MODIFICATION OF FLOWER COLOUR VIA MANIPULATION OF P450 GENE EXPRESSION IN TRANSGENIC PLANTS

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SUMMARY

Unlike animals, which synthesise cytochrome P450 enzymes mostly for the degradation of xenobiotics, plants have evolved a large number of different P450 enzymes for the synthesis of secondary metabolites. Probably the most conspicuous of these secondary metabolites are anthocyanins, which are important flower pigments. The types of anthocyanins synthesised in plants are controlled by the cytochrome P450 enzymes flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase. Cloning of flavonoid 3′,5′-hydroxylase genes has enabled the manipulation of anthocyanin synthesis in transgenic plants and enabled the production of novel pigments and flower colours.

KEY WORDS

anthocyanin, cytochrome P450, delphinidin, flavonoid 3′,5′-hydroxylase, petunia

INTRODUCTION

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. Although classical breeding techniques have been used with some success to produce a wide range of colours for most of the commercial species of flowers, this approach is limited by the constraints of a particular species' gene pool. Developments in tissue culture and molecular biology have created methods for introducing new genes from other species and enabled the

alteration of individual characteristics, such as flower colour, in a range of ornamental species.

Flower colour is predominantly due to the production of flavonoids, carotenoids or betalains. The flavonoids are the most common flower pigments and contribute to a range of colours from yellow to red to blue. The flavonoid molecules which make the major contribution to flower colour are the anthocyanins which are glycosylated derivatives of anthocyanidins, such as pelargonidin, cyanidin and delphinidin. In flowers, anthocyanins usually accumulate in vacuoles or anthocyanoplasts of the epidermal cells. The anthocyanidins differ by the degree of hydroxylation (Fig. 1) and subsequent methylation of the B-ring. Extra hydroxyl groups generally lead to a bluing of flower colour, although other factors, such as vacuolar pH, co-pigmentation and metal complexation, can play major roles in determining flower colour /1/.

FLAVONOID BIOSYNTHESIS

The biosynthetic pathway for the flavonoid pigments, illustrated in Figure 1, is well established /2,3/. The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by chalcone synthase (CHS). The product of this reaction, 2′,4,4′,6′-tetrahydroxy-chalcone, is rapidly isomerised to produce naringenin by the enzyme chalcone-flavanone isomerase (CHI). Naringenin is subsequently hydroxylated at the 3-position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The B-ring of DHK can be hydroxylated at either the 3′ or both the 3′ and 5′ positions to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. Two key enzymes involved in this pathway are flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H). The F3′H enzyme acts on DHK to produce DHQ and on naringenin to produce eriodictyol. The F3′5′H enzyme catalyses the 3′,5′-hydroxylation of naringenin and DHK and the 5′-hydroxylation of eriodictyol and DHQ, in both instances producing pentahydroxyflavanone and DHM, respectively. The various anthocyanins and flavonol co-pigments are all derived from the dihydroflavonols (dihydrokaempferol, dihydroquercetin and dihydromyricetin).

Blue and violet flowers generally contain delphinidin derivatives, whereas red and pink flowers most commonly contain pelargonidin and

