PATENTS ACT 1990 667392 COMMONWEALTH OF AUSTRALIA

NOTICE OF ENTITLEMENT

We, International Flower Developments Pty. Ltd. the Applicant/Nominated Person in respect of Application No. 46901/83 state the following:

The Nominated Person is entitled to the grant of a patent because the Nominated Person would, on the grant of a patent for the invention to inventors Timothy Albert HOLTON and Lisa Ann KEAM be entitled to have the patent assigned to it.

The Nominated Person is entitled to claim priority from the application listed in the declaration under Article 8 of the PCT because the Nominated Person is the applicant in respect of the application listed in the declaration under Article 8 of the PCT and because the application was the first application made in a convention country in respect of the invention.

DATED this 10th day of January, 1996

a member of the firm of DAVIES COLLISON CAVE for and on behalf of the applicant(s)

(DCC Ref:1721170/EJH/wm)



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(57)	Claim	
1.	An isolated nucleic acid molec	ule comprising a sequence of nucleotides encoding
or	complementary to a sequence end	coding a flavonol synthase (FLS) as hereinbefore
def	fined.	

16. An isolated nucleic acid molecule which:

- (i) encodes a FLS of plant origin; and
- (ii) hybridises under low stringency conditions to the nucleotide sequence set forth in SEQ ID No:1 or to a complementary strand thereof.

27. A transgenic plant carrying a non-indigenous genetic sequence encoding a FLS.

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(57) Abstract

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The present invention relates generally to genetic sequences encoding flavonoid pathway metabolising enzymes and in particular enzymes having flavonol synthase activity and their use such as in manipulating production of pigmentory molecules in plants. More particularly, the present invention provides genetic sequences encoding flavonol synthase (FLS).

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GENETIC SEQUENCES ENCODING FLAVONOL SYNTHASE ENZYMES AND USES THEREFOR

The present invention relates generally to genetic sequences encoding flavonoid metabolising enzymes and in particular enzymes having flavonol synthase activity and their use such as in manipulating production of pigmentory molecules in plants.

Bibliographic details of the publications referred to hereinafter in the specification are collected at the end of the description. SEQ ID No's referred to herein in relation to nucleotide and amino acid sequences are defined after the Bibliography.

The flower industry strives to develop new and different varieties of flowering plants. An effective way to create such novel varieties is through the manipulation of flower colour and classical breeding techniques have been used with some success to produce
a wide range of colours for most of the commercial varieties of flowers. 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 a full spectrum of coloured varieties. For example, the development of blue varieties of the major cut flower species such as rose, chrysanthemum, carnation, lily, tulip and gerbera would offer a significant opportunity in both the cut flower and ornamental markets.

The colours of flowers and other plant parts are predominantly due to two types of pigments: flavonoids and carotenoids. Flavonoids are the most common and the most important of the flower pigments. The most important classes of flavonoids with respect to flower colour are anthocyanins, flavonols and flavones. Anthocyanins are glycosylated derivatives of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin, and are localised in the vacuole.

One important factor for flower colour is co-pigmentation of anthocyanins with tannins
and certain flavone and flavonol glycosides (Scott-Moncrieff, 1936). When compared over a range of pH values, co-pigmented anthocyanins are always found to be bluer than the normal pigment. Co-pigmentation of anthocyanins with flavonol glycosides can also be important for the development of colour in fruit (Yoshitama *et al.*, 1992). The molar ratio of anthocyanin to co-pigment can also exert a strong influence on colour. It has recently been demonstrated that flavonol aglycones are essential for pollen germination and pollen tube growth (Mo *et al.*, 1992). The ability to control the production of co-pigments, such as flavonols, in plants could therefore have useful

applications in altering flower colour and manipulating plant fertility.

The biosynthetic pathway for the anthocyanin pigments is well established (Ebel and Hahlbrock, 1988; Hahlbrock and Grisebach, 1979; Wiering and de Vlaming, 1984; 5 Schram et al., 1984; Stafford, 1990). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA with one molecule of pcoumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase. The product of this reaction, 2',4,4',6'-tetrahydroxychalcone, is normally rapidly isomerised to produce naringenin by the enzyme chalcone-flavanone isomerase. 10 Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase to produce dihydrokaempferol (DHK). The B-ring of dihydrokaempferol can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. DHK, DHQ and DHM may be converted to coloured anthocyanins (pelargonidin 3-15 glucoside, cyanidin 3-glucoside and delphinidin 3-glucoside) by the action of at least

two enzymes (dihydroflavonol-4-reductase and flavonoid-3-glucosyltransferase).

Flavonols such as kaempferol (K), quercetin (Q) and myricetin (M) are formed from dihydroflavonols by the introduction of a double bond between C-2 and C-3
(Forkmann, 1991), as illustrated in Figure 1. Flavonols often accumulate in glycosylated forms and may also be methylated. Methylation can occur either before or after glycosylation. *In vitro* conversion of dihydroflavonols to flavonols was first observed in enzyme preparations from parsley cell cultures (Britsch et al., 1981). Flavonol synthase activity has also been detected in flower extracts from *Matthiola*(Spribille and Forkmann, 1984), *Petunia* (Forkmann et al., 1986) and *Dianthus*

(Forkmann, 1991). Flavonol synthase enzyme activity requires 2-oxoglutarate, ascorbate and ferrous ions as cofactors. In flowers of *Petunia hybrida*, the genetic locus *Fl* controls the formation of flavonols: flavonol synthesis is greatly reduced in mutants homozygous recessive for this gene (Wiering *et al.*, 1979; Forkmann *et al.*,

30 1986). In vitro enzyme assays with the flavonol synthase from petunia showed that DHK and DHQ were readily converted to the respective flavonols, whereas DHM was a poor substrate. The ability to control flavonol synthase activity in flowering plants would provide a means to manipulate petal colour by altering flavonol production, thereby enabling a single species to express a broader spectrum of flower colours. As
35 stated above, the ability to control flavonol production also has implications in respect of male fertility. Such control may be by modulating the level of production of an

indigenous enzyme or by introducing a non-indigenous enzyme

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As used herein an "indigenous" enzyme is one which is native to or naturally expressed in a particular cell. A "non-indigenous" enzyme is an enzyme not native to the cell but expressed through the introduction of genetic material into a plant cell; for example, through a transgene. An "endogenous" enzyme is an enzyme produced by a cell but which may or may not be indigenous to that cell.

In accordance with the present invention, the genetic sequences encoding flavonol synthase (hereinafter referred to as "FLS") have been identified and cloned from a number of sources and used to generate transgenic plants. These recombinant sequences permit the modulation of levels of flavonol production thereby providing a means to manipulate petal colour and male fertility. The recombinant sequences also permit the modulation of DHK metabolism as well as the metabolism of other substrates, such as DHQ and DHM. Since DHK, DHQ and DHM are precursors of the coloured anthocyanins modulation of their concentrations by expression of FLS sequences provides another means of manipulating flower colour.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a
sequence encoding a plant FLS or a functional mutant, derivative, part, fragment, homologue or analogue of said FLS. The expression "FLS" includes reference to polypeptides and proteins having FLS activity as well as any mutants, derivatives, parts, fragments, homologues or analogues of such polypeptides or proteins and which have FLS activity. A molecule having FLS activity may also be a fusion polypeptide or protein between a polypeptide or protein having FLS activity and an extraneous peptide, polypeptide or protein.

As used herein, the term "isolated nucleic acid molecule" is meant to include a genetic sequence in a non-naturally-occurring condition. Generally, this means isolated away from its natural state or formed by procedures not necessarily encountered in its natural environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments, recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids such as heterologous nucleic acids fused or operably-linked to the genetic sequences of the present invention. The term "isolated nucleic acid molecule" also extends to the genomic DNA or cDNA, or part thereof encoding FLS or a functional mutant, derivative, part, fragment, homologue or analogue of FLS, in reverse orientation

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relative to its or another promoter. It further extends to naturally-occurring sequences following at least a partial purification relative to other nucleic acid sequences. The term "isolated nucleic acid molecule" as used herein is understood to have the same meaning as a "nucleic acid isolate".

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The expression "genetic sequences" is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or via a complementary series of bases, a sequence of amino acids comprising a FLS molecule including a polypeptide or protein having FLS activity. Such a sequence of amino acids may constitute a full-length FLS such as is set forth in, for example, SEQ ID No:1 or SEQ ID No:4 or SEQ ID No:5 or an active truncated form thereof or a functional mutant, derivative, part, fragment, homologue or analogue thereof. Alternatively, the amino acid sequence may comprise part of, for example, these sequences or all or part of the sequences set forth in SEQ ID No:2 or SEQ ID No:3.

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Another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which:

(i) encodes a FLS; and

(ii) has at least 50% nucleotide sequence similarity to the nucleotide sequence set

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forth in at least one of SEQ ID No:1 or SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5.

More particularly, the present invention is directed to an isolated DNA molecule comprising a sequence of nucleotides which:

- 2.5 (i) encodes a FLS; and
 - (ii) has at least 65-75% nucleotide sequence similarity to the nucleotide sequence set forth in at least one of SEQ ID No:1 or SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5.
- 30 Preferred percentage similarities include 80%, 85%, 90%, 92-95%, 96-98% and 99-100%. Although the pecentage similarities referred to above assume an overall comparison between the sequences set forth in at least one of SEQ ID No:1 or SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5 and another genetic sequence, it is clear that there may be specific regions within the molecules being compared having less than 50% similarity. In this respect, the present invention is further defined as a nucleic acid molecule, and in particular a DNA molecule, comprising a sequence of nucleotides which:

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(i) encodes a FLS: and

(ii) has at least 50-75% nucleotide sequence similarity to one or more regions of the sequence set forth in at least one of SEQ ID No:1 or SEQ ID No:2 or SEQ ID No:3 or SEO ID No:4 or SEO ID No:5.

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The nucleic acid sequences contemplated herein also encompass oligonucleotides useful as genetic probes or as "antisense" molecules capable of regulating expression of the corresponding gene in a plant. An "antisense molecule" as used herein may also encompass a gene construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its or another promoter.

With respect to this aspect of the invention there is provided an oligonucleotide of 5-50 nucleotides having substantial similarity or complementarity to a part or region of a molecule with a nucleotide sequence set forth in at least one of SEO ID No:1 or SEO 15 ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5. By "substantial similarity or complementarity" in this context is meant a hybridizable similarity under low, alternatively and preferably medium and alternatively and most preferably high stringency conditions, as defined below. Such an oligonucleotide is useful, for example, in screening FLS genetic sequences from various sources or for monitoring 20 an introduced genetic sequence in a transgenic plant. Such an oligonucleotide is generally in the form of a primer or a probe. Preferably, the oligonucleotide is directed to a conserved FLS genetic sequence or a sequence conserved within a plant genus, plant species and/or plant strain or variety.

- 25 In one aspect of the present invention, the oligonucleotide corresponds to the 5' or the 3' end of the FLS genetic sequence. For convenience, the 5' end is considered herein to define a region substantially between the start codon of the structural gene to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural gene.
- 30 It is clear, therefore, that oligonucleotides may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such oligonucleouides.

In one embodiment, the nucleic acid sequence encoding a FLS or a functional mutant, 35 derivative, part, fragment, homologue or analogue thereof is used to reduce the activity of an indigenous FLS, such as by using co-suppression (US Patent Number 5,034,323). Alternatively, the nucleic acid sequence encoding this enzyme or various

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functional mutants, derivatives, parts, fragments, homologues or analogues thereof, is used in the antisense orientation to reduce activity of the indigenous FLS. Although not wishing to limit the present invention to any one theory, it is possible that an antisense FLS transcript or fragment or part thereof (for example, an oligonucleotide molecule) would form a duplex with all or part of the naturally-occurring mRNA specified for the enzyme thus preventing accumulation of or translation from the mRNA into active enzyme.

In another alternative, ribozymes could be used to inactivate target nucleic acid
sequences. Ribozymes are well described by Haseloff and Gerlach (1988). With respect to this embodiment, the ribozyme would preferably comprise a hybridizing portion and a catalytic portion wherein the hybridizing portion comprises one and preferably two nucleotide arms capable of hybridizing to a mRNA transcript from a gene having a nucleotide sequence substantially as set forth in at least one of SEQ ID
No:1 or SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5.

In a further embodiment, the nucleic acid sequence encoding a FLS or a functional mutant, derivative, part, fragment, homologue or analogue thereof is used to elevate the activity of an indigenous FLS above the normal endogenous or existing level, or alternatively to provide FLS activity where the normal endogenous or existing level of

20 alternatively to provide FLS activity is negligible or zero.

