention is directed to a genetically modified Kortina Chanel spray carnation line or sports thereof exhibiting tissues of a purple to blue color, the carnation comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0057] Even more particularly, the present invention provides a genetically modified Kortina Chanel (KC)/pCGP2442 (also referred to as KC/2442 or Kortina Chanel/2442) line exhibiting altered inflorescence, the line comprising an expressed genetic sequence encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0058] Examples of Kortina Chanel transgenic lines include 19890 and 19907.

[0059] Other genetically modified carnations contemplated herein include the spray carnations Cerise Westpearl, Vega and Artisan and the standard carnations Cream Cinderella, Cinderella and Rendezvous

[0060] Other genetically modified plants contemplated herein include chrysanthemums, roses, gerberas, lisianthus, tulip, lily, geranium, petunia, iris, *Torenia*, *Begonia*, *Cyclamen*, *Nierembergia*, *Catharanthus*, *Pelargonium*, orchid, grape, apple, *Euphorbia* or *Fuchsia* and other ornamental or horticultural plants

[0061] Another aspect of the present invention is directed to a genetically modified chrysanthemum exhibiting tissues of a purple to blue color, the chrysanthemum comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0062] Still another aspect of the present invention is directed to a genetically modified rose exhibiting tissues of a purple to blue color, the rose comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0063] Yet another aspect of the present invention is directed to a genetically modified gerbera exhibiting tissues of a purple to blue color, the gerbera comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0064] Yet another aspect of the present invention is directed to a genetically modified ornamental or horticultural plant exhibiting tissues of a purple to blue color, the ornamental or horticultural plant comprising expressed genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme.

[0065] The genetic sequence may be a single construct carrying the nucleotide sequences encoding the F3'5'H enzymes and the DFR enzyme or multiple genetic constructs may be employed. In addition, the genetic sequences may be integrated into the genome of a plant cell or it may be maintained as an extra-chromosomal artificial chromosome. Still furthermore, the generation of a spray carnation expressing two F3'5'H enzymes and at least one DFR enzyme may be generated by recombinant means alone or by a combination of conventional breeding and recombinant DNA manipulation. The genetic sequences are "expressed" in the sense of being operably linked to a promoter and other regulatory sequences resulting in transcription and translation to produce F3'5'H and DFR enzymes.

[0066] Hence, another aspect of the present invention contemplates a method for producing a genetically modified plant such as a spray carnation exhibiting altered inflorescence, the method comprising introducing into regenerable cells of a plant such as a spray carnation plant expressible genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme and regenerating a plant therefrom or obtaining progeny from the regenerated plant.

[0067] The plant may then undergo various generations of growth or cultivation. Hence, reference to a genetically modified spray carnation includes progeny thereof and sister lines thereof as well as sports thereof.

[0068] Another aspect of the present invention provides a method for producing a genetically modified plant such as a spray carnation line exhibiting altered inflorescence,

the method comprising selecting a plant such as a spray carnation comprising expressible genetic material encoding one of at least two F3'5'H enzymes or at least one DFR enzyme and crossing this plant with another plant such as a spray carnation comprising genetic material encoding the other of at least two F3'5'H enzymes or at least one DFR enzyme and then selecting F1 or subsequent generation plants which express the genetic material.

[0069] Nucleotide sequences encoding F3'5'H and DFR enzymes may be from any source including *Viola* sp, *Petunia* sp, *Salvia* sp, *Lisianthus* sp, *Gentiana* sp, *Sollya* sp, *Clitoria* sp, *Kennedia* sp, *Campanula* sp, *Lavandula* sp, *Verbena* sp, *Torenia* sp, *Delphinium* sp, *Solanum* sp, *Cineraria* sp, *Vitis* sp, *Babiana stricta*, *Pinus* sp, *Picea* sp, *Larix* sp, *Phaseolus* sp, *Vaccinium* sp, *Cyclamen* sp, *Iris* sp, *Pelargonium* sp, Liparieae, *Geranium* sp, *Pisum* sp, *Lathyrus* sp, *Catharanthus* sp, *Malvia* sp, *Mucuna* sp, *Vicia* sp, *Saintpaulia* sp, *Lagerstroemia* sp, *bouchina* sp, *Plumbago* sp, *Hypocalyptus* sp, *Rhododendron* sp, *Linum* sp, *Macroptilium* sp, *Hibiscus* sp, *Hydrangea* sp, *Cymbidium* sp, *Millettia* sp, *Hedysarum* sp, *Lespedeza* sp, *Asparagus* sp, *Antigonon* sp, *Pisum* sp, *Freesia* sp, *Brunella* sp or *Clarkia* sp, etc. Both F3'5'H enzymes may come from the same plant species or different plant species. For example, in one embodiment, the two F3'5'H enzymes come from *Viola* sp and *Salvia* sp. The DFR may come again from the same or different plant species.

[0070] The present invention further contemplates the use of genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme in the manufacture of a spray carnation such as a Kortina Chanel carnation or sports thereof exhibiting altered inflorescence including tissue having a purple to blue color.

[0071] In another embodiment, the present invention contemplates the use of genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme in the manufacture of a genetically modified plant selected from a rose, chrysanthemum, gerbera tulip, lily, orchid, lisianthus, begonia, torenia, geranium, petunia, nierembergia, pelargonium, iris, impatiens, cyclamen grape, apple, *Euphorbia* or *Fuchsia* or other ornamental or horticultural thereof exhibiting altered inflorescence including tissue having a purple to blue color.

[0072] Cut flowers, tissue culturable cells, regenerable cells, parts of plants, seeds, reproductive material (including pollen) are all encompassed by the present invention.

[0073] As indicated above, nucleotide sequences encoding F3'5'H and DFR enzymes may all come from the same species of plant or from two or more different species. F3'5'H nucleotide sequences from *Viola* sp and *Salvia* sp and a DFR from a *Petunia* sp are particularly useful in the practice of the present invention. The nucleotide sequences encoding the F3'5'H enzymes and the DFR enzyme and the respective amino acid sequences are defined in Table 1.

[0074] Nucleic acid molecules encoding F3'5'Hs are also provided in International Patent Application No. PCT/AU92/00334 and Holton *et al*, 1993 *supra*. These sequences have been used to modulate 3',5' hydroxylation of flavonoids in petunia (see International Patent Application No. PCT/AU92/00334 and Holton *et al*, 1993 *supra*), tobacco (see International Patent Application No. PCT/AU92/00334) and carnations (see International Patent Application No. PCT/AU96/00296). Nucleotide sequences of F3'5'H from other species such as *Viola*, *Salvia* and *Sollya* have been cloned (see International Patent Application No. PCT/AU03/01111). Any of these sequences may be used in combination with a promoter and/or terminator. The present invention particularly contemplates F3'5'H encoded by SEQ ID NOs:1, 3 and 9 and a DFR encoded by SEQ ID NO:5 or a nucleotide sequence capable of hybridizing to any of SEQ ID NOs:1, 3, 5 or 9 or a complementary form thereof under low or high stringency conditions or which has at least about 70% identity to SEQ ID NO:1 or 3 or 5 or 9 after optimal alignment. Terminator sequence SEQ ID NO:8 and promoter sequence SEQ ID NO:7 are also encompassed herein as well as sequences which hybridize thereto or have at least 70% similarity thereto

[0075] For the purposes of determining the level of stringency to define nucleic acid molecules capable of hybridizing to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or their complementary forms, low stringency includes and encompasses from at least about 0% to at least about 15% v/v formamide and from at least about 1M to at least about 2 M salt for hybridization, and at least about 1 M to at least

about 2 M salt for washing conditions. Generally, low stringency is from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace the inclusion of formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J. Mol. Biol. 5:*109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem. 46:*83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 1.0% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 1.0% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

[0076] Reference to at least 70% identity includes 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, ,94, 95, 96, 97, 98, 99 and 100% identity. The comparison may also be made at the level of similarity of amino acid sequences of SEQ ID NO:s:2, 4, 6 or 10. Hence, nucleic acid molecules are contemplated herein which encode an F3'5'H enzyme or DFR having at least 70% similarity to the amino acid sequence set forth in SEQ ID NOs:2 or 4 or 6 or 10. Again, at least 70% similarity includes 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% similarity or identity.

[0077] The nucleic acid molecule encoding the F3'5'H enzymes and the DFR includes one or more promoters and/or terminators. In one embodiment, a promoter is selected which directs expression of a F3'5'H and/or a DFR nucleotide sequence in tissue having a higher pH.

[0078] In an embodiment, the promoter sequence is native to the host carnation plant to be transformed or may be derived from an alternative source, where the region is functional in the host plant. Other sources include the *Agrobacterium* T-DNA genes, such as the promoters for the genes encoding enzymes for biosynthesis of nopaline, octapine, mannopine, or other opines; promoters from plants, such as promoters from genes encoding ubiquitin; tissue specific promoters (see, e.g, US Patent No. 5,459,252 to Conkling *et al*; WO 91/13992 to Advanced Technologies); promoters from plant viruses (including host specific viruses), or partially or wholly synthetic promoters. Numerous promoters that are potentially functional in carnation plants (see, for example, Greve, *J. Mol. Appl. Genet. 1*:499-511, 1983; Salomon *et al*, *EMBO, J. 3*:141- 146, 1984; Garfinkel *et al*, *Cell 27*:143-153, 1983; Barker *et al*, *Plant Mol. Biol. 2*:235-350, 1983); including various promoters isolated from plants (such as the Ubi promoter from the maize obi-1 gene, see, e.g, US Patent No. 4,962,028) and viruses (such as the cauliflower mosaic virus promoter, *CaMV 35S*). In other embodiments the promoter is *AmCHS 5'*, *carnANS 5'* and/or *petDFR 5'* (from *Pet gen DFR*) with corresponding terminators *petD8 3'*, *carn ANS 3'* and *petDFR 3'* (from *Pet gen DFR*), respectively.