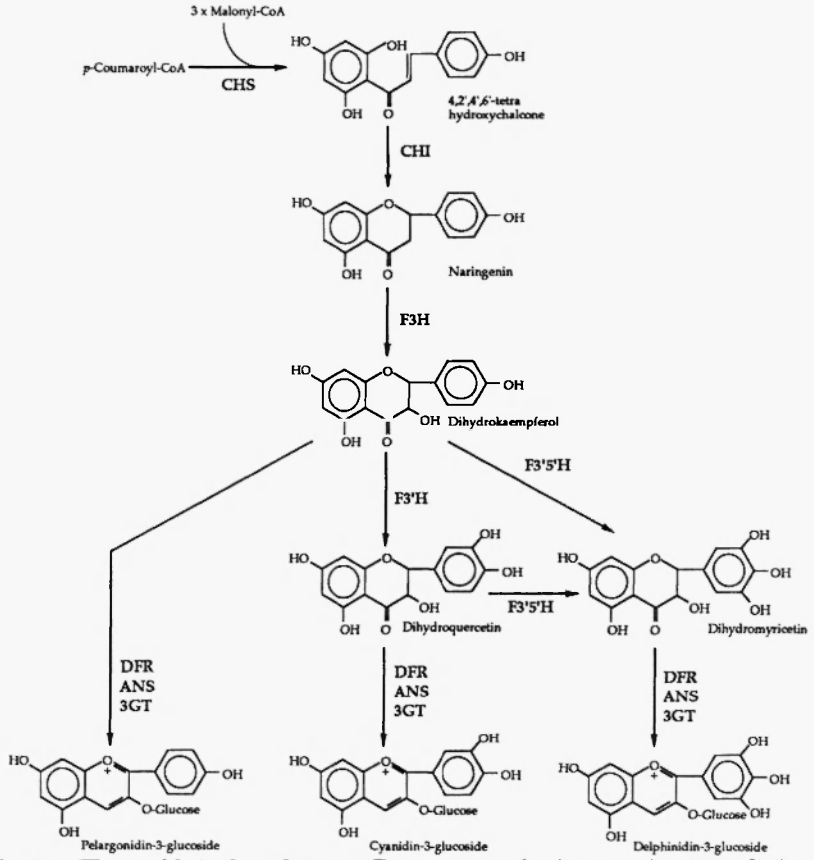

Fig. 1: Flavonoid hydroxylations. Enzymes involved in each step of the pathway are indicated as follows: PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate:CoA ligase; CHS = chalcone synthase; CHI = chalcone isomerase; F3′H = flavonoid 3′-hydroxylase; F3′5′H = flavonoid 3′,5′-hydroxylase; F3H = flavanone 3-hydroxylase; DFR = dihydroflavonol reductase; ANS = anthocyanidin synthase; 3GT = flavonoid 3-glucosyltransferase.

cyanidin derivatives. The pattern of hydroxylation of the anthocyanin B-ring therefore plays an important role in the determination of the flower colour. The ability to control the F3′H or F3′5′H activity in plants would provide a means to manipulate flower colour, thereby enabling a single species to express a broader range of flower colours.

Depending on the species, further modifications of the anthocyanins usually occur, including methylation, glycosylation and acylation.

P450 ENZYMES CONTROL FLAVONOID B-RING HYDROXYLATION

F3′H activity was first demonstrated in microsomal preparations from *Happlopappus* cell cultures /4/. Similar F3′H activity was observed in microsomal preparations obtained from *Matthiola incana* /5/ and snapdragon /6/. Later, the enzyme was characterised as a cytochrome P450-dependent monooxygenase which requires NADPH as a cofactor and molecular oxygen /7/. A F3′H enzyme from maize showed multiple substrate preferences, including flavanones, dihydroflavonols, flavones and flavonols /8/. In flower extracts of defined genotypes of *P. hybrida*, an enzyme activity was demonstrated which catalyses the hydroxylation of naringenin and dihydrokaempferol in the 3′-position /9/. Synthesis of 3′-hydroxylated flavonoids in petunia flowers is controlled by the *Ht1* locus /10/.

F3′5′H activity was first detected in microsomal preparations from flowers of *Verbena* which contain anthocyanins based on delphinidin /11/. F3′5′H activity has also been demonstrated in microsomal preparations from flowers of petunia /1/. Hydroxylation of anthocyanins at the 3′,5′-positions is controlled by the genes *Hf1* and *Hf2* in petunia /12/. *Hf1* acts in the corolla, stigma and pollen, whilst *Hf2* only acts in the corolla limb. The enzymes catalysing the flavonoid 3′,5′-hydroxylation can utilise the flavanones naringenin and eriodictyol, or the dihydroflavonols dihydrokaempferol and dihydroquercetin, as substrates. If both F3′H and F3′5′H enzymes are present in petunia, predominantly 3′,5′-hydroxylated anthocyanins are produced due largely to the substrate specificity of the enzymes involved in the conversion of dihydroflavonols to anthocyanins /13/. The F3′5′H enzyme also belongs to the cytochrome P450 superfamily /14/.

CLONING OF PETUNIA F3′5′H GENES

Isolation strategies for P450 genes have generally relied on the ability to purify the protein and assay for enzyme activity. Apart from a few exceptions, this approach has met with little success in isolating P450 genes from plants.

More than 200 different P450 sequences are available, predominantly from mammals /15/. Although the amino acid sequences of different P450s can vary enormously, all share a number of pockets of sequence similarity, including a conserved haem-binding domain in which four amino acid residues are invariant. This sequence con-

servation was used to design degenerate oligonucleotide primers to isolate petunia P450 gene sequences via PCR /14/. Clones of 18 different P450 genes which are expressed in petals were isolated using this procedure, including two different genes which were both shown to encode F3′5′H activity by expression of full-length cDNA clones in yeast. RFLP mapping showed that these genes were linked to the *Hf1* and *Hf2* loci.

Ohbayashi *et al.* /16/ have also used a PCR-based strategy to amplify, clone and sequence fragments of P450 genes expressed in petunia flowers. In this case, 17 different P450 sequences were obtained, including one sequence which was identical to the *Hf1* gene described above.