Reference herein to the altering of FLS activity relates to an elevation or reduction in activity of 30% or more, or more preferably of 30-50%, or even more preferably 50-25 75% or still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. Such elevation or reduction may be referred to as "modulation" of FLS enzyme activity. Generally, modulation is at the level of transcription or translation of FLS genetic sequences. The level of activity can be assayed using a modified method of Forkmann *et al.* (1986).

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The nucleic acids of the present invention may be ribonucleic acids or deoxyribonucleic acids, single stranded or covalently closed circular molecules. Preferably, the nucleic acid molecule is, or originates from, cDNA. The present invention also extends to other nucleic acid molecules which hybridize to the genetic sequences herein disclosed.

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According to this aspect of the present invention there is provided an isolated nucleic acid molecule comprising a sequence of nucleotides which:

- (i) encodes a FLS; and
- (ii) hybridizes to the nucleotide sequence set forth in at least one of SEQ ID No:1 or
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SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5 or a complementary respective form thereof under low stringency conditions.

For the purpose of defining the level of stringency, reference can conveniently be made to Maniatis *et al.* (1982) at pages 387-389, and especially paragraph 11, which is herein incorporated by reference. A low stringency is defined herein as being in 4-6 x SSC / 1% (w/v) SDS at 37-45°C for 2-3 hours. Depending on the source and concentration of nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions which are considered herein to be 1-4 x SSC / 0.5-1% (w/v) SDS at greater than or equal to 45°C 15 for 2-3 hours or high stringent conditions considered herein to be 0.1-1 x SSC / 0.1-1.0% (w/v) SDS at greater than or equal to 60°C for 1-3 hours.

In its most preferred embodiment, the present invention extends to a nucleic acid molecule having or comprising a nucleotide sequence set forth in at least one of SEQ 20 ID No:1 or SEQ ID No:2 or SEQ ID No:3 or SEQ ID No:4 or SEQ ID No:5 or to a molecule having at least 50%, more preferably at least 55%, even more preferably at least 60%, still more preferably at least 65-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of the nucleotide or amino acid sequence set forth, respectively, in the 25 above-referenced sequences and wherein the nucleic acid encodes or is complementary to a sequence which encodes an enzyme having FLS activity. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and yet still encode a FLS-like molecule and such molecules may still be considered within the scope of the present invention where they have 30 regions of sequence conservation.

The nucleic acid molecules contemplated herein may exist, in either orientation, alone or in combination with a vector molecule and preferably an expression-vector. The term "vector molecule" is used in its broadest sense to include any intermediate vehicle for the nucleic acid molecule, capable of facilitating transfer of the nucleic acid into the plant cell and/or facilitating integration into the plant genome. An intermediate vehicle may, for example, be adapted for use in electroporation, microprojectile bombardment, *Agrobacterium*-mediated transfer or insertion via DNA or RNA viruses. The

intermediate vehicle and/or the nucleic acid molecule contained therein may or may not need to be stably integrated into the plant genome. Such vector molecules may also replicate and/or express in prokaryotic cells. Preferably, the vector molecules or parts thereof are capable of integration into the plant genome. The nucleic acid molecule may additionally contain a promoter sequence capable of directing expression of the nucleic acid molecule in a plant cell. The nucleic acid molecule and promoter may also be introduced into the cell by any number of means such as those described above. The vector molecule may also comprise a genetic sequence encoding a ribozyme as hereinbefore defined capable of cleaving a FLS mRNA transcript.

The nucleic acid or its complementary form may encode the full-length enzyme or a derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally-occurring enzyme and which retains FLS activity. In this regard, the nucleic acid includes the naturally-15 occurring nucleotide sequence encoding FLS or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally-occurring sequence. The nucleic acid sequences of the present invention or its complementary form may also encode a "part" of a FLS, whether active or inactive, and such a nucleic acid molecule may be useful as an oligonucleotide probe, primer for polymerase chain 20 reactions or in various mutagenic techniques, or for the generation of antisense molecules or ribozyme molecules capable of regulating expression of the corresponding gene in a plant.

Amino acid insertional derivatives of the FLS of the present invention include amino 25 and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino

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acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 1, overleaf.

Where FLS is derivatised by amino acid substitution, the amino acids are generally 3.5 replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in

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the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

	Original Residue	Exemplary Substitutions
	·	
	Ala	Ser
	Arg	Lys
	Asn	Gln; His
	Asp	Glu
	Cys	Ser
	Gln	Asn; Glu
	Glu	Asp
	Gly	Pro
)	His	Asn; Gln
	Ile	Leu; Val
	Leu	lle; Val
	Lys	Arg; Gln; Glu
	Met	Leu; Ile; Val
	Phe	Met; Leu; Tyr
	Ser	Thr
	Thr	Ser
	Trp	Tyr
	Tyr	Trp; Phe
)	Val	Ile; Leu; Met

TABLE 1Suitable residues for amino acid substitutions

3 5 The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The

manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.* (1989).

- 5 Other examples of recombinant or synthetic mutants and derivatives of the FLS enzyme of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.
- 10 The terms "analogues" and "derivatives" also extend to any functional chemical equivalent of FLS and also to any amino acid derivative described above. For convenience, reference to "FLS" herein and in particular hereinafter includes reference to any functional mutant, derivative, part, fragment, homologue or analogue thereof.
- 15 The present invention is exemplified using nucleic acid sequences derived from petunia, tobacco, carnation and chrysanthemum, since these represent the most convenient and preferred sources of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants. All such nucleic acid sequences encoding directly or 20 indirectly FLS are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding FLS enzymes include, but are not limited to rose, snapdragon, lisianthus, cyclamen, grape and parsley.
- In accordance with the present invention, a nucleic acid sequence encoding a FLS may 25 be introduced into and expressed in a transgenic plant in either orientation thereby providing a means to convert DHK and/or other suitable substrates, if synthesised in the plant cell, ultimately into flavonols or derivatives of same or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing FLS activity. The production of these flavonols will modify petal colour and may contribute to the production of bluer colours via co-pigmentation with anthocyanins. Expression of the nucleic acid sequence in the plant may be constitutive, inducible or developmental and may also be tissue-specific. The term "expression" is used in its broadest sense to include production of RNA or of both RNA and protein. It also extends to partial expression of a nucleic acid molecule.

According to this aspect of the present invention there is provided a method for producing a transgenic flowering plant capable of synthesizing FLS, said method

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comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said FLS under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous FLS at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing FLS activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding a FLS activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the 15 expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing FLS activity, said method comprising altering the *Fl* gene through modification of the indigenous
sequences via homologous recombination from an appropriately altered *Fl* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

In a preferred embodiment, the present invention contemplates a method for producing 25 a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence into a FLS. Alternatively, said method may comprise stably 30 transforming a cell of a suitable plant with a nucleic acid sequence of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the indigenous or existing FLS. Preferably the altered level would be less than the indigenous or existing level of FLS activity in a comparable non-35 transgenic plant. Without wishing to limit the present invention, one theory of mode of action is that reduction of the indigenous FLS activity requires the expression of the introduced nucleic acid sequence or its complementary sequence. However,

expression of the introduced genetic sequence or its complement may not be required to achieve the desired effect: namely, a flowering plant exhibiting altered inflorescence properties.

5 In a related embodiment, the present invention contemplates a method for producing a flowering plant exhibiting altered inflorescence properties, said method comprising alteration of the *Fl* gene through modification of the indigenous sequences via homologous recombination from an appropriately altered *Fl* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant 10 from the cell.

Preferably, the altered inflorescence includes the production of white, yellow, pink, violet or blue flowers or other colour shades depending on the genotype and physiological conditions of the recipient plant.

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Accordingly, the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding a FLS or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of a FLS, said
method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, a FLS, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell. By "suitable plant" is meant a plant
capable of producing DHK, or other substrates of FLS, and possessing the appropriate physiological properties required for the development of the colour desired.

The present invention is exemplified by generation of transgenic petunia and tobacco plants containing introduced FLS genetic sequences. The use of petunia and tobacco
plants represents a particularly convenient and useful model for the generation of transgenic plants carrying genetic sequences and the results obtained from such transgenic plants are generally applicable to other plants. One skilled in the art will immediately recognise the variations applicable to this method such as increasing or decreasing the expression of the enzyme naturally present in a target plant. This would
lead to differing shades of colours. Other suitable target plants, in addition to petunia and tobacco, include but are not limited to rose, carnation, chrysanthemum, gerbera,

lisianthus, lily, iris and pelargonium.

The present invention, therefore, extends to all transgenic plants containing all or part of the nucleic acid sequence of the present invention, or antisense forms thereof and/or any homologues or related forms thereof and in particular those transgenic plants
which exhibit altered inflorescence properties. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding a FLS. Generally the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of a FLS nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to seeds from such transgenic plants. Such seeds, especially if coloured, will be useful, *inter alia*, as proprietary tags for plants. The invention further extends to fruit and to vegetable plants and leaves of, for example, ornamental plants.

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Another aspect of the present invention is directed tc mbinant forms of FLS. The recombinant forms of the enzymes will provide a source of material for research to develop, for example, more active enzymes and may be useful in developing *in vitro* systems for production of flavonols and/or coloured compounds.

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Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing a FLS or down-regulating an indigenous FLS enzyme in a plant.

- 25 Another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding a FLS extrachromasomally in plasmid form. In one embodiment, the plasmid is pCGP481 in *Escherichia coli*. The microorganism *Escherichia coli* strain DH5α containing the plasmid pCGP481 was deposited with the Australian Government Analytical Laboratories, 1 Suakin Street,
- 30 Pymble, New South Wales, 2037, Australia on August 5, 1993 under Accession Number N93/33236.

The present invention is further described by reference to the following non-limiting Figures and Examples.

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The amino acid abbreviations used throughout the specification, including in the Examples, are shown overleaf in Table 2.

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5	Amino acid	3-letter	single-letter
	L-alanine	Ala	Α
	L-arginine	Arg	R
	L-asparagine	Asn	N
0	L-aspartic acid	Asp	D
	L-cysteine	Cys	C
	L-glutamine	Gln	Q
	L-glutamic acid	Glu	E
	L-glycine	Gly	G
5	L-histidine	His	H
	L-isoleucine	lle	I
	L-leucine	Leu	L
	L-lysine	Lys	K
	L-methionine	Met	Μ
0 .	L-phenylalanine	Phe	F
	L-proline	Pro	Р
	L-serine	Ser	S
	L-threonine	Thr	Т
	L-tryptophan	Тгр	W
5	L-tyrosine	Tyr	Y
	L-valine	Val	\mathbf{V}

TABLE 2

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Table 3 provides a summary of the SEQ ID No's assigned to genetic sequences referred to herein:

Sequence	SEQ ID No
cDNA insert of pCGP481	SEQ ID No:1
cDNA insert of pCGP489	SEQ ID No:2
cDNA insert of pCGP490	SEQ ID No:3
cDNA insert of pCGP777	SEQ ID No:4
cDNA insert of pCGP874	SEQ ID No:5
Oligo #1	SEQ ID No:6
Oligo #2	SEQ ID No:7
Oligo #3	SEQ ID No:8
Oligo #4	SEQ ID No:9
Oligo #5	SEQ ID No:10
Oligo #6	SEQ ID No:11
Oligo #7	SEQ ID No:12
Oligo #8	SEQ ID No:13
Oligo #9	SEQ ID No:14
Oligo #10	SEQ ID No:15
Oligo #11	SEQ ID No:16

TABLE 3

In the Figures:

Figure 1 is a schematic representation of the conversion of dihydroflavonols to flavonols in *Petunia hybrida*. Enzymes involved in each step of the pathway are indicated as follows: F3'H = Flavonoid 3'-hydroxylase; F3'5'H = Flavonoid 3',5'-hydroxylase; FLS = flavonol synthase. DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin, K = kaempferol, Q = quercetin, M = 35 myricetin.

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Figure 2 is an autoradiograph of an RNA gel blot proted with ³²P-labelled pDIOXC3 cDNA insert. Each lane contained 20 μ g of total RNA isolated from the following - 1-5: OGB limb tissue of flowers at the five stages of development; T: OGB tube tissue from stage 3-4 flowers; L: leaf tissue from six week old OGB seedlings.

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Figure 3 shows the sequencing strategy used to obtain the complete nucleotide sequence of the cDNA insert of pCGP481. Arrows indicate the direction and length of sequences read from individual sequencing reactions. Sequencing reactions using custom-made oligonucleotide primers (Oligos 7-9; SEQ ID No's 12-14) are also shown.