[0079] The promoter sequences may include cis-acting sequences which regulate transcription, where the regulation involves, for example, chemical or physical repression or induction (e.g, regulation based on metabolites, light, or other physicochemical factors; see, e.g, WO 93/06710 disclosing a nematode responsive promoter) or regulation based on cell differentiation (such as associated with leaves, roots, seed, or the like in plants; see, e.g. US. Patent Number. 5,459,252 disclosing a root-specific promoter).

[0080] Other cis-acting sequences which may be employed include transcriptional and/or translational enhancers. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences.

[0081] The nucleic acid molecule(s) encoding at least two F3'5'H enzymes and at least one DFR, in combination with suitable promoters and/or a terminators is/are used to modulate the activity of a flavonoid molecule in a spray carnation. Reference herein to modulating

the level of a delphinidin-based molecule relates to an elevation or reduction in levels of up to 30% or more particularly of 30-50%, or even more particularly 50-75% or still more particularly 75% or greater above or below the normal endogenous or existing levels of activity.

[0082] The term "inflorescence" as used herein refers to the flowering part of a plant or any flowering system of more than one flower which is usually separated from the vegetative parts by an extended internode, and normally comprises individual flowers, bracts and peduncles, and pedicels. As indicated above, reference to a "transgenic plant" may also be read as a "genetically modified plant" and includes a progeny or hybrid line ultimately derived from a first generation transgenic plant.

[0083] The present invention also contemplates the use of genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme in the manufacture of a spray carnation such as a Kortina Chanel carnation or Cerise Westpearl carnation or sports thereof exhibiting altered inflorescence including tissue having a purple to blue color.

[0084] A cultivation business model is also provided, the model comprising generating a genetically modified spray carnation plant as described herein, providing platelets, seeds, regenerable cells, tissue culturable cells or other material to a grower, generating commercial sale numbers of plants, and providing cut flowers to retailers or wholesalers.

[0085] The present invention is further described by the following non-limiting Examples. In these Examples, materials and methods as outlined below were employed:

[0086] Methods followed were as described in Sambrook *et al, Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, 1989 or Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* 3rd edition, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, 2001 or *Plant Molecular Biology Manual* (2nd edition), Gelvin and Schilperoot (eds), Kluwer Academic Publisher, The Netherlands, 1994 or *Plant Molecular Biology Labfax*, Croy (ed), Bios scientific Publishers, Oxford, UK, 1993.

[0087] The cloning vectors pBluescript and PCR script were obtained from Stratagene, USA. pCR7 2.1 was obtained from Invitrogen, USA.

E. coli transformation

[0088] The *Escherichia coli* strains used were:

DH5α

supE44, Δ (lacZYA-ArgF)U169, (ø80lacZΔM15), hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, deoR. (Hanahan, *J. Mol. Biol. 166:*557, 1983)

XL1-Blue

supE44, hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, lac$^-$,[F'proAB, lacI^q, lacZΔM15, Tn10(tet^R)] (Bullock *et al, Biotechniques 5:*376, 1987).

BL21-CodonPlus-RIL strain

ompT hsdS(Rb- mB-) *dcm*+ Tet^r *gal endA* Hte [*argU ileY leuW* Cam^r]

M15 *E. coli* is derived from *E. coli* K12 and has the phenotype Nal^s, Str^s, Rif^s, Thi$^-$, Ara$^+$, Gal$^+$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$.

[0089] Transformation of the *E. coli* strains was performed according to the method of Inoue *et al,* (*Gene 96:*23-28, 1990).

Agrobacterium tumefaciens strains and transformations

[0090] The disarmed *Agrobacterium tumefaciens* strain used was AGL0 (Lazo *et al, Bio/technology 9:*963-967, 1991).

[0091] Plasmid DNA was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL LB culture (Sambrook *et al,* 1989 *supra*) and incubation for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% (v/v) 100 mM $CaCl_2$/15% (v/v) glycerol. The DNA-*Agrobacterium* mixture was frozen by

incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells were then mixed with 1 mL of LB (Sambrook *et al,* 1989 *supra*) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying the plasmid were selected on LB agar plates containing appropriate antibiotics such as 50 μg/mL tetracycline or 100 μg/mL gentamycin. The confirmation of the plasmid in *A. tumefaciens* was done by restriction endonuclease mapping of DNA isolated from the antibiotic-resistant transformants.

DNA ligations

[0092] DNA ligations were carried out using the Amersham Ligation Kit or Promega Ligation Kit according to procedures recommended by the manufacturer.

Isolation and purification of DNA fragments

[0093] 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.

Repair of overhanging ends after restriction endonuclease digestion

[0094] Overhanging 5' ends were repaired using DNA polymerase I Klenow fragment according to standard protocols (Sambrook *et al,* 1989 *supra*). Overhanging 3' ends were repaired using Bacteriophage T4 DNA polymerase according to standard protocols (Sambrook *et al,* 1989 *supra*).

Removal of phosphoryl groups from nucleic acids

[0095] Shrimp alkaline phosphatase (SAP) [USB] was typically used to remove phosphoryl groups from cloning vectors to prevent re-circularization according to the manufacturer's recommendations.

Polymerase Chain Reaction (PCR)

[0096] 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 minutes at 94°C, followed by 35 cycles of 94°C for 20 sec, 50°C for 30 sec and 72°C for 1 minute with a final treatment at 72°C for 10 minutes before storage at 4°C.

[0097] PCRs were performed in a Perkin Elmer GeneAmp PCR System 9600.

^{32}P-Labeling of DNA Probes

[0098] DNA fragments (50 to 100 ng) were radioactively labeled with 50 μCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on Sephadex G-50 (Fine) columns or Microbiospin P-30 Tris chromatography columns (BioRad).

Plasmid Isolation

[0099] Single colonies were analyzed for inserts by inoculating LB broth (Sambrook *et al,* 1989 *supra*) with appropriate antibiotic selection (e.g. 100 μg/mL ampicillin or 10 to 50 μg/mL tetracycline etc.) and incubating the liquid culture at 37°C (for *E. coli*) or 29°C (for *A. tumefaciens*) for ~16 hours with shaking. Plasmid DNA was purified using the alkali-lysis procedure (Sambrook *et al,* 1989 *supra*) or using The WizardPlus SV minipreps DNA purification system (Promega) or Qiagen Plasmid Mini Kit (Qiagen). Once the presence of an insert had been determined, larger amounts of plasmid DNA were prepared from 50 mL overnight cultures using the alkali-lysis procedure (Sambrook *et al,* 1989 *supra*) or QIAfilter Plasmid Midi kit (Qiagen) and following conditions recommended by the manufacturer.

DNA Sequence Analysis

[0100] DNA sequencing was performed using the PRISM (trademark) 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 University of Queensland, St Lucia, Brisbane, Australia and at The Walter and Eliza Hall Institute of

Medical Research (Melbourne, Australia) or on an automated 373A DNA sequencer (Applied Biosystems).

[0101] Sequences were analysed using a MacVector (Trade mark) application (version 9.5.2 and earlier) [MacVector Inc, Cary, North Carolina, USA].

[0102] Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85(8):*2444-2448, 1988) or BLAST programs (Altschul *et al, J. Mol. Biol. 215(3):*403-410, 1990). Percentage sequence similarities were obtained using LALIGN program (Huang and Miller, *Adv. Appl. Math. 12:*373-381, 1991) or ClustalW program (Thompson *et al, Nucleic Acids Research 22:*4673-4680, 1994) within the MacVector (Trade mark) application (MacVector Inc, USA) using default settings.

[0103] Multiple sequence alignments were produced using ClustalW (Thompson *et al,* 1994 *supra*) using default settings.

Plant transformations

[0104] Plant transformations were as described in International Patent Application No. PCT/US92/02612 incorporated herein by reference or International Patent Application No. PCT/AU96/00296 or Lu *et al, Bio/Technology 9*:864-868, 1991. Other methods may also be employed.

[0105] Cuttings of *Dianthus caryophyllus* cv. Kortina Chanel were obtained from Van Wyk and Son Flower Supply, Victoria or Propagation Australia, Queensland, Australia.

Transgenic Analysis

Color coding

[0106] The Royal Horticultural Society's Color Charts, Third and/or Fifth edition (London, UK), 1995 and/or 2007 were used to provide a description of observed color.

They provide an alternative means by which to describe the color phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colors and should not be regarded as limiting the possible colors which may be obtained.

[0107] Carnation petals consist of 3 zones, the claw, corona and limb (Glimn-Lacy and Kaufman, Botany Illustrated, Introduction to Plants, Major Groups, Flowering Plant Families, 2nd ed, Springer, USA, 2006). In general only the petal limb is colored with the claw being a green color and the corona a white shade (see Figure 4). Reference to carnation petal/flower/inflorescence color generally relates to the color of the carnation petal limb.

Chromatographic analysis

[0108] Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analysis was performed generally as described in Brugliera *et al,* (*Plant J. 5*:81-92, 1994).

[0109] In general TLC and HPLC analysis was performed on extracts isolated from the petal limbs.