ISOLATION OF F3′5′H GENES FROM OTHER SPECIES

Eggplant (*Solanum melongena*) seedlings are induced to synthesise anthocyanins when irradiated with ultraviolet-containing white light. Using a differential screening procedure, Toguri *et al.* /17/ were able to isolate a number of eggplant genes, including one P450 gene, that are induced by light. The eggplant P450 gene appears to be a homologue of the petunia F3′5′H genes, based on sequence similarity. The F3′5′H genes form the relatively new P450 gene family, CYP75 /18/. A petunia F3′5′H gene has also been used as a molecular probe to isolate homologous genes from *Eustoma russellianum* and *Campanula* (Kyowa Hakko Kogyo, international patent number W093/18155). All of the putative F3′5′H amino acid sequences share high sequence similarity (Fig. 2).

GENETIC ENGINEERING OF FLOWER COLOUR IN PETUNIA

Petunia hybrida Skr4xSw63 (*hf1hf1hf2hf2ht1ht1*) was transformed with the *Hf1* and *Hf2* cDNA clones under the control of the constitutive *MAC* promoter. In both cases there was an increase in production of 3′,5′-hydroxylated anthocyanins /14/ and a significant change in flower colour.

Petunia hybrida VR (*Hf1hf1Hf2hf2Ht1ht1*) was transformed with pCGP707, an antisense construct of *Hf1*. Transgenic flowers were pink, compared with non-transgenic flowers which were magenta. The colour difference was due to the production of delphinidin derivatives

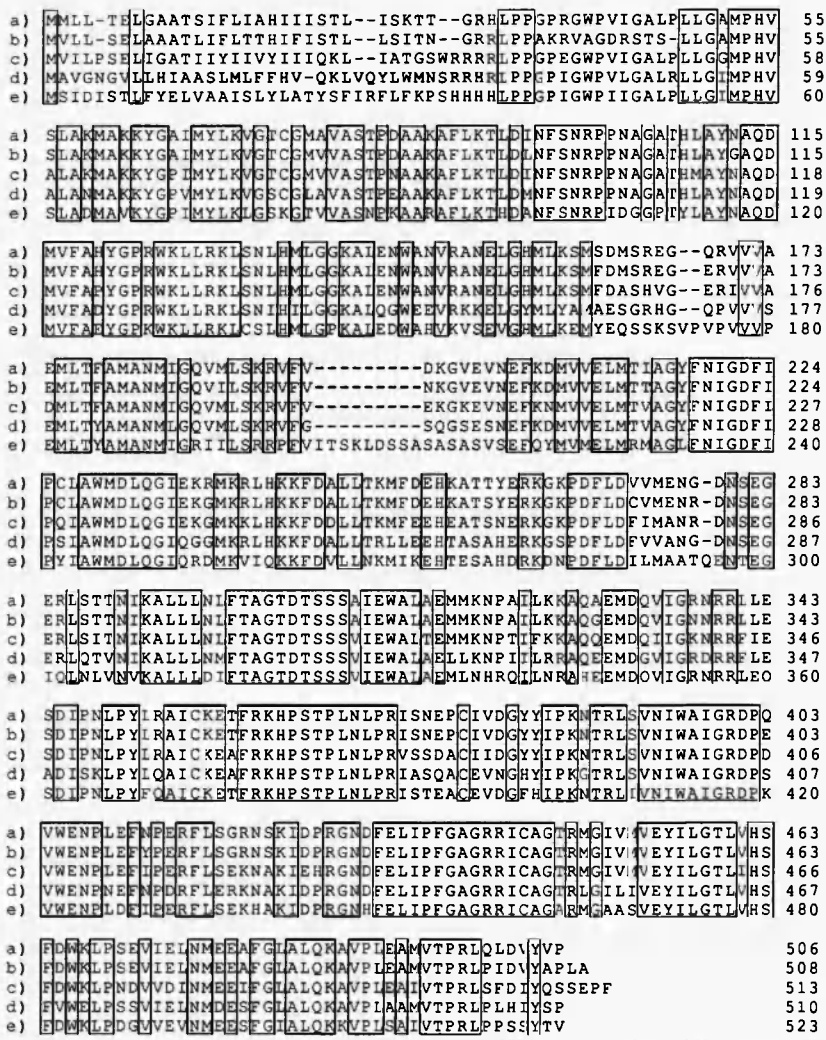

Fig. 2: Comparison of F3′5′H genes from different species. Alignment of amino acid sequences encoded by F3′5′H genes isolated from: a) *Petunia hybrida* (*Hf1*); b) *Petunia hybrida* (*Hf2*); c) *Solanum melongena*; d) *Eustoma russellianum*; e) *Campanula*. Amino acid residues that are present in every polypeptide are boxed.

in the VR flowers, compared with cyanidin derivatives in VR/pCGP707 flowers. These results would suggest that the F3′H gene does not share sufficient sequence similarity to the F3′5′H genes to be

suppressed by the transgene. The petunia line Skr4xSd5 (*hf1hf1Hf2hf2ht1ht1*) was transformed with pCGP709, an antisense gene construct of *Hf2*. Skr4xSd5 produces purple flowers due to the synthesis of delphinidin derivatives. Transgenic flowers were almost white due to an almost complete abolition of anthocyanin synthesis (unpublished results).

The F3′5′H P450 genes can therefore be used in a variety of ways to modify pigment production and flower colour in transgenic plants.

USE OF F3′5′H GENES TO EXTEND FLOWER COLOUR RANGE

Blue flowering varieties are missing from a number of important ornamental plants, including carnations, chrysanthemums and roses. None of these plants are capable of producing blue delphinidin pigments, presumably due to the absence of the gene encoding flavonoid 3′,5′-hydroxylase from their gene pool. Transformation of these species with a flavonoid 3′,5′-hydroxylase gene should overcome this limitation and allow the production of delphinidin derivatives, thereby increasing the possibility of producing blue flowers. To obtain high levels of delphinidin production the target species must produce either of the substrates of F3′5′H, dihydrokaempferol or dihydroquercetin. The target plant must also produce enzymes capable of converting dihydromyricetin (the product of F3′5′H) to delphinidin glycosides and accumulate these anthocyanins in the vacuole.

F3′5′H activity is dependent upon the transfer of electrons from P450 reductase. A potential problem may therefore arise if a heterologous F3′5′H enzyme does not interact efficiently with the endogenous P450 reductase in a target plant /19/. However, the petunia F3′5′H enzymes were shown to function in yeast using the endogenous yeast P450 reductase /14/, so it is likely that they would functionally interact with other more closely related plant P450 reductases.

Geissmann and Mehlquist /20/ reported the presence of four anthocyanin types in different coloured carnation flowers: pelargonidin-3-monoside and -3,5-dimonoside, and cyanidin-3-monoside and -3,5-dimonoside. Later experiments using less harsh extraction procedures revealed that the anthocyanins are glucosides acylated with malic acid /21/. Stich *et al.* /22/ showed by feeding experiments that carnation petals contain all the enzymes required for the conversion of dihydromyricetin into delphinidin derivatives.

Administration of dihydroquercetin and dihydromyricetin initiated the formation of cyanidin and delphinidin derivatives, respectively, in the petals, in addition to the pigments which were naturally present. DFR enzyme activity was assayed in petal extracts. Both dihydroquercetin and dihydromyricetin were converted to the respective flavan-3,4-diols at a rate about four times higher than that for dihydrokaempferol. Therefore, in the presence of F3′5′H activity, delphinidin production should out surpass pelargonidin production.

An efficient transformation protocol has been developed for a number of carnation cultivars /23/. Carnations which naturally produce pelargonidin pigments have been transformed with the petunia *Hf1* gene, under the control of a snapdragon CHS promoter (unpublished results). Flowers from the transgenic plants produced a mixture of pelargonidin and delphinidin and the colour of the transgenic flowers was shifted towards blue, as predicted. The delphinidin pigments were also shown to be acylated like the naturally occurring pelargonidin pigments. Experiments are currently under way to increase the efficiency of delphinidin production in carnation and other ornamental species to enable the production of novel blue-flowering ornamental species.

Chrysanthemums produce a much more limited range of anthocyanins: only cyanidin 3-glucoside and cyanidin 3-malonyl-glucoside have been detected /24/. Feeding experiments with dihydromyricetin have shown that chrysanthemum flowers have all the enzymes necessary for delphinidin synthesis, except for F3′5′H (Kathy Schwinn, unpublished results). An efficient transformation system for chrysanthemum has also been developed /25/.

The rose (*Rosa hybrida*) is the most important commercial cut flower. Rose pigments in species and cultivars have been studied extensively. Yokoi /26/ analysed the anthocyanin pigments contained in the flowers of 670 cultivars and 8 species of roses. The only anthocyanin pigments found in this survey were cyanidin 3-glucoside and 3,5-diglucoside, pelargonidin 3-glucoside and 3,5-diglucoside and peonidin 3,5-diglucoside.

Feeding experiments with rose petals have demonstrated that the rose enzymes are capable of converting dihydromyricetin, the product of flavonoid 3′,5′-hydroxylase, to delphinidin-glucosides /27/. Firoozabady *et al.* /28/ recently reported the stable transformation of rose plants. The introduction of the flavonoid 3′,5′-hydroxylase gene into pelargonidin- or cyanidin-producing rose cultivars should divert

the anthocyanin biosynthetic pathway towards the production of delphinidin-glucosides and the flower colour towards blue.

Cloning of the flavonoid 3',5'-hydroxylase genes from petunia and other species thus represents a major step towards the production of a range of novel flower colours in many different species, including a blue rose - a goal that has eluded traditional plant breeders for centuries.

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