Figure 4 is a diagrammatic representation of the construction of pCGP631.
pCGP631 was constructed by cloning the pCGP481 cDNA insert in a sense orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter in the
expression vector pYGA22m. The cDNA insert from pCGP481 was ligated as a <u>EcoRI/Xho</u>I fragment with the large fragment that resulted from the <u>EcoRI/SalI</u> digestion of pYGA22m. IR = inverted repeat of 2 µm plasmid, TRP1 = TRP1 gene, Ap = ampicillin resistance marker.

20 Figure 5 shows a FLS assay of yeast extracts using DHQ as substrate. The autoradiograph shows conversion of [¹⁴C]-DHQ to quercetin by enzyme extracts of yeast transformed with the plasmid pCGP631. No FLS activity was detected in untransformed yeast. C = unlabelled quercetin without yeast extract. The position of migration of unlabelled quercetin is circled.

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EXAMPLE 1 - MATERIALS

Enzymes

5 All enzymes were obtained from commercial sources and used according to the manufacturer's recommendations.

Bacterial Strains

The following *Escherichia coli* strains were used:

PLK-F' and SURE, both obtained from Stratagene; XL1-Blue (Bullock et al., 1987), and DH5α (Hanahan, 1983 and BRL, 1986). The Agrobacterium tumefaciens strain used was the disarmed AGL0 (Lazo et al., 1991).

Plant Material

1 5 The *Petunia hybrida* varieties used are indicated in Table 4, overleaf.

Flowers of *Dianthus caryophyllus* cv. Laguna were obtained from Van Wyk and Son Flower Supply, Victoria.

20 Chrysanthemum morifolium cultivars were obtained from Baguley Flower and Plant Growers, Victoria.

EXAMPLE 2 - PLANT GROWING CONDITIONS & STAGES

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Growth of plants

Petunia hybrida plants were grown in specialised growth rooms with a 14 hour day length at a light intensity of 10,000 lux and a temperature of 22 to 26°C. OGB flowers were harvested at developmental stages defined as follows:

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Stage 1: Unpigmented, closed bud (<25 mm in length).

Stage 2: Pigmented, closed bud (25-35 mm in length).

Stage 3: Dark purple bud with emerging corolla (>35 mm in length).

Stage 4: Dark purple opened flower pre-anther dehiscence (>50 mm in length).

3.5 Stage 5: Fully opened flower with all anthers dehisced.

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Plant variety	Genotype	Source/Reference
Old Glory Blue (F1 Hybrid)	An1, An2, An3, An4, An6, An8, An9, An10, An11, Hf1, Ht1, Rt, Po, Bl, Fl (inferred from phenotype)	Ball Seed, USA
V23	An1, An2, An3, An4, An6 An8, An9, An10, ph1, Hf1, Hf2, ht1, Rt, po, Bl, Fl	Wallroth <i>et al.</i> 1986 Doodeman <i>et al.</i> 1984
R51	An1, An2, An3, an4, An6, An8, An9, An10, An11, Ph1, hf1, hf2, Ht1, rt, Po, bl, fl	Wallroth <i>et al.</i> 1986 van Tunen <i>et al.</i> 1990 Doodeman <i>et al.</i> 1984
Ba20	an1, An2 an4, An6, hf1, hf2, Ht1, Ph1, Ph2, Ph5, Gf, mf1, mf2, Rt, fl	E. Farcy INRA, Dijon
V26	An1, An2, An3, an4, An6, An8, An9, An10, An11, Ht1, Hf1, hf2, Ph1, ph2, Ph5, mf1, mf2, Mt1, Mt2, po, Gf, Rt, Fl	A. Gerats Free University Amsterdam

TABLE 4

Flowers of the other petunia varieties were harvested prior to anther dehiscence, at the 30 stage of maximum pigment accumulation.

Stages of Dianthus caryophyllus flower development were defined as follows:

Stage 1: No visible flower bud.

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Stage 2: Flower buds opening: tips of florets visible.

Stage 3: Tips of nearly all florets exposed; outer florets opening, none horizontal.

Stage 4: Outer florets horizontal.

Stages of Chrysanthemum flower development were defined as follows:

Stage 0: No visible flower bud.

Stage 1: Flower bud visible: florets completely covered by the bracts.

Stage 2: Flower buds opening: tips of florets visible.

Stage 3: Florets tightly overlapped.

Stage 4: Tips of nearly all florets exposed; outer florets opening but none horizontal.

Stage 5: Outer florets horizontal.

10 Stage 6: Flower approaching maturity.

EXAMPLE 3

15 Synthesis of Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems PCR-Mate DNA synthesiser using methods recommended by the manufacturer. The oligonucleotides synthesised were, 5'-3':

Oligo 2:	TGGGGIITITT(T,C)(C,G)AIITI(A,G)TI(A,G)AICA	SEQ ID No:7
Oligo 3:	TI(A,G)TIAA(T,C)CA(T,C)GGI(A,T)TICC	SEQ ID No:8
Oligo 4:	GGI(T,C)TITT(T,C)(C,G)A(A,G)ITI(A,G)TIAA(T,C)	CA(T,C)GG
		SEQ ID No:9

	Oligo 5:	GG(T,C)TTIGG(A,G)CAIGGIGG(A,G)TA	SEQ ID No:10
	Oligo 6:	GG(A,G)CAIGGIGG(A,G)TA(A,G)TA(A,G)TT	SEQ ID No:11
	Oligo 7:	ATCAGAGTACATTAGGTC	SEQ ID No:12
30	Oligo 8:	GTCCCAAATGAAGTCCAAG	SEQ ID No:13
	Oligo 9:	TTCTTCGTTTGCTTCCCT	SEQ ID No:14
	Oligo 10	CTAGGTACCGGGCCCAAAGGATCCTCTAGAGTAC	SEQ ID No:15
	Oligo 11	: TCTAGAGGATCCTTTGGGCCCGGTAC	SEQ ID No:16

3 5 Where two nucleotides are given in parentheses, this indicates a choice of one or other of the nucleotides; the abbreviation "I" represents deoxyinosine.

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EXAMPLE 4 - CLONING OF A DIOXYGENASE FROM PETUNIA

Construction of a *petunia* cDNA library

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Total RNA was isolated from the petal tissue of *P. hybrida* cv. OGB stage 3 to 4 flowers using the method of Turpen and Griffith (1986). Poly $(A)^+$ RNA was selected from the total RNA by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, 1972).

Two micrograms of poly(A)⁺ RNA were reverse transcribed in a 20 µL volume
containing 1 x Superscript[™] reaction buffer, 10 mM dithiothreitol, 500 µM dATP, 500 µM dGTP, 500 µM dTTP, 500 µM 5-methyl-dCTP, 0.75 µg Oligo 1 (SEQ ID No:6) and 2 µL Superscript[™] reverse transcriptase (BRL). The reaction mix was incubated at 37°C for 50 minutes, 44°C for 10 minutes, then placed on ice.

- 15 Second strand reaction mix (140 μL) was added to the first strand reaction. The second strand reaction mix consisted of 21 mM Tris-HCl, 104 mM KCl, 5.3 mM MgCl₂, 171 μM β-NAD, 11.4 mM (NH₄)₂SO₄, 214 μM dATP, 642 μM dCTP, 214 mM dGTP, 214 μM dTTP, 4 mM DTT, 10 μCi ³²P-dCTP (3000 Ci/mmole), 15 units *E. coli* DNA ligase, 40 units *E. coli* DNA polymerase I (Boehringer) and 0.8 units
- 20 RNAse H. The final mixture was incubated for 150 minutes at 16°C. To make the double-stranded cDNA blunt-ended, 10 units of T4 DNA polymerase was added, and the reaction was continued for a further 15 minutes at 16°C. The reaction was stopped and the cDNA purified by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation.
- 25

EcoRI adaptors (Promega) were ligated with the cDNA and then kinased with polynucleotide kinase (Amersham) using conditions recommended by the manufacturer. The enzymes were denatured by heat (70°C for 20 minutes) and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The

- 30 cDNA was digested with 50 units <u>Xho</u>I (Boehringer) in a reaction volume of 100 μL, using conditions recommended by the manufacturer. The enzyme was heat killed (70°C for 20 minutes) and the cDNA digest passed through a Sephacryl S400 spun column (Pharmacia) which had been equilibrated in STE buffer (Sambrook *et al.*, 1989). The eluate was phenol/chloroform extracted and ethanol precipitated. After
 - 3 5 microcentrifugation at 4°C for 30 minutes the cDNA pellet was rinsed with 70% (v/v) ethanol, air dried and resuspended in 10 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

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One-quarter of the cDNA (2.5 μ L) was ligated with 1 μ g of λ ZAPII EcoRI/XhoI/ CIAP treated vector (Stratagene) in 5 µL reaction buffer consisting of 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP and 2 units of T4 DNA ligase. The reaction was incubated at 4°C for 4 days.

After incubating at room temperature for two hours, the ligation reaction mixture was packaged using the Packagene system (Promega). The total number of recombinants was 1 x 10⁶. An amount of 1 x 10⁶ plaque forming units (pfu) of the packaged cDNA was plated at 50,000 pfu per 15 cm diameter plate after transfecting E. coli PLK-F' cells. The plates were incubated at 37°C for eight hours, then stored overnight at 4°C. Phage were eluted from the plates into phage storage buffer (8 mM MgSO4, 100 mM NaCl, 0.01% (w/v) gelatin, 50 mM Tris-HCl, pH 8.0) to form an amplified cDNA library stock.

15 Design of dioxygenase oligonucleotide primers

A number of dioxygenases have been sequenced, from organisms as diverse as plants (Matsuda et al., 1991; Martin et al., 1991), fungi and bacteria (Cohen et al., 1990). A characteristic of all these enzymes is the existence of a number of small regions of sequence conservation. Amino acid sequences of a number of different plant 20 dioxygenases were aligned using the CLUSTAL programs of Higgins and Sharp (1988). The sequences used were Candi (Martin et al., 1991), hyoscyamine 6β hydroxylase (H6H) (Matsuda et al., 1991), flavanone 3-hydroxylase (F3H), E8 (Deikman and Fischer, 1988), A2 (Menssen et al., 1990) and Tom13 (Holdsworth et al., 1987). This analysis revealed two well-conserved regions, shown in Table 5:

	. · · · · · · · · · · · · · · · · · · ·			
	Candi (snapdragon)	WGVMHLINHGVP		NYYPKCPQP
	H6H (Hyoscyamus niger)	FGLFQVINHGFP	÷	NYYPPCPDP
	F3H (barley)	WGIFQVIDHGVD		NFYPRCPQP
30	E8 (tomato)	WGFFQVVNHGIP	 -	NYYPPCPQP
	A2 (maize)	WGVMHIAGHGIP		NYYPRCPQP
	Tom13 (tomato)	WGFFELVNHGIP		SNYPPCPKP
	Consensus	wGffqvinHGip		nyYPpCPqP
35		f imhlvd vd		sf r d
		v eiag f		n k k

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Oligonucleotides were designed to hybridise to genes encoding sequences similar to the consensus sequences. The sequences of each of these oligonucleotides, designated Oligo 2-6 (SEQ ID No's 7 - 11), are shown above. The inclusion of deoxyinosine (I) covered the different possibilities for codon usage where more than two codons could encode the same amino acid. Deoxyinosine base-pairs with similar efficiency to A, T, G and C (Martin et al., 1985; Ohtsuka et al., 1985).

PCR amplification of petunia dioxygenase gene fragments

10 Total RNA was isolated from stages 3-4 flowers of Ba20 (*flfl*) and V26 (*FlFl*). 25 µg of tota¹ \therefore NA was ethanol precipitated, pelleted and resuspended in 10.5 μ L water. One μ L (0.5 μ g) of Oligo 1 (SEO ID No:6) was added and the mixture was heated at 70°C for 10 minutes then placed on ice. The following were then added: 4 µL Superscript[™] reaction buffer (5x stock), 2 µL of 100 mM dithiothreitol, 0.5 µL of 5 mM dATP, 0.5 µL of 5 mM dCTP, 0.5 µL of 5 mM dGTP, 0.5 µL of 5 mM dTTP, and 0.5 μ L of $[\alpha - 3^{2}P]$ -dCTP. The mixture was incubated at 37°C for 2 minutes. Following the addition of 1 µL (200 units) of Superscript[™] reverse transcriptase, the reaction was incubated at 37°C for 60 minutes and then terminated by the addition of 80 The cDNA was purified by Sephacryl S200 spun-column µL of STE. 20 chromatography, followed by ethanol precipitation and resuspension in 100 μ L TE buffer. This cDNA was used as the template for PCR.