Extraction of anthocyanidins

[0110] Prior to HPLC analysis, the anthocyanin and flavonol molecules present in petal limbextracts were acid hydrolysed to remove glycosyl moieties from the anthocyanidin or flavonol core. Anthocyanidin and flavonol standards were used to help identify the compounds present in the floral extracts.

[0111] Anthocyanidins in the reaction mixture were analysed by HPLC *via* gradient elution using gradient conditions of 50%B to 60%B over 10 minutes, then 60% B for 10 minutes and finally 60% B to 100% B over 5 minutes where solvent A consisted of TFA:H_2O (5:995) and solvent B consisted of acetonitrile: TFA:H_2O (500:5:495). An Asahi Pac ODP-50 cartridge column (250 mm x 4.6 mm ID) was used for the reversed phase chromatographic separations. The flow rate was 1 mL/min and the temperature was 40°C.

The detection of the anthocyanidin compounds was carried out using a Shimadzu SPD-M6A three dimensional detector at 400-650 nm.

[0112] The anthocyanidin peaks were identified by reference to known standards, *viz* delphinidin, petunidin, malvidin, cyanidin and peonidin

Stages of flower development

[0113] Carnation flowers were harvested at developmental stages defined as follows:

Stage 1:	Closed bud, petals not visible.
Stage 2:	Flower buds opening: tips of petals visible.
Stage 3:	Tips of nearly all petals exposed. "Paint-brush stage".
Stage 4:	Outer petals at 45° angle to stem.
Stage 5:	Flower fully open.

[0114] For TLC or HPLC analysis, petal limbs were collected from stage 4 flowers at the stage of maximum pigment accumulation.

[0115] For Northern blot analysis, petals were collected from stage 3 flowers at the stage of maximal expression of flavonoid pathway genes.

EXAMPLE 1

Preparation of chimeric F3'5'H gene constructs

[0116] A summary of promoter, terminator and coding fragments used in the preparation of constructs and the respective abbreviations is listed in Table 2.

TABLE 2

Abbreviations used in construct preparations

ABBREVIATION	DESCRIPTION
35S 5'	promoter fragment from *CaMV 35S* gene (Franck *et al,* 1980 *supra*) with an ~60bp 5' untranslated leader sequence (CabL) from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) [Harpster *et al, MGG*, 212:182-190, 1988]
AmCHS 5'	Promoter fragment from the *Antirrhinum majus* chalcone synthase (*CHS*) gene which includes 1.2kb sequence 5' of the translation initiation site (Sommer and Saedler, *Mol Gen. Gent.*, 202:429-434, 1986)
BPF3'5'H#18	*Viola* (Black Pansy) *F3'5'H* cDNA clone #18 (International Patent Application No. PCT/AU03/01111) [SEQ ID NO:1]
BPF3'5'H#40	*Viola* (Black Pansy) *F3'5'H* cDNA clone #40 (International Patent Application No. PCT/AU03/01111 incorporated herein by reference)
CaMV 35S	~0.2 kb incorporating *Bgl*II fragment containing the promoter region from the Cauliflower Mosaic Virus 35S (*CaMV 35S*) gene - (Franck *et al, Cell* 21:285-294, 1980, Guilley *et al, Cell*, 30:763-773. 1982)
carnANS 3'	Terminator sequence of anthocyanidin synthase gene (*ANS*) from *Dianthus caryophyllus* (See International Patent Application No. PCT/GB99/02676 incorporated herein by reference) [SEQ ID NO:8]

ABBREVIATION	DESCRIPTION
carnANS 5'	Promoter sequence of anthocyanidin synthase (*ANS*) gene from *Dianthus caryophyllus* (See International Patent Application No. PCT/GB99/02676) [SEQ ID NO:7]
Pet CHS-A 5'	Promoter region of the *Petunia hybrida CHS-A* gene (Van der Meer *et al, Plant Mol Biol* 15:95-109, 1990)
Pet gen DFR	~5.3kb Petunia *DFR-A* genomic clone with it's own promoter and terminator (SEQ ID NO:5)
petD8 3'	~0.7kb terminator region from a phospholipid transfer protein gene (*D8*) of *Petunia hybrida* cv. OGB includes a 150bp untranslated region of the transcribed region of *PLTP* gene (Holton, Isolation and characterization of petal-specific genes from *Petunia hybrida*. PhD Thesis, University of Melbourne, 1992)
petHf1	Petunia *F3'5'H Hf1* cDNA clone (Holton *et al,* 1993 *supra*)
SalviaF3'5'H#2	*Salvia F3'5'H* cDNA clone #2 (International Patent Application No. PCT/AU03/01111 incorporated herein by reference)
SalviaF3'5'H#47	*Salvia F3'5'H* cDNA clone #47 (International Patent Application No. PCT/AU03/01111) [SEQ ID NO:3]
SollyaF3'5'H	*Sollya F3'5'H* cDNA clone (International Patent Application No. PCT/AU03/01111)
SuRB	Herbicide (Chlorsulfuron)-resistance gene (encodes Acetolactate Synthase) with its own terminator (*tSuRB*) from *Nicotiana tabacum* (Lee *et al, EMBO J.* 7:1241-1248, 1988)
RoseCHS 5'	~2.8kb fragment containing the promoter region from a *CHS* gene of *Rosa hybrida* (see International Patent Application No. PCT/AU03/01111 incorporated herein by reference)
nos 3'	Terminator region from the nopaline synthase (*nos)* gene of *A. tumefaciens* (Depicker *et al, J Mol. and Appl. Genetics 1*:561-573, 1982)

[0117] Kortina Chanel is a light pink colored (RHS color codes 65A, 65A, 65C, 73B, 73C, 73D) carnation of the spray type. It typically accumulates cyanidin-based pigments (0.08mg/g petal fresh weight) and a mixture of quercetin and kaempferol flavonols (5.3 mg/g and 4.6 mg/g petal fresh weight respectively). Kortina Chanel can also sport to darker purple-pink colored flower (RHS color codes 64A, 61A, 71A) [Kortina] with cyanidin derivatives typically accumulating to levels 10 fold higher than the original.

[0118] In order to produce novel purple/blue flowers in the spray carnation background of Kortina Chanel, a number of binary vector constructs were prepared utilizing the petunia, pansy, salvia, sollya and butterfly pea F3'5'H cDNA fragments and various promoter and terminator fragments. The chimeric F3'5'H genes had proved successful in other carnations and petunia leading to detectable intact F3'5'H transcripts (as detected by Northern blot analysis) and to the production of delphinidin or delphinidin-based molecules pigments. Table 3 summarizes the list of binary vector constructs containing F3'5'H cDNA fragments.

TABLE 3

Summary of chimeric F3'5'H and DFR gene expression cassettes contained in binary vector constructs used in the transformation of Kortina Chanel (see Table 2 for an explanation of abbreviations)

PLASMID	*F3'5'H & DFR* GENE(S)	SELECTABLE MARKER GENE
pCGP1452	*AmCHS 5':petHf1:petD8 3'*	*35S 5':SuRB*
pCGP1972	*AmCHS 5':BPF3'5'H#18:petD8 3'*	*35S 5':SuRB*
pCGP1973	*AmCHS 5':BPF3'5'H#40:petD8 3'*	*35S 5':SuRB*
pCGP1991	*AmCHS 5':BPF3'5'H#40:ocs 3'; Pet gen DFR*	*35S 5':SuRB*
pCGP2121	*AmCHS 5':SalviaF3'5'H#2:petD8 3'*	*35S 5':SuRB*
pCGP2122	*AmCHS 5':SalviaF3'5'H#47:petD8 3'*	*35S 5':SuRB*
pCGP2130	*AmCHS 5':SollyaF3'5'H:petD8 3'*	*35S 5':SuRB*
pCGP2205	*carnANS 5':BPF3'5'H#18:carnANS 3'*	*35S 5':SuRB*

The transformation vectors, pCGP1452, pCGP1972, pCGP1973, pCGP2121, pCGP2122, pCGP2130

[0119] The preparation of the transformation vectors pCGP1452, pCGP1972, pCGP1973, pCGP2121, pCGP2122, pCGP2130 are described in International Patent Application No. PCT/AU03/01111.

The transformation vector, pCGP1991

[0120] The transformation vector pCGP1991 contains the *AmCHS 5': BPF3'5'H#40:ocs 3'* expression cassette and the petunia genomic *DFR-A* gene along with the *35S 5':SuRB* selectable marker gene.

Construction of the intermediate plasmid, pCGP1472 (petunia DFR-A genomic clone)

[0121] A genomic library was made from *Petunia hybrida* cv. Old Glory Blue DNA in the vector λ2001 (Holton, 1992 *supra*). Approximately 200, 000 pfu were plated out on NZY plates, lifts were taken onto NEN filters and the filters were hybridized with 400, 000

cpm/mL of ^{32}P-labeled petunia *DFR-A* cDNA fragment (described in Brugliera *et al,* 1994, *supra*). Hybridizing clones were purified, DNA was isolated from each and mapped by restriction endonuclease digestion. A 13 kb *Sac*I fragment of one of these clones was isolated and ligated with *Sac*I ends of pBluescriptII to create the plasmid pCGP1472. Finer mapping indicated that an ~5.3 kb *Bgl*II fragment contained the entire petunia *DFR-A* gene (Beld *et al, .Plant Mol. Biol. 13*:491-502, 1989).

Construction of intermediate plasmid, pCGP1984 (pet gen DFR:35S 5':SuRB)

[0122] The plasmid pCGP1984 contains the petunia genomic *DFR-A* gene (*Pet gen DFR*) along with the *35S 5':SuRB* selectable marker gene in the pWTT2132 (DNAP) backbone.

[0123] The plasmid pCGP1472 (described above) was digested with the restriction endonuclease (RE) *Bgl*II to release a ~5.3kb fragment containing the entire petunia *DFR-A* gene. The resulting 5'-overhang was repaired using DNA Polymerase I (Klenow fragment) according to standard protocols (Sambrook *et al,* 1989 *supra*). The vector pWTT2132 (DNAP) was digested with the RE *Kpn*I to linearize the vector. The resulting overhanging 3'-ends of the vector were removed with Bacteriophage T4 DNA polymerase according to standard protocols (Sambrook *et al,* 1989 *supra*). The 5.3kb fragment containing the petunia *DFR-A* gene was ligated with the repaired *Kpn*I ends of the vector pWTT2132. Successful ligation of the fragment in the desired orientation in pWTT2132 was established by RE digestion of plasmid DNA isolated from tetracycline resistant *E. coli* transformants. The resulting plasmid was designated as pCGP1984.

Construction of intermediate plasmid, 1971 (AmCHS 5': BPF3'5'H#40:petD8 3')

[0124] The plasmid pCGP1971 contains a chimeric gene consisting of *AmCHS 5': BPF3'5'H#40:petD8 3'* in a pBluescript backbone. Construction of pCGP1971 is described in International Patent Application No. PCT/AU03/01111.

Construction of the transformation vector pCGP1991 (AmCHS 5': BPF3'5'H#40:petD8 3'; Pet gen DFR; 35S 5':SuRB)

[0125] The plasmid pCGP1971 was digested with REs *Eco*RV/*Not*I to release a 3.5kb fragment containing a chimeric *AmCHS 5':BPF3'5'H#40:petD8 3'* gene. The resulting 5'-

overhang was repaired using DNA Polymerase I (Klenow fragment) according to standard protocols (Sambrook *et al,* 1989 *supra*). The fragment was purified and ligated with repaired *Pst*I ends of the linearized plasmid pCGP1984 (described above). Correct insertion of the *AmCHS 5':BPF3'5'H#40:petD8 3'* fragment in pCGP1984 was established by restriction endonuclease digestion of plasmid DNA isolated from tetracycline resistant *E. coli* transformants. The resulting transformation vector was designated as pCGP1991.

The transformation vector pCGP2205 (carnANS 5': BPF3'5'H#18:carnANS 3')

[0126] The transformation vector pCGP2205 contains a chimeric *carnANS 5': BPF3'5'H#18:carnANS 3'* gene cassette in tandem with the *35S 5':SuRB* selectable marker gene cassette of the plasmid pCGP1988 (see International Patent Application No. PCT/AU03/01111).

Construction of intermediate plasmid, pCGP787. Isolation of carnation ANS genomic clone

[0127] A genomic DNA library was constructed from *Dianthus caryophyllus* cv. Laguna DNA in EMBL 3 lambda vector (STRATAGENE) using a *Sau*3A partial digest. The digested genomic DNA was size fractionated on a glycerol density gradient and fractions containing DNA fragments ranging from 15 to 20kb were pooled and ligated with *Bam*HI ends of EMBL3 λ vector. The ligation mixture was packaged using Gigapack XL Gold (PROMEGA). The total size of the library was 1 x 10^6 recombinants.

[0128] A total of 300, 000 pfu of the Laguna genomic library were screened in duplicate with ^{32}P-labeled fragments of carnation *ANS* cDNA clone from pCGP786 (see International Patent Application No. PCT/AU96/00296). Hybridization was carried using high stringency conditions (42°C, 50% formamide, 0.1%PVP, 0.1%BSA, 0.1%ficoll, 1%SDS, 100μg/mL denatured herrung sperm DNA. Washes were 2 x SSC/1%SDS and 0.2 x SSC/1%SDS at 65°C. A genomic clone (4.3) was subsequently purified and further characterized. An 8 kb *Hin*dIII fragment of the genomic clone was subcloned into pBluescript KS for further analysis. The resulting plasmid was designated pCGP787.

Construction of intermediate plasmid, pCGP1274 (ANS promoter fragment)

[0129] A ~2.5 kb fragment containing promoter sequences from the carnation *ANS* gene was released from pCGP787 (described above) upon digestion with the REs *Eco*RI and *Bam*HI. The fragment was purified and ligated with *Eco*RI/*Bam*HI ends of pBluescript KS to give the plasmid pCGP793. This 2.5kb fragment included 200bp of *ANS* coding sequence. The 3' end of the promoter fragment was then amplified by PCR using primers that introduced an *Xba*I site 5' upstream from the AUG codon. This 700 bp PCR fragment was then digested with REs *Nde*I/*Xba*I and ligated with *Nde*I/*Xba*I ends of pCGP793 (described above) to produce a 2.3kb *ANS* promoter fragment without the 200bp of *ANS* coding sequence. This new plasmid was designated as pCGP1274.

Construction of intermediate plasmid, pCGP1275 (carnANS 5': carnANS 3' cassette)

[0130] The plasmid pCGP795 (described above) was digested with the REs *Ecl*136II/*Xba*I to release a 0.7 kb *ANS* terminator fragment. The fragment was purified and ligated with *Apa*I (blunted)/*Xba*I ends of pCGP1274 to produce the plasmid pCGP1275 (containing promoter and terminator sequences of the carnation *ANS* gene (*carn ANS 5': carn ANS 3'*).

Construction of intermediate plasmid, pCGP2150 (carnANS 5': BPF3'5'H#18:carnANS 3' cassette)

[0131] The plasmid pCGP1959 (containing a *BPF3'5'H#18* cDNA clone) [described in see International Patent Application No. PCT/AU03/01111] was digested with the REs *Bam*HI and *Asp*718. The ends were repaired and the purified *BPF3'5'H#18* bearing fragment was ligated with the repaired *Pst*I/ *Xba*I ends of pCGP1275 (containing *carnANS 5':carn ANS 3'*) to produce the plasmid, pCGP2150 (containing the *carnANS 5': BPF3'5'H#18:carnANS 3'* expression cassette).

Construction of the transformation vector, pCGP2205

[0132] The chimeric *carnANS 5': BPF3'5'H#18:carnANS 3'* gene was released from pCGP2150 (described above) upon digestion with the RE *Cla*I. The *Cla*I ends of the ~4.8kb fragment were repaired and the fragment was isolated, purified and ligated with repaired *Asp*718 I ends of the plasmid pCGP1988 (see International Patent Application No. PCT/AU03/01111). Correct insertion of *carnANS 5': BPF3'5'H#18:carnANS 3'* gene in a

tandem orientation with respect to the *35S 5':SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The transformation vector was designated as pCGP2205.

[0133] Results of transgenic analysis of petals from Kortina Chanel carnations carrying the gene expression cassettes of Table 3 are shown in Table 4.

TABLE 4

Results of transgenic analysis of petals from Kortina Chanel carnations transformed with T-DNAs containing F3'5'H gene expression cassettes.

transgenes	pCGP	#tg	TLC +	HPLC +	Highest % del	RHSCC	RHSCC Group
AmCHS 5':petHf1:petD8 3'	1452	104	41/64	*nd*	3.5%	No change	Red-Purple
AmCHS 5':BP F3'5'H #18:petD8 3'	1972	26	18/20	12/12	25%	71a	Red-Purple
AmCHS 5':BP F3'5'H #40:petD8 3'	1973	26	11/15	7/8	18%	71a, 74a	Red-Purple
AmCHS 5':BP F3'5'H #40:ocs 3'; Pet gen DFR	1991	22	13/19	*3/3*	14%	74a	Red-Purple
AmCHS 5':Salvia F3'5'H #2:petD8 3'	2121	22	2/16	3/4	12.5%	74a	Red-Purple
AmCHS 5':Salvia F3'5'H #47:petD8 3'	2122	23	6/12	8/8	29%	74a	Red-Purple
AmCHS 5':Sollya F3'5'H:petD8 3'	2130	30	22/27	17/17	35%	61a, 71a	Red-Purple
carnANS 5':BP F3'5'H #18:carnANS 3'	2205	37	22/33	*14/15*	52%	74a	Red-Purple

Transgenes	=	chimeric *F3'5'H and DFR* nucleotide sequences contained on the T-DNA
pCGP	=	plasmid pCGP identification number of the transformation vector used in the transformation experiment (refer to Table 3 for details)
#tg	=	total number of transgenic carnation lines produced
TLC+	=	number of individual events in which delphinidin was detected in petals (as determined visually after TLC of petal extracts) over the total number of individual events analyzed
HPLC+	=	number of individual events in which delphinidin was detected in petals (as determined by HPLC) over the total number of individual events analyzed
Highest % del	=	Highest % delphinidin detected in the petals for the population of transgenic events
nd	=	not done
RHSCC	=	The petal color of the flower with the highest delphinidin levels using color codes according to Royal Horticultural Society Color Chart

[0134] The results suggest that all of the *F3'5'H* sequences evaluated were stable in carnation and resulted in the production of novel delphinidin-based pigments in carnation flowers. However none of the constructs led to the production of >80% delphinidin or a novel commercially viable color change in the Kortina Chanel spray carnation variety. The color changes were all in the Red-Purple group of the RHS Color Chart.

[0135] Although over 290 transgenic Kortina Chanel spray carnations were produced (Table 4) none produced flowers with a change in color to purple/blue. The most dramatic change in color was to a red/purple color (RHS color codes 74a, 78a) that was not a commercially viable novel color. Although delphinidin-based pigments were produced they were at too low a level to modify the color to a commercially viable novel Violet, Purple-Violet, Purple/Blue.