PCR reactions for amplification of petunia dioxygenase gene fragments contained 4 µL of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.01% (w/v) gelatin, 0.2 mM each dNTP, 0.4 µM each primer and 1.25 units Tag polymerase (Cetus). Reaction mixes (50 µL) were cycled 40 times: 94°C for 50 seconds; 42°C for 1 minute; 72°C for 1 minute. Fifteen microlitres of each PCR was electrophoresed on a 1.25% (w/v) agarose gel. DNA fragments in the size range 300-500 bp were collected onto NA-45 membrane. The DNA was eluted from the membrane and then ethanol 30 precipitated, pelleted by centrifugation and resuspended in 25 μ L of TE buffer. One μ L of DNA fragments from each of the V26 PCRs were pooled and ³²P-labelled using an oligo-labelling kit (BRESATEC). DNA fragments from the Ba20 PCRs were labelled in a similar manner.

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Isolation of dioxygenase homologues from a *petunia* petal cDNA library Duplicate lifts of 16,000 plaques were hybridised with 5 x 10⁵ cpm/ μ L of either the V26 probe or the Ba20 probe, and washed as follows: High stringency conditions (hybridisation: 50% (v/v) formamide, 6 x SSC, 1% (w/v) SDS at 42°C for 16 hours and washing: 2 x SSC, 1% (w/v) SDS at 65°C for 2 x 15 minutes followed by 0.2 x SSC, 1% (w/v) SDS at 65°C for 2 x 15 minutes) were used to detect sibling clones. Fourteen clones hybridised to the V26 probe but not the Ba20 probe. A further 12 clones hybridised more strongly to the V26 probe than the Ba20 probe.

10 Plasmid cDNA clones in pBluescript were rescued from λ ZAPII clones using the helper phage R408 (Stratagene).

DNA sequencing of this and other clones was performed essentially by the method of Sanger *et al.* (1977) using the Sequenase enzyme (USB, version 2.1).

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EXAMPLE 5 - NORTHERN ANALYSIS

Total RNA was isolated from tissue that had been frozen in liquid nitrogen and ground 20 to a fine powder using a mortar and pestle. An extraction buffer of 4 M guanidium isothiocyanate, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.1% (v/v) Sarkosyl, was added to the tissue and the mixture was homogenised for 1 minute using a polytron at maximum speed. The suspension was filtered through Miracloth (Calbiochem) and centrifuged in a JA20 rotor for 10 minutes at 10,000 rpm. The supernatant was 25 collected and made to 0.2 g/ mL CsCl (w/v). Samples were then layered over a 10 mL cushion of 5.7 M CsCl, 50 mM EDTA (pH 7.0) in 38.5 mL Quick- seal centrifuge tubes (Beckman) and centrifuged at 42,000 rpm for 16 hours at 25°C in a 70Ti rotor. Pellets were resuspended in TE/SDS (10 mM Tris-HCl (pH 7.5),1 mM EDTA, 0.1% (w/v) SDS) and extracted with phenol:chloroform:isoamyl alcohol (25:24:1) saturated 30 in 10 mM EDTA (pH 7.5). Following ethanol precipitation, the RNA pellets were resuspended in TE/SDS.

RNA samples (20 µg) were electrophoresed through a 2.2 M formaldehyde / 1.2% (w/v) agarose gel using running buffer containing 20 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was transferred to Hybond-N membrane (Amersham) as recommended by the manufacturer and probed with ³²P-labelled 0.9 kb <u>Eco</u>RI-XhoI

pDIOXC3 cDNA fragment (10^8 cpm/µg, 2 x 10^6 cpm/µL). Prehybridisation (one hour at 42°C) and hybridisation (16 hours at 42°C) were carried out in 50% (v/v) formamide, 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate. Degraded salmon sperm DNA ($100 \mu g/mL$) was added with the ³²P-labelled probe for the hybridisation step. Filters were washed in 2 x SSC/ 1% (w/v) SDS at 65°C for 1 to 2 hours and then 0.2 x SSC/ 1% (w/v) SDS at 65°C for 30 to 60 minutes. Filters were exposed to Kodak XAR film with an intensifying screen at -70°C for 48 hours (Figure 2).

RNA gel blot analysis revealed that the gene corresponding to the cDNA clone
pDIOXC3 was expressed at the highest level during Stage 1 of flower development
and then declined. The expression pattern is similar to that of FLS enzyme activity in
petunia flowers (Forkmann *et al.*, 1986).

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EXAMPLE 6 - RFLP MAPPING OF pDIOXC3

There is one genetic locus in *P. hybrida*, *Fl*, that controls FLS activity. It was therefore expected that a cDNA clone encoding a *P. hybrida* FLS would map to the *Fl* locus, provided that the *Fl* locus encodes the structural gene for FLS. *Fl* has been mapped to chromosome II of the *P. hybrida* genome and is linked to within 2% recombination of the *PAcl* gene (Cornu *et al.*, 1990). RFLP analysis of DNA isolated from an F_2 population of plants derived from a cross between the inbred lines V23 (*FlFl*) and R51 (*flfl*) was used to obtain linkage data for the various dioxygenase homologues.

2.5

Isolation of Genomic DNA

DNA was isolated from leaf tissue of V23 x R51 F₂ plants essentially as described by Dellaporta *et al.*, (1983). The DNA preparations were further purified by CsCl buoyant density centrifugation (Sambrook *et al.*, 1989).

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Southern blots

The genomic DNA (10 μ g) was digested for 16 hours with 60 units of <u>Xba</u>I and electrophoresed through a 0.7% (w/v) agarose gel in a running buffer of TAE (40 mM Tris-acetate, 50 mM EDTA). The DNA was then denatured in denaturing solution (1.5 M NaCl/0.5 M NaOH) for 1 to 1.5 hours, neutralised in 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl for 2 hours and the DNA was then transferred to a Hybond-N (Amersham) filter in 20 x SSC.

DNA fragments (50 to 100 ng) were radioactively labelled with 50 μ Ci of [α -³²P]dCTP using an oligolabelling kit (Bresatec). Unincorporated [α -³²P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column. A <u>PAc</u>1 probe was synthesised from a 2.7 kb <u>HindIII/Bam</u>HI fragment of vPAc1 (Baird and Meagher, 1987). A pDIOXC3 cDNA probe was synthesised from a 0.9 kb <u>Eco</u>RI-XhoI fragment of pDIOXC3.

Duplicate Southern blots of genomic DNA digested with <u>Xba</u>I were hybridised with
either the pDIOXC3 probe or the <u>PAc1</u> probe, to detect RFLP patterns. For 40 out of the 42 plants analysed there was co-segregation of the V23, VR and R51 RFLP patterns for <u>PAc1</u> with the corresponding RFLP patterns of pDIOXC3, demonstrating the corresponding genes are closely linked (4.7% recombination).

1 5 These data provided strong evidence that the gene corresponding to pDIOXC3 is linked to the *Fl* locus. This linkage, as well as the Northern analysis, provided circumstantial evidence that the pDIOXC3 cDNA might encode FLS.

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EXAMPLE 7 - ISOLATION OF FULL-LENGTH SIBLING cDNA CLONES OF pDIOXC3

From preliminary sequence analysis it was shown that pDIOXC3 did not represent a full-length clone of the corresponding transcript. To obtain a full-length version of 25 pDIOXC3, approximately 20,000 recombinants from the cDNA library were screened for clones that hybridised to the 0.9 kb EcoRI-XhoI fragment from pDIOXC3. Six clones produced strong hybridisation signals and were chosen for further analysis. A number of clones appeared to be full-length based on agreement between the size of the cDNA insert and the mRNA. The complete sequence of the cDNA insert from one of 30 these clones, designated pCGP481, was determined by compilation of sequence from different pBluescript subclones obtained using standard cloning procedures (Sambrook et al., 1989). For some regions it was necessary to synthesise specific oligonucleotide primers (Oligos 7-9; SEQ ID No's 12 - 14) to obtain overlapping sequence data. The complete nucleotide sequence and deduced amino acid sequence of pCGP481 is shown 35 as SEQ ID No:1.

EXAMPLE 8 - EXPRESSION OF PCGP481 cDNA IN YEAST

Construction of the yeast expression vector pYGA22m

M13-mp18 was digested with <u>Eco</u>RI and <u>BgI</u>II to produce a 700 bp fragment that contained a multicloning site. This fragment was ligated with the 9 kb <u>Eco</u>RI-<u>BgIII</u> fragment from pYGA2269 (Ashikari *et al.*, 1989). The resulting construct, designated pYGA22m, contained the multicloning site inserted downstream of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter.

10 Construction of pCGP631

A 1.3 kb <u>EcoRI-Xho</u>l fragment that included the entire cDNA insert from pCGP481 was ligated with the 9 kb <u>EcoRI/Sal</u>I fragment from pYGA22m. The resulting plasmid, designated pCGP631 (Figure 4), contained the pCGP481 cDNA fragment ligated in a sense orientation behind the glyceraldehyde-3-phosphate dehydrogenase promoter.

Yeast transformation

The yeast strain G-1315 (Matα, trpl) (Ashikara *et al.*, 1989) was transformed with pCGP631 according to Ito *et al.*, (1983). The transformants were selected by their ability to restore G-1315 to tryptophan prototrophy.

Preparation of yeast extracts for assay of FLS activity

Single isolates of G-1315/pCGP631 and a G-1315 revertant that grew on media lacking tryptophan were used to inoculate 10 mL of YNBC {1.2% (w/v) yeast nitrogen base without amino acids (Difco) and 0.3% (w/v) Casamino acid (Difco)} and incubated with shaking for 2 days at 30°C.

Yeast cells were harvested by centrifugation, washed once with TE buffer and resuspended in 100 µL of buffer B (10 mM Tris-HCl (pH 7.5), 1.2 M sorbitol, 0.1
30 mM DTT, 0.1 mM EDTA) containing zymolyase (0.1 mg/mL) (Seikagakukogyo, Japan) and kept at 30°C for 1 hour. Spheroplasts were collected by centrifugation and resuspended in 500 µL of 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol and 1 mM PMSF. The suspension was then vortexed with glass beads (diameter = 0.4 mm) for 2 minutes. The supernatant after centrifugation 35 was used as a crude extract.

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FLS assay of yeast enzyme extracts

FLS activity was measured by the method of Forkmann et al. (1986) with modifications. The reaction mixture contained in a total of 200 μL: 0.1 M potassium
phosphate (pH 7.0), 1.4 mM 2-mercaptoethanol, 250 μM 2-oxoglutarate, 5 mM ascorbic acid, 50 μM ferrous sulphate, 5000 cpm of ¹⁴C-DHQ and 40 μL of crude extract. Incubation was carried out for 10 minutes or 1 hour at 30°C. The mixture was immediately extracted with 500 μL of ethyl acetate and chromatographed on a cellulose plate (Merck Art 5577, Germany) with Forestal (acetic acid: HCl: water = 30: 3: 10)
along with un. belled DHQ and quercetin. Radioactivity was localised by

- autoradiography. An enzyme extract prepared from G-1315/ pCGP631 was shown to have FLS activity while an equivalent fraction prepared from non-transformed yeast had no activity (Figure 5).
- 15 The yeast expression results confirmed that the cDNA insert from pCGP481 encoded an FLS enzyme. Forkmann *et al.* (1986) suggested that two enzymes, a 2-hydroxylase and a dehydratase, are necessary for the conversion of dihydroflavonols to flavonols. However, the results show that expression the enzyme encoded by the petunia FLS cDNA clone in yeast is sufficient for this conversion, suggesting that only one enzyme is required for the conversion of dihydroflavonols to flavonols.

EXAMPLE 9 - MANIPULATION OF FLAVONOL AND ANTHOCYANIN SYNTHESIS IN TRANSGENIC PLANTS

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Binary constructs

The binary expression vector pCGP478 was constructed by replacing the <u>XbaI-KpnI</u> fragment of the multiple cloning site from pCGP293 (Brugliera *et al.*, 1993) with a synthetic polylinker containing sites for <u>XbaI</u>, <u>BamHI</u>, <u>ApaI</u> and <u>Asp</u>718. The synthetic polylinker was made by annealing the two oligonucleotides Oligo 10 and Oligo 11 (SEQ ID No's 15 and 16). The order of the restriction enzyme sites between the *MAC* promoter and the *mas* terminator of pCGP478 facilitates direct subcloning of cDNA inserts from directional λ ZAPII clones in an antisense orientation.

3 5 A 1.2 kb <u>Xba</u>I/<u>Asp</u>718 fragment containing the complete cDNA from pCGP481 was cloned in an antisense orientation between the *MAC* promoter and *mas* terminator of pCGP478 to create pCGP479. The plasmid pCGP479 was introduced into

Agrobacterium tumefaciens strain AGL0 (Lazo et al., 1991) using the method of Gynheung et al. (1988). Cells of A. tumefaciens carrying pCGP479 were selected on MG/L agar plates containing 100 μ g/mL gentamycin.