[0136] Even the use of a chimeric *F3'5'H* and a petunia *DFR* gene (contained in pCGP1991, see Table 4) as has been used successfully in the modification of white carnations (deficient in DFR) [see International Patent Application No. PCT/AU96/00296] did not lead to the production of >80% delphinidin based pigments or a novel Violet, Purple-Violet, Purple-Blue color in the Kortina Chanel background.

EXAMPLE 2

Delphinidin-based anthocyanins

[0137] In order to increase the levels of delphinidin-based anthocyanins and therefore increase the chance of violet/purple/blue color in the Kortina Chanel spray carnation flowers, a novel construct was prepared that included the use of 2 (two) *F3'5'H* chimeric genes and a petunia *DFR* gene.

[0138] The *DFR* genomic fragments used in this application were isolated from petunia. The petunia DFR enzyme is only capable of using dihydroquercetin and dihydomyricetin as a substrate, but not dihydrokaempferol (Holton and Cornish, 1995 *supra*). This ensures that most or all of the anthocyanidin produced is delphinidin.

[0139] The *F3'5'H* coding sequences in the chimeric genes used in the new construct were from pansy (*carnANS 5':BP F3'5'H #18: carnANS 3' in* pCGP2205) and salvia (*AmCHS 5': Salvia F3'5'H #47: petD8 3' in* pCGP2122) [Table 3] as these represent the two expression cassettes that were the most efficient in producing the highest levels of delphinidin in the Kortina Chanel spray carnation so far (Table 4).

The transformation vector, pCGP2442

[0140] The transformation vector pCGP2442 (Figure 2) contains a chimeric *AmCHS:Salvia F3'5'H#47: petD8 3'* gene in tandem with a petunia genomic *DFR-A* gene, a chimeric *carnANS: BPF3'5'H#18:carnANS 3'* gene and the *35S 5':SuRB* selectable marker gene cassette of the plasmid pWTT2132 (see International Patent Application No. PCT/AU03/01111).

Construction of the intermediate plasmid, pCGP2221 (5.3kb petunia DFR-A genomic clone)

[0141] The plasmid pCGP2221 contains the petunia *DFR-A* gene (*Pet gen DFR*)from pCGP1472 (described above) in the cloning vector pNEB193 (NEW ENGLAND BIOLABS).

[0142] The plasmid pCGP1472 (described above in Example 1) was digested with the RE *Bgl*II to release a ~5.3kb fragment containing the *Pet gen DFR* gene. The resulting 5'-overhang was partially repaired according to standard protocols (Sambrook *et al,* 1989 *supra*). The cloning vector pNEB193 (DNAP) was digested with *Sal*I to linearize the vector. The resulting overhanging ends of the vector were partially repaired according to standard protocols (Sambrook *et al,* 1989 *supra*). The 5.3kb fragment bearing the *Pet gen DFR* gene was ligated with the partially repaired ends of the vector pNEB193. Successful ligation in the desired orientation of the insert in pNEB193 was established by restriction endonuclease digestion of plasmid DNA isolated from ampicillin-resistant *E. coli* transformants. The resulting plasmid was designated as pCGP2221.

Construction of the intermediate plasmid, pCGP2117 (AmCHS 5': Salvia F3'5'H#47:petD8 3' in pBluescript)

[0143] The plasmid pCGP2117 contains a *AmCHS 5': Salvia F3'5'H#47:petD8 3'* expression cassette in the cloning vector, pBluescript KSII. (Stratagene, USA).

[0144] The petunia *F3'5'H* (*petHf1*) cDNA clone in pCGP725 (contains *AmCHS 5': petHf1:petD8 3'* in a pBluescript KS backbone) [described in International Patent Application No. PCT/AU03/01111] was released by initially digesting the plasmid pCGP725 with the RE *Bam*HI. The overhanging ends were repaired and the linearized plasmid was further digested with the RE *Xba*I. The ~4.9kb fragment containing the vector with the *AmCHS 5'* and *petD8 3'* sequences was purified and ligated with the repaired ends of ~1.6kb *Xho*I/*Eco*RI fragment from the plasmid pCGP1999 (containing the salvia *F3'5'H* #47 cDNA clone, described in International Patent Application No. PCT/AU03/01111), to produce the plasmid, pCGP2117.

Construction of the intermediate plasmid, pCGP2122 (AmCHS 5': salvia F3'5'H#47:petD8 3'; 35S: SuRB)

[0145] The plasmid pCGP2122 contains the *AmCHS 5': salvia F3'5'H#47:petD8 3'* expression cassette in tandem with the *35S 5':SuRB* selectable marker gene.

[0146] The plasmid pCGP2117 (described above) was firstly linearized by digestion with the RE *Not*I. The ends of the linearized plasmid were repaired and then the *AmCHS 5': salvia F3'5'H#47:petD8 3'* cassette was released upon digestion with the RE *Eco*RV. The ~3.6kb purified fragment was then ligated with the repaired *Asp*718 ends of the plasmid pCGP1988 (described in International Patent Application No. PCT/AU03/01111). Successful ligation of the fragment in the desired orientation was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2122.

Construction of the intermediate plasmid, pCGP2438

[0147] The construct pCGP2438 contains *AmCHS 5': SalviaF3'5'H#47:petD8 3'* , *carnANS 5': BPF3'5'H#18:carnANS 3' and 35S 5':SuRB* cassettes.

[0148] The *carnANS 5': BPF3'5'H#18:carnANS 3'* cassette contained in pCGP2150 (described above) was released upon digestion with the RE *ClaI* . The fragment was isolated, purified and ligated with *Sal*I "T-filled" ends of pCGP2122 (containing *AmCHS: SalviaF3'5'H#47:petD8 3' and 35S 5':SuRB* cassettes) [described above]. Successful ligation of the fragment in the desired orientation was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2438.

Construction of the transformation vector, pCGP2442

[0149] The transformation vector pCGP2442 contains the chimeric *AmCHS:Salvia F3'5'H#47:petD8 3'* gene in tandem with *Pet gen DFR,* and *carnANS 5': BPF3'5'H#18:carnANS 3'* genes and the *35S 5':SuRB* selectable marker gene.

[0150] The *Pet gen DFR* clone from the plasmid pCGP2300 (described above) was released upon digestion with the RE *Asc*I. The purified 5.3kb fragment was then ligated with *Asc*I ends of the plasmid pCGP2438 (described above). Successful ligation of the fragment in the desired orientation was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting transformation vector was designated pCGP2442 (Figure 2).

TABLE 5

Chimeric F3'5'H and DFR genes contained in pCGP2442

PLASMID	*F3'5'H and DFR GENES*	*SELECTABLE MARKER GENE*
pCGP2442	*Pet gen DFR;* *carnANS 5':BPF3'5'H# 18:carnANS 3';* *AmCHS 5':Salvia F3'5'H#47:pet D8 3'*	*CaMV 35S 5':SuRB*

[0151] The two chimeric *F3'5'H* genes and a *DFR* gene contained within the T-DNA of the transformation vector pCGP2442 was introduced into the spray carnation variety Kortina Chanel *via Agrobacterium*-mediated transformation.

[0152] Of 74 transgenic lines produced, eight produced flowers of a significant shift in color (RHSCC 71A, 71C, 72A, 81A, 86A, 87A; see Table 6). HPLC analysis of these lines revealed 30-94% delphinidin (up to 3.4 g delphinidin per gram fresh weight tissue). However, of the eight only three lines (19890, 19907 and 19898) [Table 6] produced flowers with a shift in color into the violet, purple/violet range and represented a commercially valuable, novel phenotype in a spray carnation (Kortina Chanel) background.

TABLE 6

Transgenic Kortina Chanel lines and the RHS color code of the petal limb

ACCESSION NUMBER	RHSCC NUMBER (PETAL LIMB COLOR)	RHSCC GROUP
19890	**86A**	**VIOLET**
19942	71A	RED-PURPLE
19898	**87A**	**VIOLET**
19912	72A	RED-PURPLE
19900	71A	RED-PURPLE
19940	71A	RED-PURPLE
19923	71C	RED-PURPLE
19907	**81A**	**PURPLE-VIOLET**

Description of phenotypes

[0153] The phenotype of two transgenic spray carnation varieties designated 19890 and 19907 were further examined. The colors of flowers from the parent line and the two transgenic lines are shown in Figures 3 to 5. Lines19890 and 19907 were essentially similar to the parent in the morphological aspects of the flower (Table 7), but could be further distinguished from the parent through out the accumulation of pigment in the filaments and anthers of the flower, which was a new phenotype of the transgenic lines. Some styles and anthers of both transgenic lines also had a shift in color to light purple, whereas the styles and anthers from flowers of the parent line were a cream-white color (Figure 5).

TABLE 7

Summary of flower measurements (mean values)

Measurement	Parent	19890	19907
Calyx height, mm	22	22	22
Corolla height, mm	18	18	17
Flower diameter, mm	44	43	44
Petal number	27	29	26
Number of styles	2	2	2
Style length, mm	20	23	18
Number of filaments	11	13	10
Stamen length, mm	20.3	23.1	17.6
Number of anthers	3	5	2
Anther length, mm	6.0	6.7	6.0
Anther width, mm	2.8	2.5	2.3
Anther width X length, mm^2	17.3	16.6	14.1

Introduction of the transformation vector pCGP2442 into other carnation varieties

[0154] Due to the success in obtaining high delphinidin levels in the carnation variety, Kortina Chanel using two chimeric *F3'5'H* genes and a *DFR* gene, the same genes are introduced into other carnation cultivars such as but not limited to Cream Cinderella, Cinderella, Cerise Westpearl, Vega, Artisan, Rendezvous.

[0155] The transgenic plants are assessed for flower color as described above and lines with novel flower color (as compared to controls) are selected for commercialization.

Introduction of two F3'5'H genes and a DFR gene into chrysanthemum.

[0156] A construct is prepared based on the expression cassettes that are functional in chrysanthemum.

[0157] An example would include the use of two *F3'5'H* genes such as *RoseCHS:BPF3'5'H#18:nos* and/or a genomic cineraria *F3'5'H* gene and/or *carnANS 5':*

BPF3'5'H#18:carnANS 3' and a genomic petunia *DFR* gene (under the control of its own promoter and terminator) [described above].

[0158] Transformation of chrysanthemum is achieved as described_in International Patent Application No. PCT/US91/05805, US Patent No. 251,392 (US Registration No. 5,567,599) or by any other method well known in the art. For example, Aida *et al*, *Breeding Science*, *54*:51-58, 2004; Dolgov *et al*, *Acta Hort 447*:329-334, 1997, Toguri *et al*, *Plant Biotechnology*, *20*:121-127, 2003, Ledger *et al*, *Plant Cell Reports*, *10*:195-199, 1991 or

[0159] For example. the transformation vector pCGP3440 contains the chimeric *RoseCHS 5': BPF3'5'H#18:nos 3'* gene (described in International Patent Application PCT/AU03/0111) along with a genomic cineraria *F3'5'H* gene (under the control of its own promoter and terminator) [SEQ ID NO:9] and a genomic petunia *DFR* gene (under the control of its own promoter and terminator) [SEQ ID NO:5] (described above).

[0160] The two *F3'5'H* genes and the *DFR* gene contained in the transformation vector pCGP3440, were introduced into the chrysanthemum cultivar, Improved Reagan.

[0161] A total of 52 transgenic plants were sent to soil. The plants are flowered and a selection is made on the basis of shift in color from pink to violet/purple/blue color. The delphinidin levels are measured as described above.

Introduction of two F3'5'H genes and a DFR gene into rose.

[0162] A construct is prepared based on the expression cassettes that are functional in rose. See International Patent Application No. PCT/AU03/01111 and International Patent Application PCT/JP2004/011958)

[0163] An example would include the use of two *F3'5'H* genes such as *CaMV 35S: BPF3'5'H#18:ocs 3'* and/or *CaMV 35S: BPF3'5'H#40:ocs 3'* and/or *CaMV 35S: SalviaF3'5'H#47:ocs 3'* (as described in International Patent Application No.

PCT/AU03/01111) and a *DFR functional* in rose such as *CaMV 35S: iris DFR:ocs 3'* (as described in International Patent Application PCT/JP2004/011958).

[0164] Transformation of rose is achieved as described in International Patent Application PCT/JP2004/011958 or International Patent Application No. PCT/US91/04412 or Robinson and Firoozabady (*Scientia Horticulturae*, *55*:83-99, 1993), Rout *et al*, (*Scientia Horticulturae*, *81*:201-238, 1999) or Marchant *et al*, (*Molecular Breeding 4*:187-194, 1998) or by any other method well known in the art.

Introduction of two F3'5'H genes and a DFR gene into other floral crops.

[0165] pCGP2442 or its functional equivalent is used to generate genetically modified plants with altered inflorescence. Plants selected include other carnation lines such as Kortina, Royal Red Kortina, Cerise Kortina and Dusty Kortina. Non-carnation plants include chrysanthemum, rose, gerbera and other ornamental or horticultural plants such as but not limited to lisianthus, lily, geranium, *Torenia*, *Begonia*, *Cyclamen*, *Nierembergia*, *Catharanthus*, *Pelargonium*, orchid, grape, apple, *Euphorbia* or *Fuchsia*.

[0166] Methods used to transform and regenerate plants are as described herein or in *Scientia Horticulturae:* Transformation of Horticultural Crops, *55* (eds, Germing and Reid) Elsevier, Amsterdam, 1993, Deroles *et al*, In *Biotechnology of Ornamental Plants* (eds, Geneve, Preece and Markle), CAB International, Wallingford, 87-119, 1997, Tanaka *et al*, 2005, *supra*.. Progeny of the genetically modified plants are capable of expressing at least two F3'5'H enzymes and at least one DFR. Color changes include a red--purple, purple, purple-violet, violet, violet-blue and blue (according to RHS color charts). Flowers may be severed from the plant and packaged for sale.

[0167] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A genetically modified plant exhibiting altered inflorescence said plant or its progeny comprising expressed genetic material encoding at least two flavonoid 3',5' hydroxylase (F3'5'H) enzymes and at least one dihydroflavonol-4-reductase (DFR) enzyme.

2. The genetically modified plant wherein the plant is selected from a carnation, chrysanthemum, rose, gerbera and an ornamental or horticultural plant.

3. The genetically modified plant of Claim 1 or 2 wherein the plant is a carnation.

4. The genetically modified plant of any one of Claims 1 to 3 wherein the altered inflorescence is a color in the range of red-purple to blue.

5. The genetically modified plant of Claim 4 wherein the altered inflorescence is a purple to blue color.

6. The genetically modified plant of Claim 5 wherein the altered inflorescence is in a flower, petal, anther or style of carnation plant.

7. The genetically modified plant of Claim 3 wherein the carnation is in the spray carnation *Dianthus caryophyllus* cv. Kortina Chanel genetic background or a sport thereof.

8. The genetically modified plant of Claim 7 wherein the sport is selected from Kortina, Royal Red Kortina, Cerise Kortina and Dusty Kortina.

9. The genetically modified plant of Claim 3 wherein the carnation has the genetic background of Cerise Westpearl, Vega, Artisan, Cream Cinderella, Cinderella or Rendezvous.

10. The genetically modified plant of any one of Claims 1 to 9 wherein the F3'5'H enzymes are respectively from *Viola* sp and *Salvia* sp.

11. The genetically modified plant of Claim 10 wherein the F3'5'H enzymes are encoded by SEQ ID NOs:1 and 3, respectively or a nucleotide sequence capable of hybridizing to a complementary form of SEQ ID NO:1 or 3 under low stringency conditions.

12. The genetically modified plant of Claim 10 or 11 wherein the F3'5'H enzymes are encoded by SEQ ID NO:1 and 3.

13. The genetically modified plant of any one of Claims 1 to 12 wherein the DFR is from *Petunia* sp.

14. The genetically modified plant of Claim 13 wherein the DFR is encoded by SEQ ID NO:5 or a nucleotide sequence capable of hybridizing to a complementary form of SEQ ID NO:5 under low stringency conditions.

15. The genetically modified plant of Claim 14 wherein the DFR is encoded by SEQ ID NO:5.

16. The genetically modified plant of Claim 4 or 5 wherein the red-purple/blue color is in the range 81A, 86A and 87A according to the Royal Horticultural Society color chart or a color in between or proximal to the ends of this range.

17. The genetically modified plant of Claim 16 wherein the plant is Kortina Chanel/2442.

18. Progeny, reproductive material, cut flowers, tissue culturable cells and regenerable cells from the genetically modified plant of any one of Claims 1 to 17.

19. Use of genetic sequences encoding at least two F3'5'H enzymes and at least one DFR enzyme in the manufacture of a carnation such as a Kortina Chanel carnation or sports thereof exhibiting altered inflorescence including tissue having a purple to violet to blue color.

20. A method for producing a carnation exhibiting altered inflorescence, said method comprising introducing into regenerable cells of a carnation plant expressible genetic material encoding at least two F3'5'H enzymes and at least one DFR enzyme and regenerating a plant therefrom or obtaining progeny of the regenerated plant.

21. A method for producing a carnation line exhibiting altered inflorescence, the method comprising selecting a spray carnation comprising genetic material encoding one of at least two F3'5'H enzymes or at least one DFR enzyme and crossing this plant with another carnation comprising genetic material encoding the other of at least two F3'5'H enzymes or at least one DFR enzyme and then selecting F1 or subsequent generation plants which express the genetic material.