A 1.2 kb <u>Xbal/Asp</u>718 fragment containing the cDNA from pCGP481 was cloned in a sense orientation between the *MAC* promoter and *mas* terminator of pCGP293 to create pCGP482. The plasmid pCGP482 was introduced into *A. tumefaciens* strain AGL0 (Lazo *et al.*, 1991) using the method of Gynheung *et al.* (1988). Cells of *A. tumefaciens* carrying pCGP482 were selected on MG/L agar plates containing 100 μg/mL gentamycin.

Production of transgenic plants

Petunia cv. VR (Flfl) plants were transformed by co-cultivation of leaf discs with AGL0/pCGP479 using the method of Horsch et al. (1985). Transgenic plants were
grown to flowering and scored for altered flower colour compared with non-transformed VR flowers. Four out of 12 transgenic plants produced redder flowers than non-transgenic controls. Apart from the change in flower colour in the transgenic petunias no other effects of antisense expression of the FLS cDNA were observed.

20 Petunia cv. Old Glory Blue plants were transformed by co-cultivation of leaf discs with AGL0/pCGP482 using the method of Horsch et al. (1985). Transgenic plants were grown to flowering and scored for altered flower colour compared with non-transformed Old Glory Blue flowers. Three out of 15 transgenic plants produced redder flowers than non-transgenic controls. Non-transgenic Old Glory Blue flowers
25 are generally blue-violet while the colours of the Old Glory Blue flowers transformed with pCGP482 ranged in colour from blue-violet to purple.

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) were transformed by co-cultivation of leaf discs with AGL0/pCGP479 using the method of Horsch *et al.* (1985). Tobacco
flowers are normally light pink and produce low levels of cyanidin derivatives in the limb of the corolla. Transformation of tobacco with the antisense FLS gene construct caused a reduction in flavonol production and lead to the production of red flowers. Red pigmentation was also increased in the filaments. The red flower colour was due to a three-fold increase in anthocyanin production in the corolla limb.

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The colour changes observed may be described in terms of the numbers from the Royal Horticultural Society's Colour Chart as shown in Table 6, overleaf:

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29

TABLE 6

MODIFICATION OF FLOWER COLOUR IN TRANSGENIC PLANTS

Control: Petunia cv.VR Purple-violet 80Å Transgenic: VR/pCGP479 Red-purple 74A Control: Petunia cv. Old Glory Blue Violet-blue 89A - 89C Transgenic: OGB/pCGP482 Violet 83A - 83B Control: Nicotiana tabacum cv. Xanthi Red-purple 65B Transgenic: Xanthi/pCGP479 Red 51B

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It should be noted, however, that other biochemical and physiological conditions will affect the individual outcome and the citing of the specific colour change achieved by expression of the FLS sense and antisense constructs in transgenic plants should not be interpreted as limiting the possible range of colour changes which may be observed.

20

Extraction and analysis of flavonoids from flowers

Flavonol aglycones were isolated from petunia flowers by boiling a single corolla in 1 mL of 2 M HCl for 30 minutes and extracting the flavonoids with 150 μ L ethyl acetate. For thin layer chromatography (TLC), 4 μ L of ethyl acetate extracts and 4 μ L flavonol standards (kaempferol, quercetin and myricetin) were applied to a TLC plate and developed as described above. Flavonols were visualised under UV light after fuming with ammonia.

25

Flavonols extracted from petuniaVR and petunia VR/pCGP479 flowers were analysed
 by thin layer chromatography. The red VR/pCGP479 flowers produced markedly less
 flavonols than non-transgenic VR flowers. Flowers from tobacco plants transformed
 with pCGP479 were similarly analysed and found to have reduced flavonol content.

Anthocyanins were extracted from petunia and tobacco flowers with 0.5% HCl in 35 methanol. Anthocyanin concentrations were estimated from A₅₃₀ measurements of the extracts as described by Gerats (1985).

EXAMPLE 10 - ISOLATION OF cDNA HOMOLOGUES FROM NICOTIANA

Construction and screening of a Nicotiana alata cDNA library

5 A cDNA library in the vector λ ZAP was made from RNA isolated from styles of S6S6 Nicotiana alata as described by Chen et al. (1992).

Approximately 36,000 cDNA clones were hybridised with 32 P-labelled pCGP481 cDNA fragment in 6 x SSC, 35% (v/v) formamide, 1% (w/v) SDS for 16 hours at

- 10 42°C. The filters were washed under medium stringency conditions in 2 x SSC, 1% SDS at 65°C and then autoradiographed. Hybridising plaques were picked off into PSB to allow the phage to elute. Eight plasmid clones were rescued using the single-stranded helper phage VCSM13 (Stratagene). The clone containing the largest cDNA insert, pCGP489, was sequenced (SEQ ID No:2) and showed 88% similarity to the
- 15 *Petunia* FLS genetic sequence at the nucleotide level, and 91% similarity over 241 amino acids to the *Petunia* FLS sequence encoded by pCGP481.

Construction and screening of a Nicotiana sylvestris cDNA library

A cDNA library in the vector λ ZAPII was made from RNA isolated from styles of *Nicotiana sylvestris* using methods described by Chen *et al.* (1992).

Approximately 120,000 cDNA clones were hybridised with ³²P-labelled pCGP481 cDNA fragment in low stringency hybridisation buffer (6xSSC, 35% (v/v) formamide, 1% (w/v) SDS) for 16 hours at 42°C. The filters were washed in 2xSSC, 1% (w/v)
2 5 SDS at 65°C and then autoradiographed. Hybridising plaques were picked off into PSB to allow the phage to elute. Three plasmid clones were rescued using the single-stranded helper phage VCSM13. The clone containing the largest cDNA insert, pCGP490, was sequenced (SEQ ID No:3) and showed 84% similarity to the ^Detunia FLS genetic sequence at the nucleotide level, and 93% similarity over the 45 amino acid sequence to the *Petunia* FLS sequence encoded by pCGP481.

A comparison of the amino acid sequences of the two *Nicotiana* cDNA clones pCGP489 (SEQ ID No:2) and pCGP490 (SEQ ID No:3) with the amino acid sequence of the petunia FLS cDNA clone (SEQ ID No:1) is shown in Table 7, overleaf.

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TABLE 7

COMPARISON OF SEQUENCES FROM NICOTIANA WITH pCGP481

	70 *	80 *	90	100 *	110 *	1
	RDPDENKMVKLIAD	ASKEWGIFQL	INHGIPDEAI	ADLQKVGKEF		
pCGP489 ²	·				VPQEEK	•
			4.5.0			
	130	140	150 *	160 *	170	:
PetFLS pCGP489	GSNDIEGYGTSLQK GSqnIEGYGTSLQK					
	••	. •		•		•
	190	200	210	220	230	:
	*	*	*	*	*	
PetFLS	EVVDRTEKSLSLG	GLEGHEMTEA	AGGDEIVVII	KINYYPPOP	PDLALGVVA	нтом
PetFLS pCGP489	EVVDRIFKSLSLGL EVaekmFKSLSLGL					
pCGP489	EVaekmFKSLSLGL ••••• 250 *	GLEaHEMMEA • • 260 *	AGGedIVYLL •• 270 *	KINYYPPCPF 280 *	290	HTDM
pCGP489 PetFLS	EVaekmFKSLSLGL	GLEaHEMMEA 260 * *	AGGedIVYLL •• 270 * TIPNALIVHIG	KINYYPPCPF 280 * EDQVEILSNGF	290 290	HTDM VNKDI
pCGP489 PetFLS	EVaekmFKSLSLGL 250 TILVPNEVQGLQVF	GLEaHEMMEA 260 * *	AGGedIVYLL •• 270 * TIPNALIVHIG	KINYYPPCPF 280 * EDQVEILSNGF	290 290	HTDM VNKD
pCGP489 PetFLS	EVaekmFKSLSLGL •••• 250 * TILVPNEVQGLQVF TILVPNEVQGLQVF	GLEaHEMMEA 260 * YKDGHWYDVKY YKDGHWYDVnY	AGGedIVYLI •• 270 * TIPNALIVHIG	KINYYPPCPF 280 * DQVEILSNGF DQ1EILSNGF	290 290	HTDM VNKD
pCGP489 PetFLS	EVaekmFKSLSLGL 250 TILVPNEVQGLQVF	GLEaHEMMEA 260 * *	AGGedIVYLL •• 270 * TIPNALIVHIG	KINYYPPCPF 280 * EDQVEILSNGF	290 290	HTDM VNKD
PetFLS pCGP489 PetFLS pCGP489	EVaekmFKSLSLGL 250 * TILVPNEVQGLQVF TILVPNEVQGLQVF 310 * MSWPVFLEPPSEF MSWPVFLEPPSEF	GLEaHEMMEA 260 * KDGHWYDVKY KDGHWYDVNY 320 # HEVGPIPKLLS HEVGPIPKLVT	AGGedIVYLL 270 * TIPNALIVHIC 330 * EEANPPKFKTF	KINYYPPCPF 280 * DQVEILSNGF DQIEILSNGF * 340 * KYKDYVYCKI KYKDYVYCKI	290 290 * XYKSVYHRTT XYKSVYHRTT LNKLPQ LNKLPQ	HTDM VNKD
pCGP489 PetFLS pCGP489 PetFLS	EVaekmFKSLSLGL 250 * TILVPNEVQGLQVF TILVPNEVQGLQVF 310 * MSWPVFLEPPSEF MSWPVFLEPPSEF	GLEaHEMMEA 260 * * * * * * * * * * * * * * * * * * *	AGGedIVYLL 270 * TPNALIVHIC JPNALIVHIC 330 * EANPPKFKTH	KINYYPPCPF 280 * DQVEILSNGF DQIEILSNGF * 340 * KYKDYVYCKI KYKDYVYCKI	290 290 * XYKSVYHRTT XYKSVYHRTT LNKLPQ LNKLPQ	HTDM VNKD

² Nicotiana alata sequence (SEQ ID No:2)

4 5 ³ Nicotiana sylvestris sequence (SEQ ID No:3)

• Amino acid mismatches in the compared sequences; all other amino acids are identical over the sequence lengths compared.

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EXAMPLE 11 - ISOLATION OF cDNA HOMOLOGUE FROM *DIANTHUS*

5 Construction of a Dianthus cDNA library

Total RNA was isolated from the petal tissue of *D. caryophyllus* cv. Laguna stage 3 flowers, using the method of Turpen and Griffith (1986). Poly(A)⁺ RNA was selected from the total RNA by Oligotex dT-30 (Takana, Japan) following the manufacturer's protocol.

10

cDNA was made and a library constructed in λ ZAPII according to the protocol used for the *petunia* library.

The primary library, which contained 150,000 pfu, was plated at 37,500 pfu per 15 cm
1 5 diameter plate after transfecting *E. coli* SURE cells. The plates were incubated at 37°C for eight hours, then stored overnight at 4°C. Phage were eluted from the plates into phage storage buffer (8 mM MgSO4, 100 mM NaCl, 0.01% (w/v) gelatin, 50 mM Tris-HCl, pH 8.0) to form an amplified cDNA library stock.

20 Isolation of a FLS homologue from a Dianthus cDNA library

A total of 100,000 plaques were screened, in duplicate, with ³²P-labelled 1.1 kb <u>EcoRI-HindII pDIOXC3 cDNA fragment (5 x 10⁵ cpm/µL)</u>. Hybridisation was carried out in a low stringency buffer (6 x SSC, 0.5% (w/v) SDS, 5 x Denhardt's solution, 0.01 M EDTA, 100 µg/ml) for 16 hours at 42°C. The filters were washed in 2 x SSC/1% (w/v) SDS at 65°C and then autoradiographed. Hybridised cDNA clones were rescued from λZAPII using the single-stranded helper phage Exassist (Stratagene) according to the manufacturer's instructions.

When sequenced, one of the isolated clones, pCGP777 (SEQ ID No:4), revealed 65%
30 similarity at both the nucleotide level and the amino acid level to the *Petunia* FLS sequence encoded by pCGP481.

EXAMPLE 12 - ISOLATION OF cDNA HOMOLOGUE FROM CHRYSANTHEMUM

Construction of a Chrysanthemum cDNA library

- 5 Total RNA was isolated from the petal tissue of *Chrysanthemum morifolium* cv. Dark Pink Pompom (Reference Number 5999), stages 2 and 3 flowers, again using the method of Turpen and Griffith (1986). An amount of 30 µg of the total RNA was used as template for cDNA synthesis.
- 10 Following fractionation and ligation, the cDNA reaction mixture was packaged using the Packagene system (Promega). The titre of the unamplified library was 3.7 x 10⁴ pfu/ml.