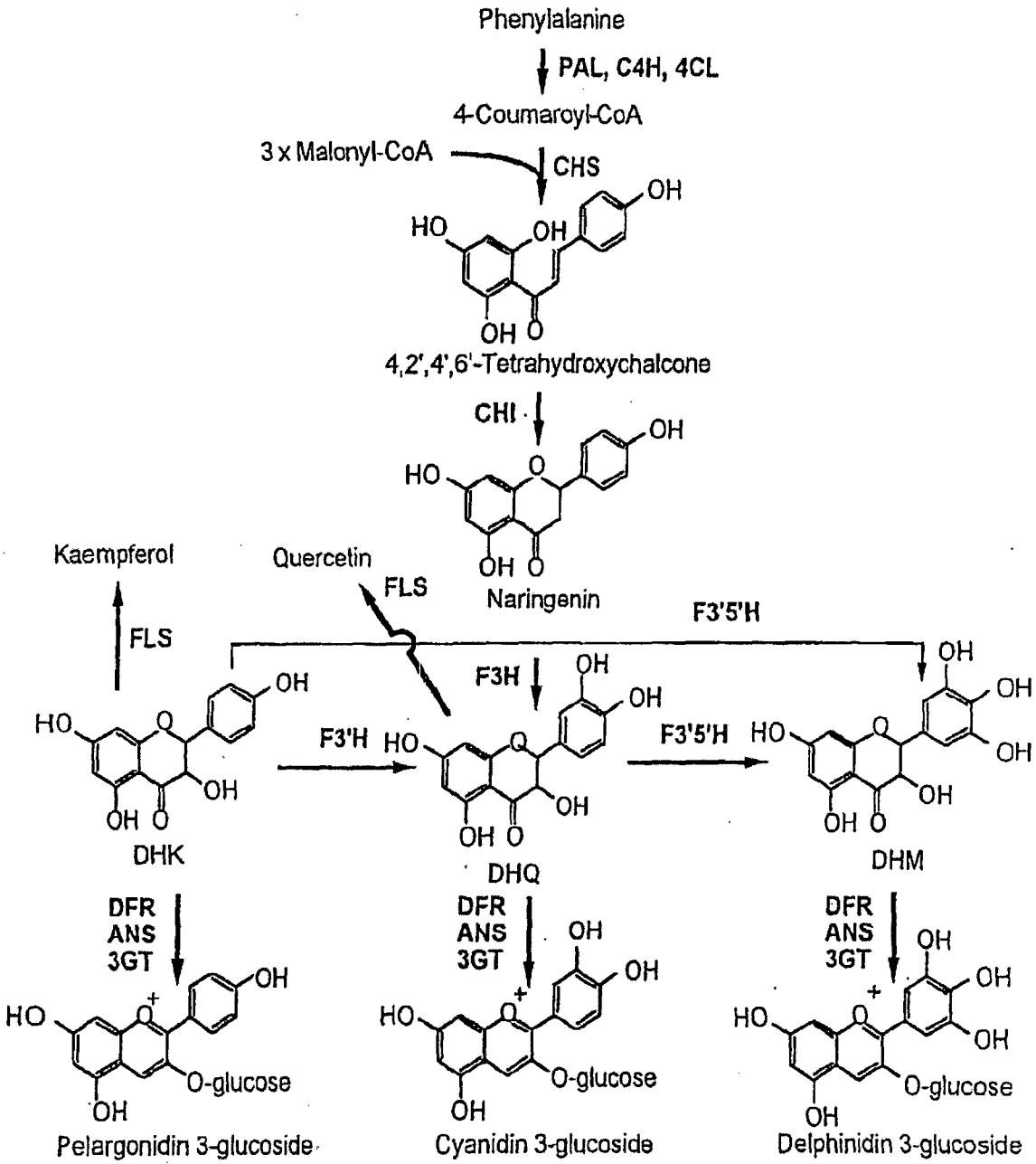

Phenylalanine
PAL, C4H, 4CL
4-Coumaroyl-CoA
3 x Malonyl-CoA
CHS
4,2',4',6'-Tetrahydroxychalcone
CHI
Naringenin
Kaempferol
Quercetin
FLS
FLS
F3'5'H
F3H
F3'H
F3'5'H
DHK
DHQ
DHM
DFR
ANS
3GT
DFR
ANS
3GT
DFR
ANS
3GT
O-glucose
O-glucose
O-glucose
Pelargonidin 3-glucoside
Cyanidin 3-glucoside
Delphinidin 3-glucoside

Figure 1

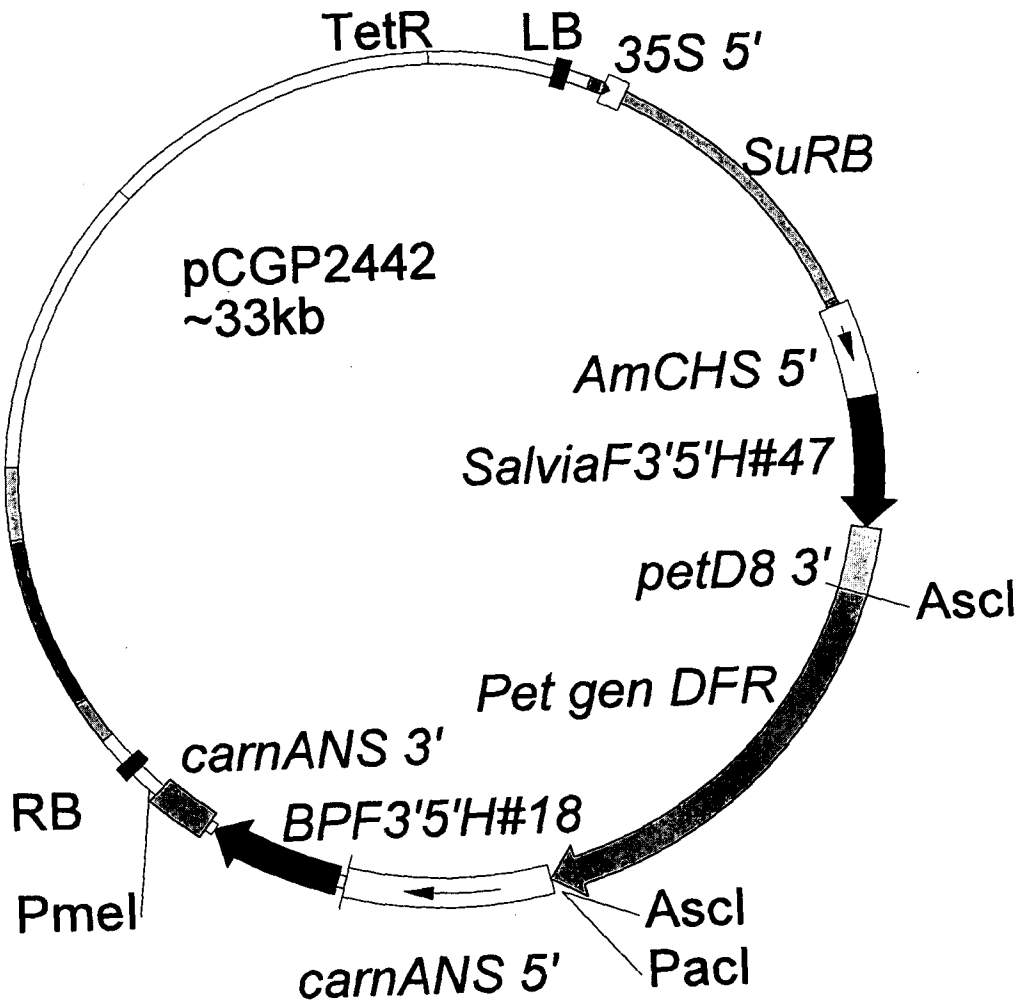

TetR
LB
35S 5'
SuRB
pCGP2442
~33kb
AmCHS 5'
SalviaF3'5'H#47
petD8 3'
AscI
Pet gen DFR
carnANS 3'
RB
BPF3'5'H#18
PmeI
AscI
PacI
carnANS 5'