Isolation of a FLS homologue from a Chrysanthemum cDNA library

- 1 5 An amount of 90,000 pfu (of amplified library; 2.6 x 10⁷ pfu/ml) of the packaged cDNA was plated at 10,000 pfu per 15 cm diameter plate after transfecting XL1-Blue cells. The plates were incubated at 37°C overnight, then stored at 4°C. Duplicate lifts were taken onto Colony/Plaque Screen[™] filters (DuPont), treated as recommended by the manufacturer, and screened with the ³²P-labelled EcoRI-XhoI pCGP481 cDNA
 2 0 fragment. Hybridisation was carried out in a low stringency buffer (6 x SSC, 1.0% (w/v) SDS, 20% (v/v) for namide) for 16 hours at 42°C. The filters were washed twice for 30 minutes in 2 x SSC/1% SDS at 65°C, and then autoradiographed.
- The isolated clone pCGP874 (SEQ ID No:5), when sequenced, revealed 70% 25 similarity at the nucleotide level and 72% similarity at the amino acid level to the *Petunia* FLS sequence encoded by pCGP481.

EXAMPLE 13 - EXPRESSION OF PCGP874 cDNA IN YEAST

- 30 Construction of the yeast expression vector pYGA22m and pCGP492 The yeast expression vector pYGA22m was contructed as described in Example 8 above. A 1.3 kb EcoRI-XhoI fragment that included the entire cDNA insert from pCGP874 was ligated with the 9 kb EcoRI/SalI fragment from pYGA22m, in the same manner as for the construction of pCGP 631, described above and shown in Figure 4.
- 3 5 The resulting plasmid, designated pCGP492, contained the pCGP874 cDNA fragment ligated in a sense orientation behind the glyceraldehyde-3-phosphate dehydrogenase promoter.

5

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Yeast transformation and assay of FLS activity

Transformation of yeast and preparation of extracts for assay of FLS activity were carried out as described above in Example 8. FLS activity was again measured by the method of Forkmann *et al.* (1986) with modifications, using unlabelled DHK and DHQ as substrates. The results confirmed that the pCGP874 cDNA encodes a functional chrysanthemum FLS enzyme.

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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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT (other than US): INTERNATIONAL FLOWER DEVELOPME..IS PTY LTD

(US only): HOLTON, T.A., KEAM, L.A.

(ii) TITLE OF INVENTION: GENETIC SEQUENCES ENCODING FLAVONOL SYNTHASE ENZYMES AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: DAVIES COLLISON CAVE

(B) STREET: 1 LITTLE COLLINS STREET

(C) CITY: MELBOURNE

(D) STATE: VICTORIA

(E) COUNTRY: AUSTRALIA

(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: AU INTERNATIONAL

(B) FILING DATE: 05-AUG-1993

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: SLATTERY, JOHN M.

(C) REFERENCE/DOCKET NUMBER: EJH/JMS/EK

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 61 3 254 2777

(B) TELEFAX: 61 3 254 2770

(C) TELEX: AA 31787

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3

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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1211 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 59..1101

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Leu Gln Lys Val Gly Lys Glu Phe Phe Glu His Val Pro Gln Glu Glu 100 105 110

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TGG CCT AAA AAC CCT CCT TCA TAC AGG GAA GCA AAC GAA GAA TAT GGA 586 Trp Pro Lys Asn Pro Pro Ser Tyr Arg Glu Ala Asn Glu Glu Tyr Gly 165 170 175

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CCC GAT TTG GCT CTT GGT GTT GTG GCC CAT ACG GAC ATG TCA TAT ATC778Pro Asp Leu Ala Leu Gly Val Val Ala His Thr Asp Met Ser Tyr Ile225230235240

ACC ATT CTT GTC CCA AAT GAA GTC CAA GGC CTC CAA GTG TTC AAG GAT 826 Thr Ile Leu Val Pro Asn Glu Val Gln Gly Leu Gln Val Phe Lys Asp 245 250 255

GGC CAT TGG TAT GAT GTC AAG TAC ATA CCA AAT GCC TTA ATT GTC CAT 874 Gly His Trp Tyr Asp Val Lys Tyr Ile Pro Asn Ala Leu Ile Val His 260 265 270

ATT GGT GAC CAA GTT GAG ATT CTT AGC AAT GGC AAA TAC AAG AGT GTA922Ile Gly Asp Gln Val Glu Ile Leu Ser Asn Gly Lys Tyr Lys Ser Val275280285

TAC CAT AGG ACA ACG GTG AAC AAG GAC AAG ACA AGA ATG TCA TGG CCG 970Tyr His Arg Thr Thr Val Asn Lys Asp Lys Thr Arg Met Ser Trp Pro290295300

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 890 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..725

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 5
 10
 15

CAG AAT ATT GAA GGG TAT GGT ACA TCT TTG CAG AAG GAA GTT GAA GGG95Gln Asn Ile Glu Gly Tyr Gly Thr Ser Leu Gln Lys Glu Val Glu Gly20202530

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AAA AGA GGT TGG GTT GAT CAT TTG TTT CAT AAG ATT TGG CCT CCT TCT 143 Lys Arg Gly Trp Val Asp His Leu Phe His Lys Ile Trp Pro Pro Ser 35 40 45 GCC ATC AAT TAT CGT TAT TGG CCT AAA AAC CCT CCT TCC TAC AGG GAA 191 Ala Ile Asn Tyr Arg Tyr Trp Pro Lys Asn Pro Pro Ser Tyr Arg Glu 50 55 60 GCA AAT GAG GAA TAT GCA AAG AGG CTG CGA GAA GTT GCG GAG AAG ATG 239 Ala Asn Glu Glu Tyr Ala Lys Arg Leu Arg Glu Val Ala Glu Lys Met 70 65 75 TTT AAG AGC TTA TCA CTT GGG CTT GGG TTA GAA GCC CAT GAA ATG ATG 2.87 Phe Lys Ser Leu Ser Leu Gly Leu Gly Leu Glu Ala His Glu Met Met 80 85 90 95 GAG GCA GCA GGT GGT GAA GAT ATA GTT TAC TTG TTG AAG ATC AAT TAT 335 Glu Ala Ala Gly Gly Glu Asp Ile Val Tyr Leu Leu Lys Ile Asn Tyr 100 105 110 TAC CCA CCA TGC CCA AGG CCT GAT TTG GCA CTT GGA GTT GTG GCC CAT 3.83 Tyr Pro Pro Cys Pro Arg Pro Asp Leu Ala Leu Gly Val Val Ala His 115 120 125 ACA GAC ATG TCC CAT ATA ACC ATT CTT GTC CCA AAT GAA GTC CAA GGC 431 Thr Asp Met Ser His Ile Thr Ile Leu Val Pro Asn Glu Val Gln Gly 130 135 140 CTC CAA GTC TTC AAG GAT GGC CAT TGG TAT GAT GTC AAC TAC ATA CCA 479 Leu Gln Val Phe Lys Asp Gly His Trp Tyr Asp Val Asn Tyr Ile Pro 150 145 155 AAT GCC CTA ATT GTC CAC ATT GGT GAC CAA CTT GAG ATC CTT AGC AAT 527 Asn Ala Leu Ile Val His Ile Gly Asp Gln Leu Glu Ile Leu Ser Asn 160 165 170 175 GGG AAA TAC AAG AGT GTG TAT CAT AGG ACA ACA GTG ACA AAG GAT AAG 575 Gly Lys Tyr Lys Ser Val Tyr His Arg Thr Thr Val Thr Lys Asp Lys 180 185 190 ACA AGA ATG TCA TGG CCA GTT TTC TTG GAG CCA CCA TCA GAG CAT GAA 623 Thr Arg Met Ser Trp Pro Val Phe Leu Glu Pro Pro Ser Glu His Glu 200 205 195

GTT GGG CCA ATT CCT AAG CTG GTT AAT GAG GCC AAT CCA CCC AAA TTC 671 Val Gly Pro Ile Pro Lys Leu Val Asn Glu Ala Asn Pro Pro Lys Phe 210 215 220

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AAG ACC AAG AAG TAC AAG GAT TAT GTC TAT TGT AAG CTT AAC AAG CTT719Lys Thr Lys Lys Tyr Lys Asp Tyr Val Tyr Cys Lys Leu Asn Lys Leu225230235

CCT CAG TGAAGAAACT CCTCTATATA TGTTTGGCAG CGATTAGCTA CTATATGTTC 775 Pro Gln 240

GTCAGTATTA TATTATGGTT TGTACTATCC TTACCAACAG ATGTCTTATT ATGATTAAGG 835 ACTATATATT TACACTTAAA ACTTTTTGAT ACTAGCTAAT AACTGACTTA TTAAG 890

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 236 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..135

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCA GTA TTC TTG GAG CCA CCA TCA GAG CAT GAA GTA GGG CCA ATT TCT 48 Pro Val Phe Leu Glu Pro Pro Ser Glu His Glu Val Gly Pro Ile Ser 1 5 10 15 AAG CTG GTT AAT GAG GCC AAT CCA CCC AAA TTC AAG ACC AAG AAG TAC 96 Lys Leu Val Asn Glu Ala Asn Pro Pro Lys Phe Lys Thr Lys Lys Tyr 20 25 30 AAG GAT TAT GTT TAT TGT AAG CTT AAC AAG CTT CCT CAG TGAAGAAACC 145 Lys Asp Tyr Val Tyr Cys Lys Leu Asn Lys Leu Pro Gln 35 40 45

CCTCTATATA TGTTTGGCAG CGAATAGCTA GTATATTCGT GAGTACTAAA TTATGGTTTG 205

TACTATCCTT ACCAAGAGAT GTCTTATTAT G

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(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1236 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 70..1068

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

60 AATAAAAGT ATG GGC ATA TCT TCA GAA AGA GTA CAA AAC ATA GCC TTA 108 Met Gly Ile Ser Ser Glu Arg Val Gln Asn Ile Ala Leu 5 10 1 AAA TCC CAG AAC ATA GAC GAC ATT CCA CCC GAA TAC ATA AGA TTA GAG 156 Lys Ser Gln Asn Ile Asp Asp Ile Pro Pro Glu Tyr Ile Arg Leu Glu 15 20 25 GAT GAA CAA CCA GCA ATC ACA ACA GTC CTC GAC ACG GTT CTC GAG GTC 204 Asp Glu Gln Pro Ala Ile Thr Thr Val Leu Asp Thr Val Leu Glu Val 30 35 40 45 CCT GCG ATC GAC CTC AGC CTC GAG GAA GAC GAC GTT GTA AAA CTC GTC 252 Pro Ala Ile Asp Leu Ser Leu Glu Glu Asp Asp Val Val Lys Leu Val 50 55 60 300 TTG AGT GCA AGC AAA GAG TGG GGA CTG TTT CAG GTC ACC AAC CAC GGA Leu Ser Ala Ser Lys Glu Trp Gly Leu Phe Gln Val Thr Asn His Gly 65 70 75 ATT CCG ACT GAA GTC ATT GAA AAA TTG CAA AAA GTC GGT AAG ATG TTT 348 Ile Pro Thr Glu Val Ile Glu Lys Leu Gln Lys Val Gly Lys Met Phe 80 85 90

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				GCA												396
Phe		Ala	Pro	Ala	Glu		Lys	Glu	Thr	Ile		Lys	Pro	Glu	Gly	
	95					100					105					
GGT	GTT	GAA	GGG	ТАТ	GGG	ACC	ATG	TTG	CAA	AAG	GÀG	ATT	CAA	GGG	AGA	444
				Tyr												
110					115					120				_	125	
																•
				GAT												492
Lys	GIY	Trp	Val	Asp 130	His	Leu	Phe	His	Lys 135	Val	Trp	Pro	Pro		Val	
				130					122					140		
ÄТТ	AAC	TAC	CAA	TGG	TGG	ССТ	AAG	ACT	сст	TCT	тат	AGG	GAG	GCG	AAC	540
Ile	Asn	Tyr	Gln	Trp	Trp	Pro	Lys	Thr	Pro	Ser	Tyr	Arg	Glu	Ala	Asn	
			145					150					155			
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				AAG Lys												588
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				GGA												636
Cys		Ser	Lys	Gly	Leu		Leu	Glu	Glu	Asp		Val	Lys	Lys	Ser	
	175					180					185					
TGT	GGC	ААТ	GAÀ	GAC	ATA	GTG	TAC	CTT	CTC	AAG	ATC	AAC	TAC	TAC	CCG	684
				Asp												
190					195					200					205	
				CCC												732
P10	Cys	Pro	Arg	Pro 210	Asp	Leu	Ala	Leu	215	val	AIA	AIA	HIS	220	Asp	
														220		
TTG	AGC	ĠTC	ATC	ACC	ATT	CTT	GTŤ	CCG	AAT	GAT	GTT	GCC	GGT	ĊTT	CAG	780
Leu	Ser	Val		Thr	Ile	Leu	Val	Pro	Asn	Asp	Val	Ala	Gly	Leu	Gln	
			225					230					235			
GTC	ጥርጥ	202	GAC	GGA	ĊGT	TCC	ጥልሮ	CDT	CTTC	סעע	መእር	יחית א	CCT	እእጥ	CC)	828
				Gly					-							020
		240	-	-	- 5	- •	245			-4		250				•
				GTT												876
Leu		Ile	His	Val	Gly		Gln	Met	Glu	Ile		Ser	Asn	Gly	Glu	
	255					260		÷.			265					
TAC	AAG	GCG	GTG	ĊTT	CAT	AGG	TCG	ACA	GTG	AAC	AAA	GAA	AGA	ACA	AGG	924
				Leu												
270					275					280					285	

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ATA TCG TGG CCC GTG TTC CTG GAA CCG CCA TCA GAC TTT GCA GTC GGG 972 Ile Ser Trp Pro Val Phe Leu Glu Pro Pro Ser Asp Phe Ala Val Gly 290 295 300 CCT ATT CCA AAG CTC ATT AGT GAT GAA AAG CCA GCC AAG TAT AAG ACG 1020 Pro Ile Pro Lys Leu Ile Ser Asp Glu Lys Pro Ala Lys Tyr Lys Thr 305 310 315 AAG GTG TTT TCC GAG TAC AAG TAT TGT AAG CTG AAC AAG CTA CCT ATG 1068 Lys Val Phe Ser Glu Tyr Lys Tyr Cys Lys Leu Asn Lys Leu Pro Me'. 320 325 330 TGAAGACTGA AGATGTTTAA TATTAGTCTT ATATGTTTAA TAAAGGCTAT TGTTGGTTAT 1128 CAGTTATCAC CTTGTTATTT GAATGTGCCA CTAAAATCAC TGTTAATTAA GGTGATATGG 1188 1236

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1250 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 11..1065

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCACACACA ATG GAG GTG GAA AGA GTT CAA GAA ATA GCA ACA CTC TCA Met Glu Val Glu Arg Val Gln Glu Ile Ala Thr Leu Ser 1 5 10

97

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AAC CTA AAT GGC ACA ATC CCA AGT GAG TTC ATA AGA CTG GAA AAC GAAAsn Leu Asn Gly Thr Ile Pro Ser Glu Phe Ile Arg Leu Glu Asn Glu152025

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CAA CCA GCA ACG ACC ACT CTC CAT GGC GTC TTG TTG GAG GTT CCA GTG Gln Pro Ala Thr Thr Thr Leu His Gly Val Leu Leu Glu Val Pro Val ATC GAT CTT AGC CAG GCG GAT AAC GAA TCC TTG GTC GCT TTG ATA TCC Ile Asp Leu Ser Gln Ala Asp Asn Glu Ser Leu Val Ala Leu Ile Ser AAA GCG AGC AAG GAT TGG GGT ATT TTT CAA GTG GTG AAC CAT GGG ATA Lys Ala Ser Lys Asp Trp Gly Ile Phe Gln Val Val Asn His Gly Ile CCA AGT GAA CTC ATT AGC AAG TTA CAA AAT GTT GGA AAA GAG TTC TTT Pro Ser Glu Leu Ile Ser Lys Leu Gln Asn Val Gly Lys Glu Phe Phe GAG CTA CCA CAA GAA GAA AAA GAA GTC ATT GCT AAA CCA GAT GGT TAC Glu Leu Pro Gln Glu Glu Lys Glu Val Ile Ala Lys Pro Asp Gly Tyr AAA GGT GTT GAA GGG TAT GGA ACA AAG CTT CAA AAA GAA GTT CAA GGC Lys Gly Val Glu Gly Tyr Gly Thr Lys Leu Gln Lys Glu Val Gln Gly AAG AAA GGA TGG GTG GAT CAT TTG TTT CAT ATA GTT TGG CCA CCT TCT Lys Lys Gly Trp Val Asp His Leu Phe His Ile Val Trp Pro Pro Ser TTT ATT AAC TAT CAA TTT TGG CCA AAG AAC CCT CCT TCT TAC AGA GAT Phe Ile Asn Tyr Gln Phe Trp Pro Lys Asn Pro Pro Ser Tyr Arg Asp ACA AAT GAG GAA TAC ACA CAA AGC TTG ATA GGG GTG GCA AAT AAG TTG Thr Asn Glu Glu Tyr Thr Gln Ser Leu Ile Gly Val Ala Asn Lys Leu CTT GGG CTG TTG TCA AAA GGG CTT GGA CTG GAA GAA GAT GAA GTG AAG Leu Gly Leu Leu Ser Lys Gly Leu Gly Leu Glu Glu Asp Glu Val Lys CAA GCG TTG GGT GGC GAA GAC TTG ATC TAC ATG TTG AAA ATA AAC TAC Gln Ala Leu Gly Gly Glu Asp Leu Ile Tyr Met Leu Lys Ile Asn Tyr TAC CCA CCA TGT CCA TGC CCC GAG CTT GCT CTT GGG GTA GCC CCA CAT Tyr Pro Pro Cys Pro Cys Pro Glu Leu Ala Leu Gly Val Ala Pro His

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ACC GAC ATG TCT TCA ATC ACC ATA CTT GTC CCG AAT GAA GTT CAA GGT 721 Thr Asp Met Ser Ser Ile Thr Ile Leu Val Pro Asn Glu Val Gln Gly 225 230 235 CTA CAA GTC TTT AAA GAT GGT CAA TGG TAT GAT GTT GCT TAC ATT CCT 769 Leu Gln Val Phe Lys Asp Gly Gln Trp Tyr Asp Val Ala Tyr Ile Pro 240 245 250 AAT GCT CTC ATT ATT CAC ATT GGT GAC CAG ATT GAG ATA CTG AGC AAT 817 Asn Ala Leu Ile Ile His Ile Gly Asp Gln Ile Glu Ile Leu Ser Asn 255 260 265 GGA AAA TAT AAG AGT GTG TAT CAC AGA TCA ACT GTG AAT AAG GAG AAA 865 Gly Lys Tyr Lys Ser Val Tyr His Arg Ser Thr Val Asn Lys Glu Lys 270 275 280 285 ACA AGA ATG TCG TGG CCA GCA TTT TTG GAG CCA CCG CCA GAG TTT GAG 913 Thr Arg Met Ser Trp Pro Ala Phe Leu Glu Pro Pro Glu Phe Glu 290 295 300 GTT GGT CCA ATT CCA AAG CTC GTC AAT AAA GAC GAT CCA CCA AAA TAC 961 Val Gly Pro Ile Pro Lys Leu Val Asn Lys Asp Asp Pro Pro Lys Tyr 305 310 315 AAG ACC AAG AAG TAC AAA GAC TAT GTC TAT TGC AAG CTA AAT AAG CTT 1009 Lys Thr Lys Lys Tyr Lys Asp Tyr Val Tyr Cys Lys Leu Asn Lys Leu 320 325 330 CCG CAG TGA GGG ATT TTA GAT ATT ATC TGC AAA CAT ATA TGT ATT TCT 1057 Pro Gln 335 GAA CGC AT GCGTGTGTAA GCTGTTGAGT TATTTGAATG TGGGATTGTA ATATGAGACT 1115 CTGATCGAAT TTAGTTCAAA GCTATATGAA CATACAATAA GAGGAGTAAC TCATTCATCC 1175 ACTTTGGCAT TTTCATCCTT TATATTCGAT ATTAGGTATG TATCATGCTT TACAAGGTAA 1235 ΑΑΑΑΑΑΑΑΑΑ ΛΑΑΑΑ 1250

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(ix) FEATURE:

ų,

(A) (X,Y) = either X or Y
(B) I = inosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGGGGIITTT T(T,C)(C,G)AIITI(A,G)T I(A,G)AICA

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(ix) FEATURE:

- (A) (X,Y) = either X or Y
- (B) I = inosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TI(A,G)TIAA(T,C)CA (T,C)GGI(A,T)TICC

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(ix) FEATURE:

(A) (X,Y) = either X or Y
(B) I = inosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGI(T,C)TITT(T,C)(C,G) A(A,G)ITI(A,G)TIAA (T,C)CA(T,C)GG - 26

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- (2) INFORMATION FOR SEQ ID NO:10:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(ix) FEATURE:

(A) (X,Y) = either X or Y
(B) I = inosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CJ(T,C)TTIGG(A,G)C AIGGIGG(A,G)TA

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

- (ix) FEATURE:
 - (A) (X,Y) = either X or Y
 - (B) I = inosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GG(A,G)CAIGGIG G(A,G)TA(A,G)TA(A,G)TT

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGACAAGGAG GATAATAATT

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCAGAGTAC ATTAGGTC

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCCCAAATG AAGTCCAAG

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(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: Oligonucleotide DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTCTTCGTTT GCTTCCCT

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTAGGTACCG GGCCCAAAGG ATCCTCTAGA GTAC

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a flavonol synthase (FLS) as hereinbefore defined.

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2. An isolated nucleic acid molecule according to claim 1 wherein said nucleic acid is DNA.

10 3. An isolated nucleic acid molecule according to claim 1 or 2 wherein the plant is selected from the group consisting of petunia, snapdragon, tobacco, rose, carnation, chrysanthemum, lisianthus, cyclamen, parsely and grape.

4. An isolated nucleic acid molecule according to claim 3 wherein the plant is 15 selected from the group consisting of petunia, tobacco, carnation and chrysanthemum.

5. An isolated nucleic acid molecule according to claim 4 having a nucleotide sequence or complementary nucleotide sequence which comprises the sequence as set forth in SEQ ID No:1 or having at least 50% similarity thereto.

6. An isolated nucleic acid molecule according to claim 4 having a nucleotide sequence or complementary nucleotide sequence which comprises the sequence as set forth in SEQ ID No:2 or having at least 50% similarity thereto.

25 7. An isolated nucleic acid molecule according to claim 4 having a nucleotide sequence or complementary nucleotide sequence which comprises the sequence as set forth in SEQ ID No:3 or having at least 50% similarity thereto.

An isolated nucleic acid molecule according to claim 4 having a nucleotide
 sequence or complementary nucleotide sequence which comprises the sequence as set
 forth in SEQ ID No:4 or having at least 50% similarity thereto.



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9. An isolated nucleic acid molecule according to claim 4 having a nucleotide sequence or complementary nucleotide sequence which comprises the sequence as set forth in SEQ ID No:5 or having at least 50% similarity thereto.

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5 10. An isolated DNA molecule comprising a sequence of nucleotides which

(i) encodes a FLS of plant origin; and

 (ii) has at least 50% nucleotide sequence similarity to the sequence set forth in SEQ ID No:1.

10 11. An isolated DNA molecule according to claim 10 having a nucleotide sequence as set forth in SEQ ID No:1.

12. An isolated DNA molecule according to claim 10 having a nucelotide sequence as set forth in SEQ ID No:2.

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13. An isolated DNA molecule according to claim 10 having a nucelotide sequence as set forth in SEQ ID No:3.

14. An isolated DNA molecule according to claim 10 having a nucleotide sequence20 as set forth in SEQ ID No:4.

15. An isolated DNA molecule according to claim 10 having a nucleotide sequence as set forth in SEQ ID No:5.

25 16. An isolated nucleic acid molecule which:

(i) encodes a FLS of plant origin; and

 (ii) hybridises under low stringency conditions to the nucleotide sequence set forth in SEQ ID No:1 or to a complementary strand thereof.

30 17. An isolated nucleic acid molecule according to claim 16 having a nucleotide sequence as set forth in SEQ ID No:1.

18. An isolated nucleic acid molecule according to claim 16 having a nucleotide sequence as set forth in SEQ ID No:2.

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19. An isolated nucleic acid molecule according to claim 16 having a nucleotide5 sequence as set forth in SEQ ID No:3.

20. An isolated nucleic acid molecule according to claim 16 having a nucleotide sequence as set forth in SEQ ID No:4.

10 21. An isolated nucleic acid molecule according to claim 16 having a nucleotide sequence as set forth in SEQ ID No:5.

22. A vector comprising the nucleic acid molecule according to claim 1 or 10 or 16.

15 23. A vector according to claim 22 wherein the nucleic acid molecule is operably linked to a promoter.

24. A vector according to claim 23 capable of replication and expression in a eukaryotic cell.

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25. A vector according to claim 23 capable of replication and expression in a prokaryotic cell.

26. An oligonucleotide capable of hybridising under low stringency conditions to part of the nucleotide sequence or its complementary form set forth in at least one of the sequences selected from the list consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4 and SEQ ID No:5.

27. 30

28. A transgenic plant according to claim 27 wherein the genetic sequence is capable of expression and said expression is optionally regulatable.

A transgenic plant carrying a non-indigenous genetic sequence encoding a FLS.

29. A transgenic plant according to claim 28 wherein the expression is developmentally regulated.

30. A transgenic plant according to claim 27 or 28 wherein the FLS is from a plant
5 selected from the group consisting of petunia, snapdragon, tobacco, rose, carnation, chrysanthemum, lisianthus, cyclamen, parsely and grape.

31. A transgenic plant according to claim 27 or 28 wherein the FLS is from a plant selected from the group consisting of petunia, tobacco, carnation and chrysanthemum.

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32. A transgenic plant according to claim 27 or 28 wherein said plant is selected from the group consisting of petunia, rose, carnation, chrysanthemum, gerbera, tobacco, lisianthus, lily, iris and pelargonium.

15 33. A transgenic plant according to claim 27 or 28 wherein the FLS is encoded by a nucleotide sequence as set forth in SEQ ID No:1 or having at least 50% similarity thereto.

34. A transgenic plant according to claim 27 or 28 wherein the FLS is encoded by20 a nucleotide sequence as set forth in SEQ ID No:2 or having at least 50% similarity thereto.

35. A transgenic plant according to claim 27 or 28 wherein the FLS is encoded by a nucleotide sequence as set forth in SEQ ID No:3 or having at least 50% similarity
25 thereto.

36. A transgenic plant according to claim 27 or 28 wherein the FLS is encoded by a nucleotide sequence as set forth in SEQ ID No:4 or having at least 50% similarity thereto.

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37. A transgenic plant according to claim 27 or 28 wherein the FLS is encoded by a nucleotide sequence as set forth in SEQ ID No:5 or having at least 50% similarity

thereto.

38. A transgenic plant selected from the group consisting of petunia, rose, carnation, chrysanthemum, gerbera, tobacco, lisianthus, lily, iris and pelargonium carrying a nonindigenous genetic sequence encoding a FLS, said genetic sequence optionally capable of being expressed and wherein said FLS is encoded by a DNA molecule comprising a DNA strand capable of hybridising under low stringency conditions to a nucleic acid molecule comprising all or part of the sequence of nucleotides set forth in at least one of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4 and SEQ ID No:5.

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39. A cut flower from a transgenic plant according to any one of claims 27 to 38.

40. A method for producing a transgenic flowering plant capable of exhibiting altered inflorescence properties, said method comprising introducing into a cell of a suitable
15 plant the nucleic acid molecule according to claim 1 or 10 or 16, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit expression of the nucleic acid sequence into FLS.

41. A method according to claim 40 wherein the transgenic plant is selected from the
20 group consisting of petunia, rose, carnation, chrysanthemum, gerbera, tobacco, lisianthus,
lily, iris and pelargonium.

42. A method according to claim 41 wherein the introduced nucleic acid is DNA and encodes FLS having the nucleotide sequence substantially as set forth in at least one of
25 the sequences selected from the list consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4 and SEQ ID No:5.

43. A method for producing a transgenic flowering plant capable of exhibiting altered inflorescence properties, said method comprising introducing into a cell of a plant
30 carrying an indigenous FLS, the nucleic acid according to any one of claims 1 or 10 or 16 under conditions to induce co-suppression of said indigenous FLS.



44. A method according to claim 43 wherein the transgenic plant is selected from the list consisting of petunia, rose, carnation, chrysanthemum, gerbera, tobacco, lisianthus, lily, iris and pelargonium.

- 5 45. A method according to claim 44 wherein the introduced nucleic acid is DNA and encodes FLS and has the nucleotide sequence substantially as set forth in at least one of the sequences selected from the list consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4 and SEQ ID No:5.
- 10 46. An isolated nucleic acid molecule according to any one of claims 1 to 21 or a vector according to any one of claims 22 to 25 or an oligonucleotide according to claim 26 or a transgenic plant according to any one of claims 27 to 38 or a cut flower according to claim 39 or a method according to any one of claims 39 to 42 substantially as described herein with reference to the Figures and/or Examples.
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Dated this 10th day of January, 1995 INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD by DAVIES COLLISON CAVE Patent Attorneys for the Applicant.





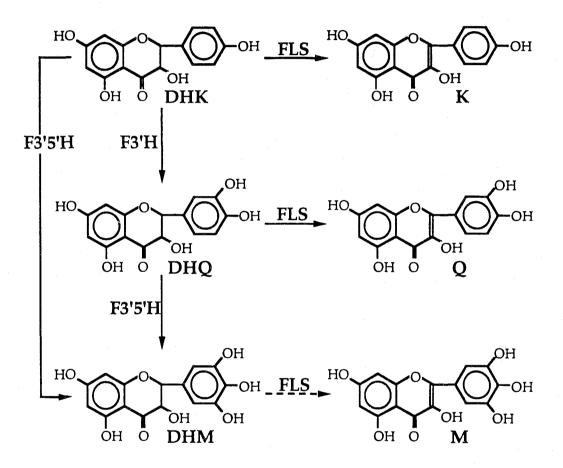


Figure 1

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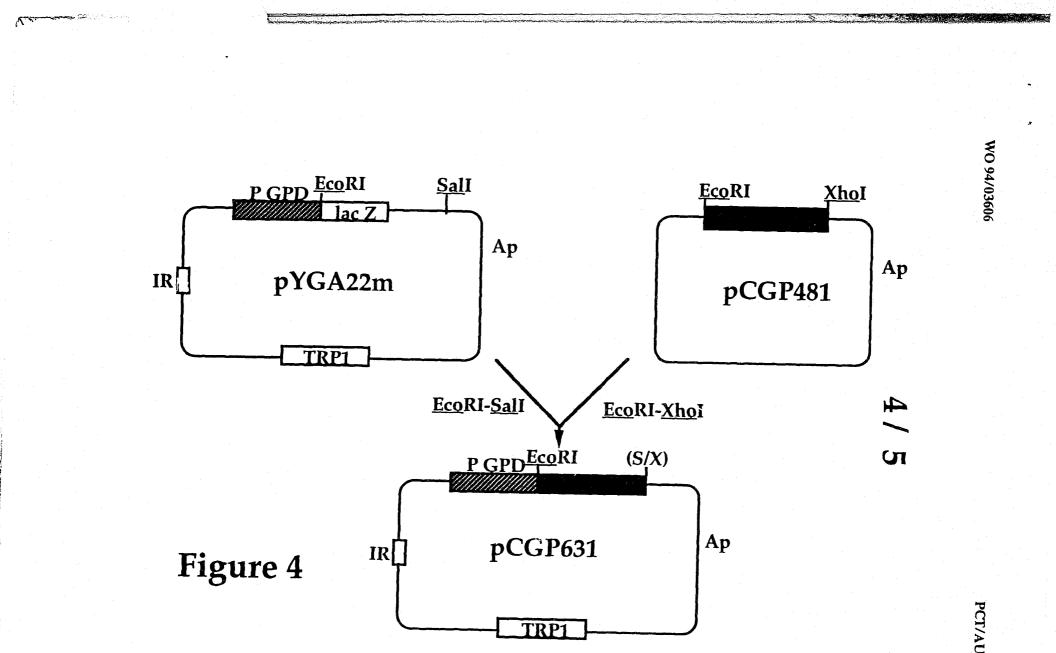
FIGURE 2

EcoRI 0 kb 0.2 kb 0.4 kb 0.6 kb 0.8 kb 1.0 kb 1.2 kb Oligo 8 Oligo 9 Oligo 9 Oligo 9

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Figure 3

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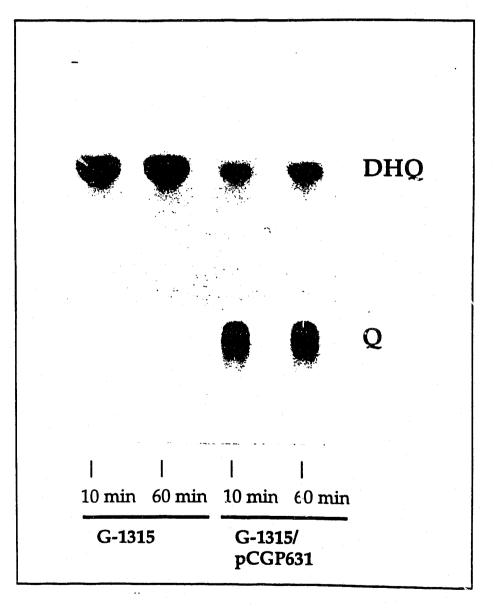


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Figure 5



INTERNATIONAL SEARCH REPORT

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International application No. PCT/AU 93/00400

	CLASSIFICATION OF SUBJECT MATTER 2N 15/52, 15/53, 9/02, 9/04, A01H 5/00, 5/02	2	
According to	International Patent Classification (IPC) or to both a	national classification and IPC	
B.	FIELDS SEARCHED	<u></u>	
	cumentation searched (classification system followed C12N 15/52, 15/53, A01H 5/00, 5/02, C12N 9		
Documentatio	on searched other than minimum documentation to the	he extent that such documents are included i	n the fields searched
WPAT; CA #,SYNTHA	ta base consulted during the international search (na SA; BIOT : KEYWORDS : FLS, FLAVONO SE #, FLOWER #, PETAL #, PETUNEA #, I , FLAVON:, DIHYDROFLAVON:, DIHYDR N;	L(S) SYNTHASE #, DEOXYGENASE DIANTHUS, CHRYSANTH:, CARNA	#, OXYGENASE 110N #, TOBACCO,
С.	DOCUMENTS CONSIDERED TO BE RELEVA	NT	
Category	Citation of document, with indication, where a	ppropriate, of the relevant passages	Relevant to Claim No.
A A	Plant Molecular Biology 13 (1989) p.287-29 manipulation of floral pigmentation genes". Journal of Biosciences Vol 42 No 4 (1987) p "Induction and Characterisation of a NADPH Cell Cultures of Soybean".	. 343-348 Kochs G and Guisebach H,	
X Furth in the	er documents are listed continuation of Box C.	X See patent family annex	S.
"A" docu not c "E" carlie intern "L" docu or W	al categories of cited documents : ment defining the general state of the art which is onsidered to be of particular relevance er document but published on or after the lational filing date ment which may throw doubts on priority claim(s) nich is cited to establish the publication date of her citation or other special reason (as specified) ment referring to an oral disclosure, use,	filing date or priority d with the application but principle or theory und document of particular invention cannot be cor	cited to understand the erlying the invention relevance; the claimed isidered novel or cannot be n inventive step when the e relevance; the claimed isidered to involve an
"O" docu exhit "P" docu	bition or other means ment published prior to the international filing date ater than the priority date claimed	inventive step when the with one or more other	such documents, such ous to a person skilled in
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"P" docu exhit docu but b Date of the a 13 October Name and m	bition or other means ment published prior to the international filing date atter than the priority date claimed (ctual completion of the international search 1993 (13.10.93) (ailing address of the ISA/AU AN INDUSTRIAL PROPERTY ORGANISATION (CT 2606	inventive step when the with one or more other combination being obvi- the art "&" document member of the Date of mailing of the international search	such documents, such ious to a percon skilled in ne same patent family

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International application No. PCT/AU 93/00400

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Category	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.		
A	The Embo Journal Vol 7 No 5 (1988) p. 1257-1263 Turner A J et al "Cloning of the two chalcone flavanone isomerase genes from Petunia hybrida : coordinate, light-regulated and differential expression of flavonod genes".			
Α	AU 54123/90 (DNA PLANT TECHNOLOGY CORPORATION) 18 October 1990 (18.10.90)			
PA	AU,B, 19530/92 (639393) (INTERNATIONAL FLOWER DEVELOPMENTS) 21 January 1993 (21.01.93)			
A	EP 0335451 (VERENIGING VOOR CHRISTELIJK WETENSCHAPPELIJK ONDERWIJS) 4 October 1989 (04.10.89)			
A	Journal of Biosciences Vol 47, No 7/8 (1992) p. 553-560 Stich K. et al "Flavonal Synthase Activity and the Regulation of Flavonol and Anthocyonin Biosynthesis during Flower Development in Dianthus caryophyllus L. (Carnation)			

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International application No. PCT/AU 93/00400

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.

AU 90	54123		EP US	4655 50343	72 23	JP US	4504800 5231020	WO	901208	.4	
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