Figure 2

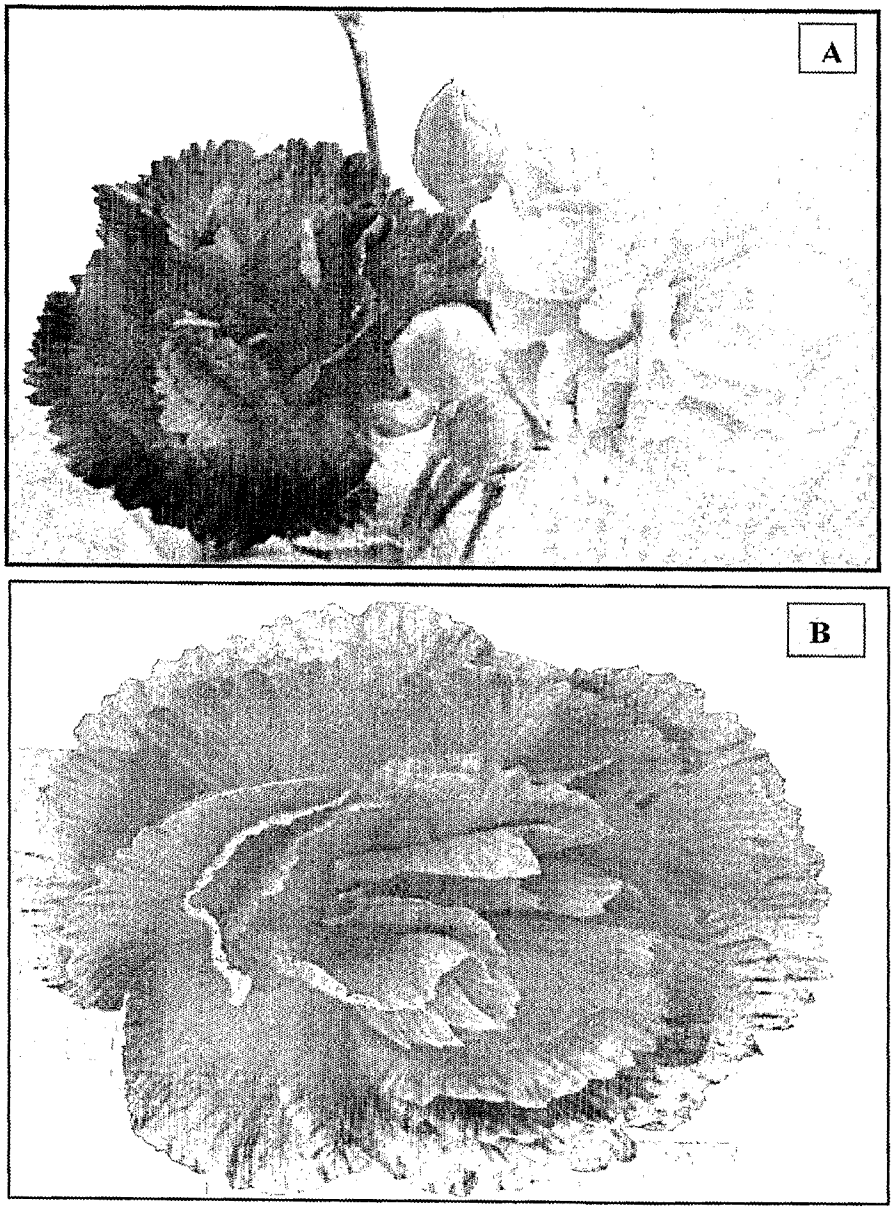


Figure 3

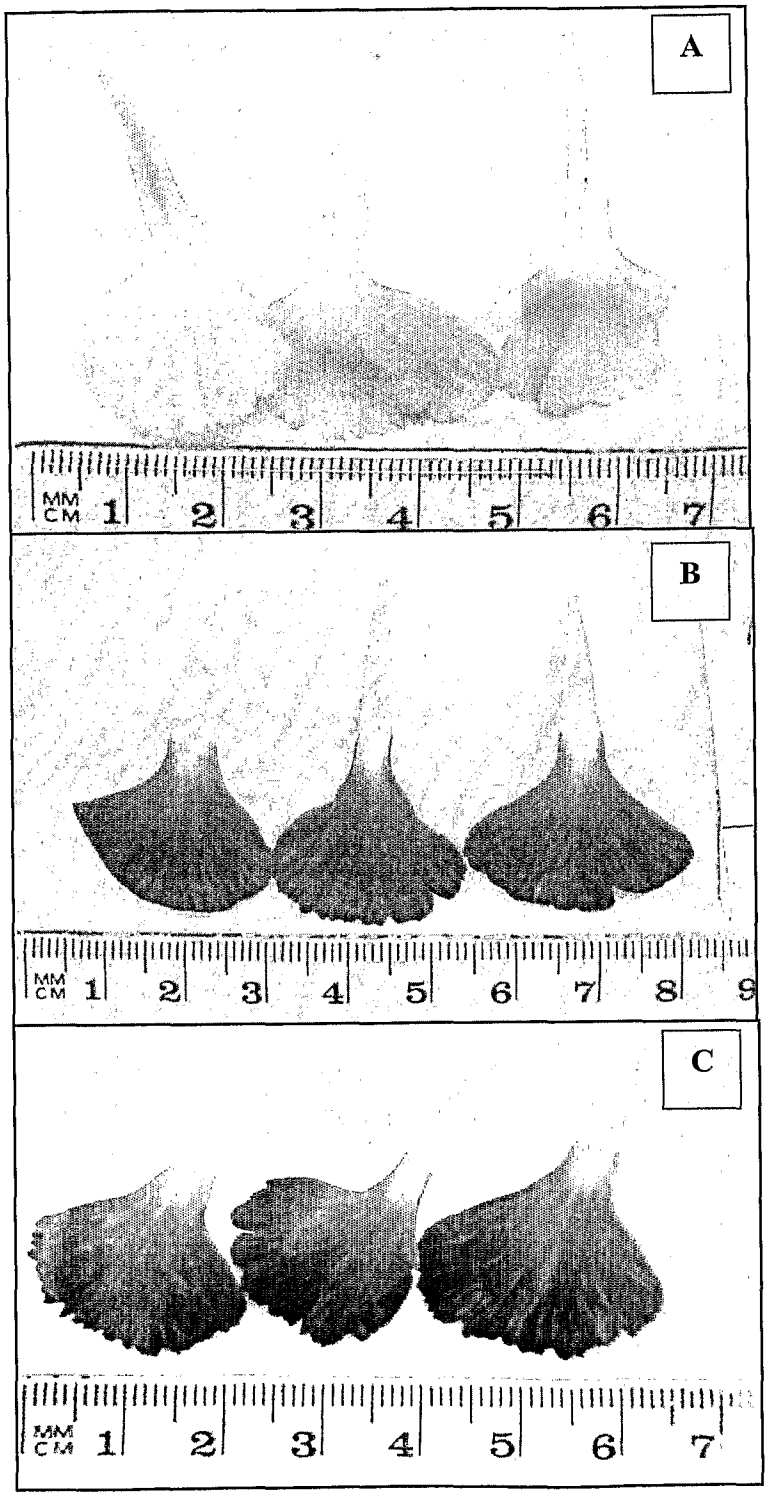


Figure 4

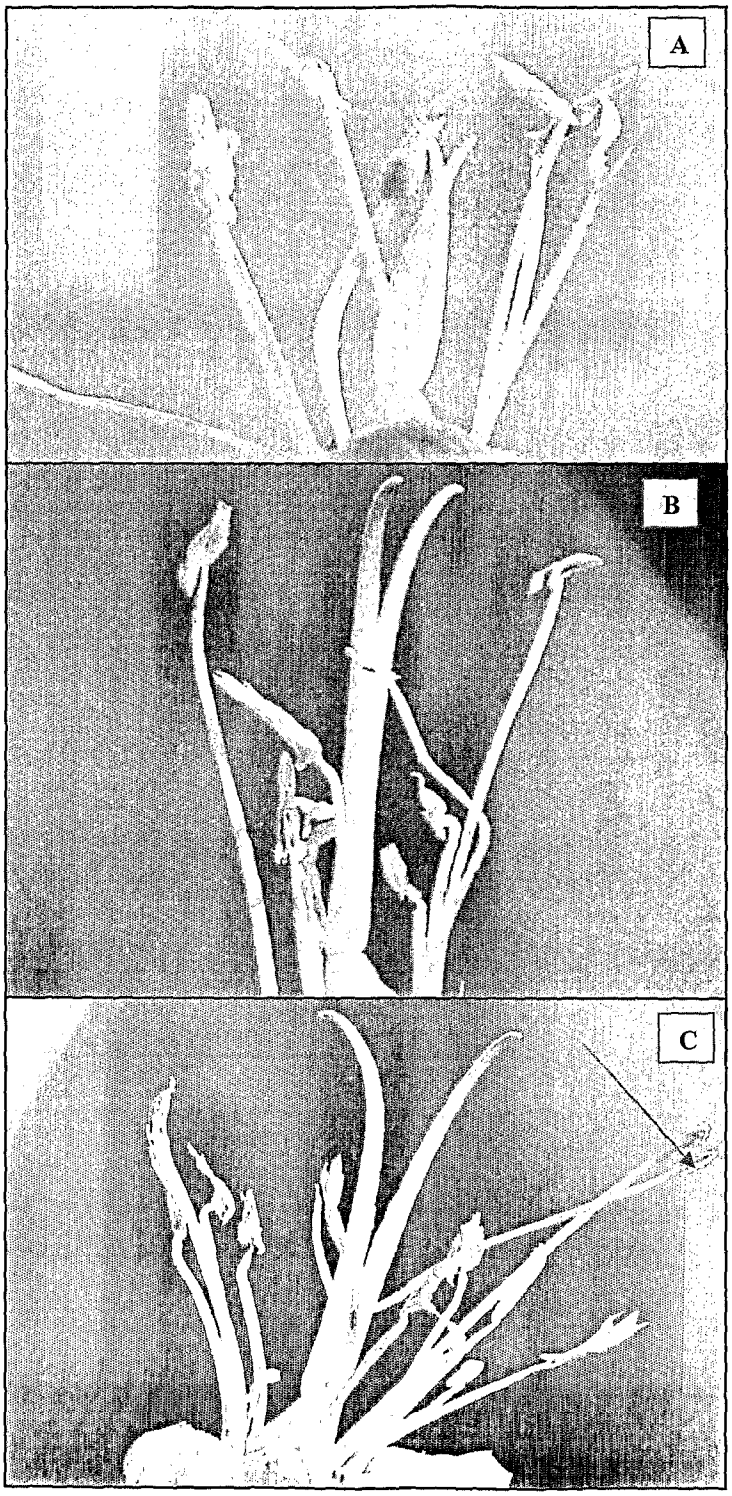


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001700

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A01H 5/02 (2006.01) *C12N 5/10* (2006.01) *C12N 15/53* (2006.01)
A01H 4/00 (2006.01) *C12N 9/02* (2006.01) *C12N 15/82* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI EPODOC BIOSIS AGRICOLA HCA : Inflorescence, flower, colour, violet, blue, flavonoid 3',5' hydroxylase, dihydroflavonol-4-reductase, delphinidin, carnation, rose, chrysanthemum, and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO1994/003591 A1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD) 17 February 1994 See the whole document, particularly page 41, lines 23-28; pages 19-20, example 1; page 34, example 12; example 13, tables 5-6	1-2, 4-5, 13-15, 18
X	WO1996/036716 A1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD) 21 November 1996 See the whole document, particularly examples 9-16	1-21
A	WO2004/020637 A1 (INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD) 11 March 2004	
A	EP1652916 (INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD) 3 May 2006	

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2009	03 FEB 2009
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **DANIEL SHEAHAN** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 7969

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/001700

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	1994003591	AU	45519/93	AU	75464/96	CA	2140637
		EP	0656940	NZ	254013	US	5859334
WO	1996036716	AU	56396/96	CA	2202668	EP	0873410
		HU	9802555	NZ	307119	US	6080920
WO	2004020637	AU	2003254410	CN	1678746	EP	1546340
		US	2007033674				
EP	1652916	AU	2004264488	CA	2537065	CN	1836039
		KR	20060081400	SG	146636	WO	2005017147

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX