3

Modification of Flower Colour using Genetic Engineering

PAULA ELOMAA[1] AND TIMOTHY HOLTON[2]

[1] *Institute of Biotechnology, University of Helsinki, Finland and* [2] *Florigene Pty Ltd, Collingwood, Victoria, Australia*

Introduction

Plant breeding, besides producing better crop plants, has also focused on species that are attractive and have aesthetically valuable characteristics. Today, a whole new area of the flower industry has evolved and rapidly expanded world-wide (Mol, Stuitje and van der Krol, 1989). Traditionally, breeding of ornamental plants has been based mainly on continuous crossing and selection. Several decades of traditional breeding have combined commercially important characteristics into élite genotypes. Developments in tissue culture and methods of molecular genetics now give an alternative approach to change single characteristics in these genotypes. Furthermore, possibilities of producing completely new characteristics are no longer limited by the natural genetic variation existing in the target species, since genes of any origin can be transferred into the pre-existing gene pool. This is not possible by using traditional methods.

Breeding of ornamental species via genetic engineering focuses on many characteristics that are the same as in traditional breeding programmes. One of the most important goals from the flower industry's perspective is to modify production characteristics such as flower productivity, timing and synchrony of flowering, disease and pest resistance and vase life. Nevertheless, flower morphology and flower colour are of the greatest importance in directing the markets in the flower industry, as characteristics that are judged by the consumer. Ever-changing preferences and fashion increase the demand for rapid production of new characteristics in ornamental plants.

Abbreviations: AMT, anthocyonin methyltransferase; An13, anthocyonin 13; ANS, anthocyanidin synthase; CaMV, Cauliflower mosaic virus; cDNA, complementary DNA; C4H, cinnamate-4-hydroxylase; CHI, chalcone-flavanone isomerase; CHS, chalcone synthase; 4CL, 4-coumaryl-CoA ligase; DFR, dihydroflavonol- 4-reductase; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3', 5'-hydroxylase; FLS, flavonol synthase; FS, flavone synthase; GA, gibberellic acid; 3GT, UDPG-flavonoid-3-*O*-glucosyltransferase; GUS, β-glucuronidase; PAL, phenylalanine ammonialyase; PCR, polymerase chain reaction; 3RT, anthocyanidin-3-glucoside rhamnosyltransferase; T-DNA, transfer DNA; UV, ultraviolet.

Biotechnology and Genetic Engineering Reviews — Vol. 12, December 1994
0264–8725/94/12/63–88 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

There are still many limitations and problems to be overcome before molecular methods can be used effectively in breeding. Molecular flower-breeding requires transformation and regeneration methods for the target species. The gene to be transferred has to be isolated, characterized and suitable constructs with selectable marker genes have to be made. The gene must then be stably integrated into the genome of the target plant and expressed in a correct manner and have the desired, and stable, effect on the phenotype. The main limitation to the widespread use of molecular breeding is that there is no general transformation method suitable for all species. This is because regeneration and transformation capacities vary strongly in different genotypes, and even in various explants. Also, many target characteristics are encoded by several genes. Molecular breeding will, by no means, replace traditional breeding methods, but instead it may provide additional tools to introduce new, desirable characteristics into pre-existing varieties.

Modification of flower colour is one of the first examples of how genetic engineering can be applied in breeding new varieties. This has been possible through detailed knowledge of the genetics and biochemistry of the phenylpropanoid pathway leading to synthesis of anthocyanin pigments, and isolation of genes encoding the enzymes of the pathway. Although huge natural variation exists with respect to flower colour in several important ornamental plants, no plant species can produce varieties in the full spectrum of colours (Mol, Stuitje and van der Krol, 1989). Blue and purple varieties are still missing from important ornamental species such as rose, carnation, chrysanthemum, gerbera and tulip. There is also an absence of yellow colour in pelargonium, cyclamen and saintpaulia, as well as bright red varieties in cyclamen, iris, saintpaulia and lisianthus (Forkmann, 1991). Molecular flower breeding provides an attractive approach to change flower pigmentation in a highly controlled way, in otherwise superior genotypes achieved through traditional breeding programmes.

Besides producing new flower colours in ornamental plants, transformation with genes affecting the flower pigmentation can be used as phenotypic markers to visualize transgene regulation in general and thereby provide additional information for other transformation applications.

This chapter describes the present state in development of transformation methods in ornamental species and outlines factors influencing pigmentation. The main focus of this chapter is to introduce the information considering applications of genetic engineering in connection to flower colour. Colour modification in *Petunia hybrida*, which has been used as a model plant in this field, will be presented, together with the latest achievements in the most important ornamental plants.

Transformation of ornamental species

Development of a transformation method for any plant species or cultivar requires an effective tissue culture technique in order to regenerate the transformed tissue. Regeneration capacity is the main limitation for transformation in many species. However, regeneration media for the commercially most important ornamental species, rose, carnation, chrysanthemum and gerbera, have been developed in recent years (Hutchinson *et al.*, 1992). This has enabled the use of *Agrobacterium*-mediated transformation in these species, though with variable efficiency.

Agrobacterium-mediated transformation systems have so far been the most suc-

cessful for dicotyledonous plants. The mechanism and interactions underlying this method have been under extensive study and have been reviewed comprehensively by Zambryski (1988), Hooykaas (1989) and Winans (1992). Therefore, we will only briefly describe some basic details of the method.

Transformation using the *Agrobacterium* vector system is based on the capability of a naturally occurring Gram-negative soil bacterium to infect the target plant and introduce part of its DNA (T-DNA) from the Ti-plasmid into wounded plant cells. Variable copies of this DNA are integrated into random positions in the plant genome (Zambryski, 1988). In nature, infection by *Agrobacterium tumefaciens* results in crown gall tumour formation in most dicotyledonous plants. The T-DNA directs plant hormone synthesis, leading to the transformation of plant cells into tumour cells. Some of the genes in the T-DNA region encode for enzymes that synthesize opines. These compounds are excreted from the transformed plant cells and consumed by the tumour-inducing *Agrobacterium*. The T-DNA region is surrounded by 25 bp direct repeats that have been shown to define the transferred DNA. Separate from the T-DNA region, virulence (*vir*) genes encode *trans*-acting products that are associated with the DNA transfer process. The transfer mechanism is complicated and it is assumed that the T-DNA enters the plant nuclear genome as a single strand and integrates at random positions. The T-DNA can be integrated as a single copy or multiple copies, either tightly linked or sometimes dispersed throughout the genome (reviewed by Winans, 1992).

The use of the *Agrobacterium* system for gene transfer has been made possible by removing the tumour-inducing genes from the T-DNA (disarmed vectors) and inserting foreign genes to be transferred. There are two kinds of vector systems – binary and co-integrate. In co-integrate vectors the introduced DNA is in the *cis*-position in respect of *vir* genes, in place of the T-DNA (Zambryski *et al.*, 1983; Deblaere *et al.*, 1985). In binary vectors the T-DNA is in a separate plasmid from the *vir* genes needed for the transformation (An *et al.*, 1985). Both vector systems have been used to transform ornamental species.

The major limitation of this system is that it is applicable only to those species that are susceptible to infection by *Agrobacterium*. Activation of the virulence system is caused by phenolic compounds formed in the wounding of the plant tissue (Bolton, Nester and Gordon, 1986). Deficiency of these inducers in monocotyledonous plants has been proposed to be the reason that they are not susceptible to agro-infection. However, it has been shown that *Agrobactium* infection does occur in a few monocots, including species of Liliaceae and Amaryllidaceae (Hooykaas-Van Slogteren, Hooykaas and Schilperoort, 1984).

Another limitation of the *Agrobacterium*-mediated transformation system is that it requires an efficient regeneration method. Although there is a lot of literature on regeneration methods for different ornamental species, there are still only a few published transformation protocols using the *Agrobacterium* method in ornamental plants. Most of the methods rely on various modifications of the leaf-disc transformation protocol (Horsch *et al.*, 1985).

Lu *et al.* (1991) published a transformation protocol for the carnation (*Dianthus caryophyllus* L.) cultivars White, Red and Crowley Sim, using two marker genes encoding NPTII and GUS. Transgenic plants were regenerated from stem and petal explants with relatively high frequency and without any differences between the

cultivars tested. It was also noticed that adding 20 μM acetosyringone to the co-cultivation medium for induction of *vir* genes increased the transformation frequency threefold.

A similar system was used to transfer the *nptII* and *uidA* marker genes into chrysanthemum (*Dendranthema indicum* L. Korean genotype) by Ledger, Deroles and Given (1991), using leaf explants. A total of 80 kanamycin-resistant plants were produced using the *nos-nptII* gene, and three transformed shoots using *nptII* and *uidA*. Van Wordragen *et al.* (1991) used an *Agrobacterium*-mediated method to produce transgenic calli from chrysanthemum (*Dendranthema grandiflora* cv. Parliament) that showed NPTII and GUS activity. The susceptibilities of various genotypes and *Agrobacterium* strains were studied and it was concluded that the transformation of chrysanthemum strongly depends on the genotype and bacterial strain used.

Transformation methods using the *Agrobacterium* system have been developed for four cultivars of the increasingly popular cut flower, lisianthus (*Eustoma grandiflorum* Grise.) and, despite a low transformation frequency (1–3% of the inoculated leaf pieces), kanamycin-resistant shoots were regenerated (Deroles *et al.*, 1993). Eleven cultivars of pelargonium were inoculated with two *Agrobacterium* strains containing plasmids with the *uidA* marker gene. Leaf discs of all cultivars showed GUS activity, with at least one strain/plasmid combination. Development of regeneration systems for three of these cultivars is in progress (Boase and Smith, 1994).

An *Agrobacterium* co-cultivation method was used to transform pieces of petioles of *Gerbera hybrida*, variety Terra Regina (Elomaa *et al.*, 1993), and also quite recently a detailed transformation protocol for various chrysanthemum varieties has been submitted by Robinson *et al.*, as reported by Courtney-Gutterson *et al.* (1994). These methods were used to alter the floral pigmentation in the ornamental species. These cases will be discussed further in connection with colour modification.

Transformation of ornamental species has, in most reported cases, relied on the *Agrobacterium* system. However, many important ornamental species, such as tulip, freesia, iris and daffodil, are monocots and therefore may not be susceptible to *Agrobacterium* infection. Development of the particle bombardment method (Klein *et al.*, 1987), based on the direct transformation of foreign genes into embryogenic tissue, has turned out to be successful in the transformation of many crop plants (Christou, 1992). This method may also be an alternative approach in ornamental plants, and has already been used to introduce the phosphinothricin (Basta) resistance gene and *uidA* marker gene into tulips. Transgenic shoots were formed from immature flower-stem segments (Wilmink, van de Ven and Dons, 1994). The expression of the *bar* and *uidA* genes was enhanced using monocot promoters.

Other alternative approaches to transform plant cells are being studied. Silicon carbide fibres have been used to deliver plasmid DNA into embryogenic maize suspension-culture cells, and fertile transgenic maize plants were recovered (Thompson *et al.*, 1994). The possibilities of using yeast artificial chromosomes in order to introduce large DNA inserts into plants are also under study (Mullen *et al.*, 1994; van Wordragen *et al.*, 1994).

The flavonoid pathway

Flavonoids are phenolic compounds common to all higher plants. They have several

unrelated roles in plants (van Tunen and Mol, 1991; Koes, Quattrocchio and Mol, 1994). Coloured anthocyanins are important flower pigments, where their major role is undoubtedly to attract pollinators. Flavonoids also have a role in protection against UV light and pathogens (phytoalexins), and they function as signal molecules in symbiotic plant–microbe interactions, such as nodulation in legumes. They are important in the formation of various plant structures and in sexual reproduction.

The structure, biochemistry and synthesis of flavonoids has been studied extensively (Forkmann, 1991; Stevenson, 1991; van Tunen and Mol, 1991). Also, the genetics of the flavonoid pathway in maize, petunia and snapdragon are thoroughly known, and have been reviewed extensively (Dooner, Robbins and Jorgensen, 1991; Martin and Gerats, 1993). Therefore, only the main steps of the flavonoid biosynthesis and their role in flower pigmentation will be outlined here.

The colours of flowers are mainly formed by three chemically different types of natural pigments: flavonoids, carotenoids and betalains. These compounds can, in many cases, produce very similar colours, and very often contribute in mixtures to flower colour (Forkmann, 1991). Carotenoids are lipid-soluble pigments located in plastids, and account for the majority of yellow hues in flowers. Betalains are water-soluble nitrogenous pigments which contribute to flower colours from ivory and yellow and various shades of orange and red to violet (Forkmann, 1991).

Flavonoids, which accumulate in vacuoles of epidermal cells as water-soluble glycosides, are the most common and most important pigments found in most vascular plants. The basic structure of flavonoids consists of two aromatic rings (A and B) and a central heterocycle (C) (*Figure 1*). The degree of oxidation of the central C-ring determines to which class of flavonoids the compound belongs.

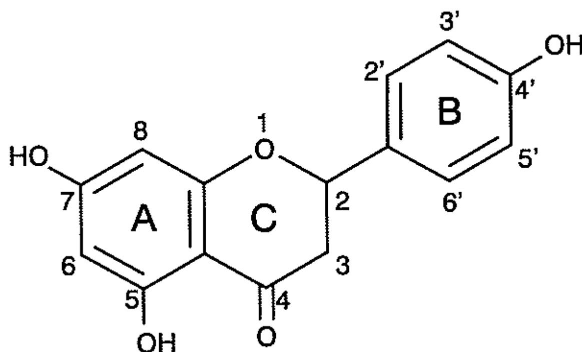
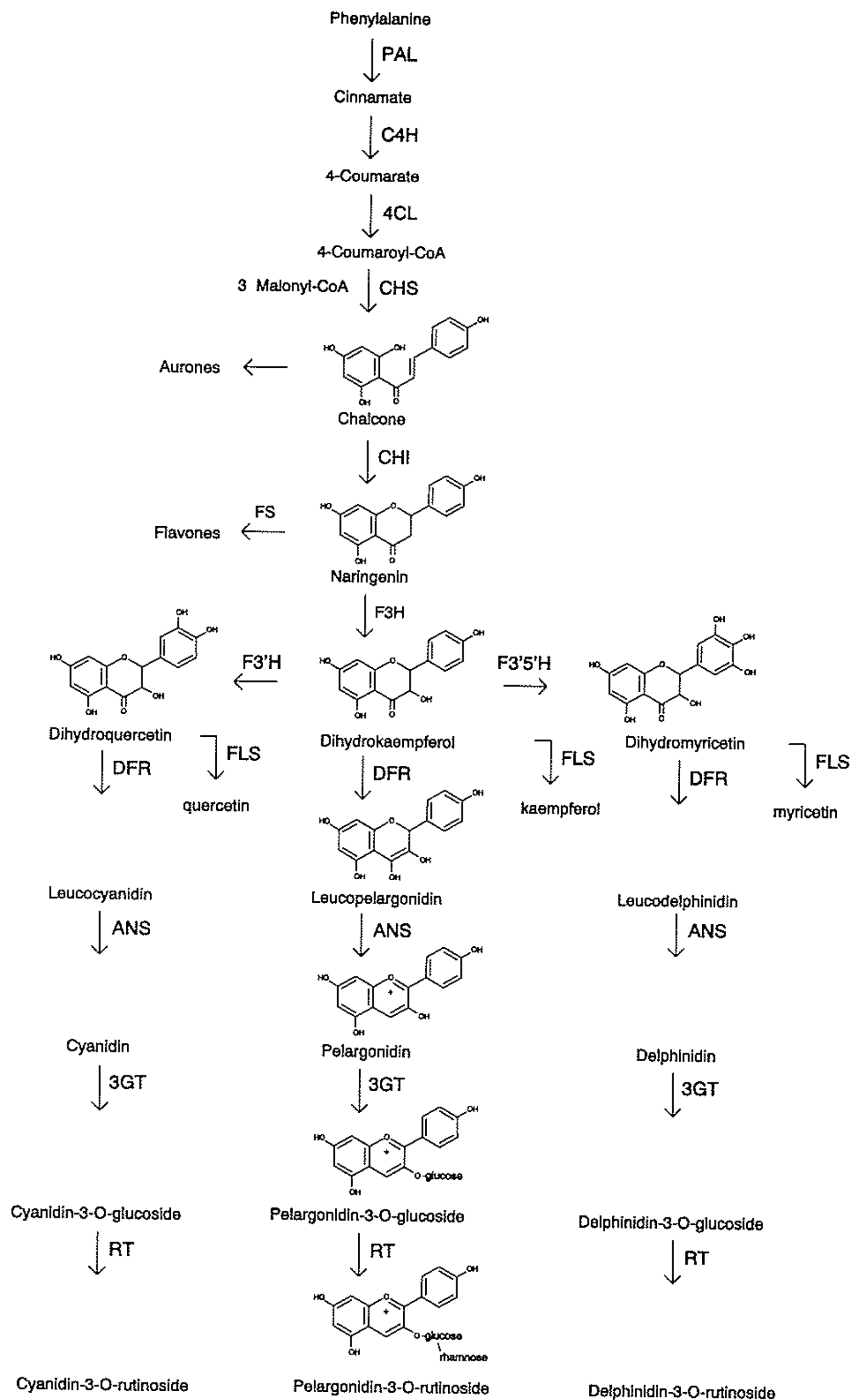


Figure 1. The basic structure of flavonoids consists of two aromatic rings (A and B) and a central heterocycle (C).

Flavonoids are synthesized via the phenylpropanoid pathway, starting from phenylalanine and leading to synthesis of coloured anthocyanins (*Figure 2*). Phenylalanine is converted through a series of enzymatic reactions catalysed by phenylalanine ammonialyase (PAL), cinnamate- 4-hydroxylase (C4H) and 4-coumaryl-CoA ligase (4CL) to 4-coumaryl-CoA, which then participates in a reaction catalysed by chalcone synthase (CHS), the first enzyme dedicated to the anthocyanin pathway.

Chalcone synthase has been extensively studied in many plant species. Reaction of 4-coumaryl-CoA with three molecules of malonyl-CoA leads to the formation of yellow chalcone. At the next step, chalcone–flavanone isomerase (CHI) catalyses the


Phenylalanine
PAL
Cinnamate
C4H
4-Coumarate
4CL
4-Coumaroyl-CoA
3 Malonyl-CoA
CHS
Aurones
Chalcone
CHI
FS
Flavones
Naringenin
F3H
F3'H
F3'5'H
Dihydroquercetin
Dihydrokaempferol
Dihydromyricetin
FLS
DFR
quercetin
kaempferol
myricetin
Leucocyanidin
Leucopelargonidin
Leucodelphinidin
ANS
Cyanidin
Pelargonidin
Delphinidin
3GT
Cyanidin-3-O-glucoside
Pelargonidin-3-O-glucoside
Delphinidin-3-O-glucoside
RT
Cyanidin-3-O-rutinoside
Pelargonidin-3-O-rutinoside
Delphinidin-3-O-rutinoside

formation of the colourless flavanone, naringenin, which is then hydroxylated at the 3 position of the C-ring by flavanone 3-hydroxylase (F3H) and converted to dihydrokaempferol (DHK).

Hydroxylation of the B-ring of DHK by 3'-hydroxylase and 3'5'-hydroxylase results in formation of dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. The synthesis of these compounds determines the type of anthocyanins produced later. Dihydromyricetin, with a 3'4'5'-hydroxylation pattern in the B-ring, is converted to blue- and purple-coloured delphinidins, while dihydroquercetin, hydroxylated at 3' and 4' positions, is the precursor of red-coloured cyanidins and 4'-hydroxylated dihydrokaempferol is the precursor of orange-coloured pelargonidins. The synthesis of the coloured anthocyanins is complex and requires several enzymes. Dihydroflavonols are first reduced by dihydroflavonol-4-reductase (DFR) to corresponding leucoanthocyanidins, which are then converted by one or two, as yet uncharacterized, enzymes to unstable anthocyanidins. They are stabilized by glycosylation at the 3 position of the C-ring by UDPG-flavonoid-3-*O*-glucosyltransferase (3GT). Finally, anthocyanidin 3-glucosides can be further modified in ordered sequence by glycosylation (e.g. glucose or rhamnose added), acylation and methylation. Methylation results in three common groups of flavonoids (peonidins, petunidins and malvidins), and has a reddening effect on colour (Forkmann, 1991). The type of modification pattern varies among different species and even varieties, determining the type of anthocyanin that is produced (van Tunen and Mol, 1991).

In addition to anthocyanins, other flavonoids are also produced via the phenylpropanoid pathway. These compounds have several distinct roles in plants and also affect floral pigmentation by acting as co-pigments. Yellow aurones are synthesized from chalcones, but little is known of the enzymatic reactions involved (van Tunen and Mol, 1991). Flavone synthase (FS) catalyses the formation of flavones, which have been shown to be part of plant waxes (van Tunen and Mol, 1991). Flavonol synthase (FLS) catalyses the formation of flavonols (kaempferol, quercetin and myricetin) from corresponding dihydroflavonols. Flavonols are also important co-pigments and have an important role in pollen germination and pollen tube growth (Mo, Nagel and Taylor, 1992).

Although the flower colour is mainly determined by the type and concentration of anthocyanin present in the vacuoles, other factors also affect the final hue. Co-pigmentation of anthocyanins with the colourless flavonols and flavones is an important factor influencing pigmentation. The relative amounts of anthocyanin to co-pigments can have a strong effect on colour (Forkmann, 1991). Also, metal ions, such as iron, aluminium and magnesium, can form complexes with anthocyanins and modify colour. The pH of the vacuoles of the epidermal cells has an effect, especially on blue pigmentation. The higher the pH, the bluer the colour, presuming that delphinidin pigments are present (van Tunen and Mol, 1991). Yellow colours found

---

Figure 2. The phenylpropanoid pathway leading to the synthesis of coloured anthocyanins. The enzymes involved in the reactions are: PAL, phenylalanine ammonialyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase, CHI, chalcone–flavanone isomerase, F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; 3GT, UDPG-flavonoid-3-*O*-glucosyltransferase; 3RT, anthocyanidin-3-glucoside rhamnosyltransferase; FS, flavone synthase; FLS, flavonol synthase.

in nature are the result of accumulation of chalcones, aurones or flavonols, but also of carotenoids. For a more detailed review see Forkmann (1991).

Several genes encoding the enzymes of the pathway have been cloned using various techniques (Dooner, Robbins and Jorgensen, 1991; Stevenson, 1991; van Tunen and Mol, 1991; Martin and Gerats, 1993). Many of the genes have now been isolated from *Petunia hybrida* and from the most important ornamental plants (*Table 1*).

Table 1 Flavonoid-specific cDNAs and genes cloned from petunia and other important ornamental plants

Species	Gene	Reference
Carnation	CHS	Forkmann (1991)
	CHI	Forkmann (1991)
	F3H	Britsch *et al.* (1993)
	DFR	Forkmann (1991)
	FLS	patent, PCT/AU93/00400
	ANS	Keam (unpublished)
Chrysanthemum	CHS	Courtney-Gutterson *et al.* (1994)
	DFR	Barri-Rewell (unpublished)
	FLS	patent, PCT/AU94/00400
	ANS	Barri-Rewell (unpublished)
Gerbera	CHS	Elomaa *et al.* (1993)
	DFR	Helariutta *et al.* (1993)
Lisianthus	CHS	Davies *et al.* (1993)
	CHI	Davies *et al.* (1993)
	DFR	Davies *et al.* (1993)
	F3'5'H	Nielsen (unpublished)
	FLS	Nielsen (unpublished)
Rose	CHS	Courtney-Gutterson (unpublished)
	DFR	Tanaka (unpublished)
	FLS	Tanaka (unpublished)
	ANS	Tanaka (unpublished)
Petunia	CHS	Reif *et al.* (1985)
	CHI	van Tunen *et al.* (1988)
	F3H	Britsch, Ruhnau-Britsch and Forkmann (1992)
	F3'H	patent, PCT/AU93/00127
	F3'5'H	Holton *et al.* (1993)
	FLS	Holton, Brugliera and Tanaka (1993)
	DFR	Beld *et al.* (1989)
	ANS	Weiss *et al.* (1993)
	An13	Quattrocchio *et al.* (1993)
	3RT	Brugliera *et al.* (1994), Kroon *et al.* (1994)
	AMT	Quattrocchio *et al.* (1993)

Genetic modification of the pathway

There are several possibilities of altering flower pigmentation by using genetic engineering. By suppressing the expression of certain genes of the phenylpropanoid pathway, anthocyanin synthesis can be blocked at several steps and a range of paler colours or altered colour hues can be produced. This can be done by affecting the key reactions in the pathway, but also by modifying the expression of the genes affecting the synthesis of co-pigments or later modification steps of the anthocyanin molecules.

Another strategy is to over-express a specific gene in order to intensify pigmentation. It is also possible to introduce genes of heterologous origin, encoding enzyme activities missing from the target plant. This would allow formation of novel pigmentation, not found naturally in this species. Expression of regulatory genes that affect the expression of several biosynthetic genes, can also turn on anthocyanin synthesis in normally acyanic organs, and thereby new pigmentation patterns can be achieved. These approaches and success in using them in various applications are presented next.

MODIFICATION OF THE FLOWER COLOUR INTENSITY

To date, suppression of gene expression at several different steps of the pathway has been successful in petunia, a model plant in this area, and in several important ornamental species, such as gerbera, chrysanthemum, carnation and rose. In the antisense approach, the gene to be introduced to the target plant is placed in reverse physical orientation under the promoter directing its expression. The exact mechanism of the antisense effect is still unknown, and it is possible that the suppression occurs in different ways in different cases. It has been suggested that an RNA duplex is formed between mRNA and antisense RNA. This duplex is then rapidly degraded, or processing, transport of the mRNA or its translation is prevented (van der Krol, Mol and Stuitje, 1988). The latest results from work on petunia suggest that genomic position may be important in the interaction between antisense and endogenous genes, occurring either via RNA or DNA, and that the mechanism operates post-transcriptionally (de Lange, 1994; van Blokland, 1994).

Attempts to intensify flower pigmentation by over-expressing either *chs* or *dfr* in petunia have so far been unsuccessful. Introduction of additional copies of flavonoid genes into target plants has, in fact, led to an opposite effect – reduction of pigmentation was detected. This phenomenon has been termed co-suppression because the expression of both the transgene and the endogenous gene is suppressed in these transformants.

Increased red pigmentation has been obtained in petunia and tobacco by suppressing the formation of flavonol co-pigments (Holton, Brugliera and Tanaka, 1993). It has also been possible to alter pigmentation by suppressing the expression of genes involved in further modification of flavonoid pigments by rhamnosylation (Brugliera *et al.*, 1994; Kroon *et al.*, 1994).

Petunia

The first modification of flower colour intensity using genetic engineering was done in *Petunia hybrida* (van der Krol *et al.*, 1988). Constitutive expression of a full-length antisense chalcone synthase gene (*chsA*, isolated from petunia) altered the flower pigmentation in petunia and also in a heterologous plant system, tobacco. Normal petunia (VR hybrid) flowers have uniformly coloured corollas, tubes and anthers, while, unexpectedly, some of the antisense transformants showed variable phenotypes, with sectored pigmentation patterns in corolla tissue or ring patterns starting from the tube region (van der Krol *et al.*, 1988). Also, fully white transformants were obtained from both petunia and tobacco. Northern and Western

analysis of these transformants showed that steady-state *chs* mRNA and CHS protein levels correlated well with the observed phenotypes. The chalcone synthase gene family of petunia comprises 8–10 members, two of which are expressed in flower tissue (*chsA* and *chsJ*) (Koes, Spelt and Mol, 1989). These genes share 86% homology. In white tissues of antisense *chs* transformants transcripts of both these genes were absent (van der Krol *et al.*, 1990b). In addition, reduction was specific for *chs* mRNA and no effect on *chi* or *dfr* mRNA levels were detected. Steady-state antisense *chs* RNA levels were very low and did not correlate with the observed phenotypes (van der Krol *et al.*, 1990b). In fact, low levels of antisense *chs* were detected in transformants that had a strong effect on phenotype and, on the other hand, similar antisense levels detected in leaf tissue could lead to different phenotypes. Further study of the variable phenotypes indicated that pigmentation in these plants can be influenced by plant hormones and light (van der Krol *et al.*, 1990b). Spraying gibberellic acid (GA) on the plants with variable phenotypes resulted in an increase in pigmented area, while spraying with B9 (which inhibits endogenous GA synthesis) decreased the pigmented sector area. Light intensity also affected the floral pigmentation pattern. Transformants given extra light started to form fully white flowers. Similar effects have been reported in the natural *chs* mutant, Red Star (van der Krol *et al.*, 1990b).

Van Blokland (1994) introduced antisense *chs* or *dfr* genes into petunia to study the mechanism of antisense inhibition, in order to get more information about why inhibition occurred in some plants but not in others. Reduced flower pigmentation was detected in 1 out of 10 antisense *chs* transformants and in 6 out of 26 *dfr* transformants, correlating with reduced *chs* or *dfr* mRNA levels. Also, the expression of antisense *chs* gene in the white tissue was repressed with the endogenous *chs* genes. To study the expression of antisense genes, transcription rates in various transformants were measured by nuclear run-on transcription assays. Interestingly, antisense *chs* or *dfr* transcription rates were equal in unpigmented and pigmented tissues, thus showing no correlation with the phenotype. Even a minimal amount of antisense RNA was sufficient to repress *chs* and *dfr* activity, while excess of antisense RNA was sometimes ineffective (van Blokland, 1994). As the transcription rates of endogenous *chs* genes were not affected in the white tissues, it was concluded that antisense inhibition must involve a post-transcriptional mechanism. The exact mechanism, possibly involving some kind of interaction between homologous sequences, is still to be determined.

At the same time as the first antisense transformations of petunia were carried out, attempts to enhance the flower pigmentation by over-expressing the genes of the flavonoid pathway were made by two groups independently of each other (Napoli, Lemieux and Jorgensen, 1990; van der Krol *et al.*, 1990a). Both groups obtained similar results: addition of extra copies of *chs* (van der Krol *et al.*, 1990a; Napoli, Lemieux and Jorgensen, 1990) or *dfr* (van der Krol *et al.*, 1990a) genes under control of the 35S promoter or using genomic *chs* clones (van der Krol *et al.*, 1990a) resulted in blockage of anthocyanin biosynthesis. White and patterned phenotypes, different from those produced in antisense experiments, were obtained. No transformants with darker flowers than the parental genotype were obtained. In white flowers, suppression of both the introduced gene and the endogenous gene were detected. Further characterization of the *dfr* transformants showed that neither the copy number of the

integrated transgene nor the expression levels detected in leaf tissue correlated with the phenotype (van der Krol *et al.*, 1990a).

The basic mechanisms of suppression of gene expression are still unknown but are under active investigation. Recent results of van Blokland (1994) suggest that co-suppression of *chs* genes in petunia is post-transcriptional. Introduction of extra copies of *chs* genes into petunia led to a reduction of steady-state *chs* mRNA levels. However, as in the case of antisense inhibition, transcription of both introduced and endogenous genes (measured by nuclear run-on assays) were not affected, showing that the promoters of these genes were active and not inactivated by methylation and co-suppression resulting from increased *chs* RNA turnover. Also, elongation of transcripts of co-suppressed *chs* genes was shown to be normal. However, it was found that splicing of transcripts from the suppressed genes was, to some extent, impaired. The ratio of unspliced to spliced transcripts was dependent on the degree of suppression. As a conclusion, van Blokland suggested that direct interaction between homologous sequences can lead to changes in chromatin structure. This could then inhibit RNA processing or transport, leading to transcript destabilization.

A group in the DNA Plant Technology Corporation (USA) has studied sense suppression, focusing especially on the stability of the modified phenotype for commercial applications. They have modified the flower colour in chrysanthemum and rose using this approach but also used petunia as a model to assay the possible changes in suppression in primary transformants during prolonged growth and among progeny of these transformants (Courtney-Gutterson, personal communication). Three white-flowering lines where *chs* expression had been suppressed were analysed in the experiments. Over 12 000 progeny with very similar flower phenotypes were obtained by crossings from the primary transformants (Courtney-Gutterson, personal communication). During an extended growth period (over 1 year), no revertant branches were obtained for either line, and it was concluded that the stability of the suppression phenotypes was sufficient for commercial purposes.

Holton, Brugliera and Tanaka (1993) demonstrated the modification of flower colour intensity in petunia and tobacco by introducing a flavonol synthase (FLS) gene which affects the synthesis of flavonols. Flavonols are important co-pigments but also involved in pollen germination and pollen tube growth. Flavonol synthase belongs to the 2-oxoglutarate-dependent dioxygenase gene family and a full-length cDNA was isolated from petunia by a combination of differential screening and polymerase chain reaction (PCR) amplification using degenerate primers. Introduction of antisense FLS cDNA in petunia (VR) plants resulted in redder flowers in 4 out of 12 transformants. Red transformants showed markedly less flavonols than non-transformed controls, analysed by thin-layer chromatography. Similarly, the same construct caused a reduction in flavonol production in tobacco, and red flowers, with, in some cases, levels of anthocyanins more than three times higher than control flowers, were detected (Holton, Brugliera and Tanaka, 1993). Obviously, enzymes involved in flavonol and anthocyanin synthesis compete for common dihydroflavonol substrates and, thereby, reduction of flavonol synthesis led to enhanced production of anthocyanins. These types of interactions between the flavonoid classes can make it difficult to predict the final flower pigmentation in advance.

It has also been possible to change flower pigmentation in petunia by altering the expression of genes affecting the further modification steps of the anthocyanin

molecules, such as rhamnosylation. A rhamnose group can be added to the 3-*O*-bound glucose of the anthocyanidin-3-glucoside molecule by 3RT (anthocyanidin-3-glucoside rhamnosyltransferase) to produce anthocyanidin-3-rutinosides. Brugliera *et al.* (1994) isolated a cDNA clone corresponding to the *Rt* locus of petunia using a differential screening technique. The functionality of the isolated cDNA was shown by complementation of a *Rtrt* mutant line. Antisense expression of the cDNA clone in purple petunia VR plants (*Rtrt*) plants resulted in altered flower colour in 7 out of 12 transformants. In most cases, uniformly pink flowers were detected but also flowers with purple and red sectors. The antisense transformants had distinct pigment profiles, with reduced levels of malvidin and higher levels of petunidin compared to control plants, and thereby altered phenotypes were produced. Similar results were obtained by Kroon *et al.* (1994).

Chrysanthemum

A recent article reported on flower colour modification in florist's chrysanthemum by introducing a chalcone synthase cDNA in both antisense and sense orientation, using *Agrobacterium*-mediated transformation of leaf explants (Courtney-Gutterson *et al.*, 1994). As in the case of petunia, a full-length CHS coding sequence was used under the control of the CaMV (cauliflower mosaic virus) 35S promoter. Both approaches were comparable in producing white-flowering transgenics from the cultivar Moneymaker, although the frequency of obtaining *chs* suppression was rather low (3/133 sense and 3/83 antisense individuals). In contrast to petunia, no pattern formation was detected. The white-flowering transformants were shown to accumulate CHS precursors, analysed by thin-layer chromatography of flower extracts. RNase protection assay of the white-flowering sense transformants showed reduction of endogenous *chs* message levels in leaves and flowers (Courtney-Gutterson *et al.*, 1994).

To study the stability of the phenotype, a small-scale field trial was performed in three different locations in the USA, using one vegetatively propagated white-flowering sense and one antisense line (200 cuttings from both at each site). Some variation in flower colour was detected during the growth in various sites, caused by environmental influences on the pigmentation, but this is common to white-flowering cultivars in many ornamentals. General growth characteristics were not altered. The overall conclusion of this experiment was that both approaches can be applied equally well for colour modification in chrysanthemum. Plant Variety Rights have been applied for in respect of three of the Moneymaker transformants.

Gerbera

Gerbera hybrida is an important ornamental plant and was ranked sixth in sales through Dutch auctions in 1991. Modification of flower colour has been obtained by transformation with both antisense chalcone synthase cDNA (nearly full length) and antisense dihydroflavonol-4-reductase cDNA into the red-flowering variety, Terra Regina (Elomaa *et al.*, 1993). Although the transformation frequency using the *Agrobacterium* co-cultivation method was low, a range of new phenotypes was obtained. With the antisense *chs* construct one pink- and one cream-flowering

transformant were obtained, and with antisense *dfr* two different pink transformants, with slightly different shades of colour, were obtained. The reduction of flower pigmentation correlated with the reduction of either *chs* or *dfr* mRNA levels, respectively. Transformation with one antisense gene did not affect the expression of the other of the two genes of the pathway. Also, in the case of antisense chalcone synthase transformants, the enzyme activity was shown to be reduced as well. Very little antisense RNA was detected in transformants with altered phenotypes (unpublished results). The mutual absence of both antisense and sense transcripts have been detected in other cases (Smith *et al.*, 1988; van der Krol *et al.*, 1988; Hamilton, Lycett and Grierson, 1990). Some variation in the extent of the reduced pigmentation in these transformants was noticed during their growth in greenhouses, reflecting effects of environmental conditions on anthocyanin synthesis.

Rose

Rosa hybrida is the most important commercial cut-flower. At the DNA Plant Technology Corporation, USA, transformation of a dark-red flowering variety, Royalty, was performed using chalcone synthase cDNA in sense orientation under the 35S promoter (Courtney-Gutterson, personal communication). The constructs were introduced into embryogenic calli using *Agrobacterium*-mediated transformation and 15 transgenic plants (out of about 100) with reduced pigmentation were obtained. The reduction in anthocyanin amount ranged from 39% to 97%. Thin-layer chromatography showed the accumulation of CHS precursors and Northern analysis showed specific reduction of *chs* mRNA levels in transformants. However, no white-flowering transformants were obtained. This could be for several reasons. As known from petunia, some antisense fragments are more effective in altering the phenotype than others (van der Krol *et al.*, 1990c; de Lange *et al.*, 1993). In this case, only a fragment of *chs* coding sequence was used. It is also possible that the introduced *chs* has not suppressed the expression of all members of the chalcone synthase gene family in rose (Courtney-Gutterson, personal communication). The colour changes in the pink transformants were stable.

BRINGING NEW COLOURS TO THE EXISTING POOL

There are already many examples of altering flower colour intensity by using either antisense or co-suppression approaches. Thereby, it has been possible to produce a range of paler or acyanic flower colours from red-flowering varieties, as described above. In order to bring totally new hues into the colour selection of certain species, more knowledge of the genetics and chemistry of the particular species is required. Introduction of the maize *A1* or gerbera *dfr* gene into a petunia mutant resulted in production of orange pelargonidins not occurring naturally in petunia. Pelargonidin pigments are also missing from chrysanthemum and lisianthus. The possibilities of producing novel red and yellow pigmentation in ornamentals are discussed. Also, progress in bringing the missing enzymes needed for the route to blue pigments into the most important cut-flowers is presented. In fact, all of the genes that should be necessary for the production of blue flowers have now been isolated (Holton and Tanaka, 1994).

Maize and gerbera dfr genes in petunia RL01

Transformation of petunia by Meyer *et al.* (1987) using a heterologous gene from maize was the first case of using genetic engineering to alter flower colour. Orange-coloured pelargonidin pigments are not produced in naturally occurring varieties of petunia, due to the substrate specificity of the petunia dihydroflavonol-4-reductase. Petunia DFR cannot reduce dihydrokaempferol, but uses instead dihydromyricetin and dihydroquercetin as substrates, leading to the synthesis of the corresponding leucoanthocyanidins (Meyer *et al.*, 1987). The previously characterized petunia mutant, RL01, accumulates dihydrokaempferol due to lack of both flavonoid 3'-hydroxylase and 3',5'-hydroxylase activity. The flower colour of this mutant is therefore nearly white, and it accumulates only traces of cyanidin and delphinidin derivatives. By transforming the *A1* gene from maize, encoding the maize dihydroflavonol-4-reductase, under the control of the CaMV 35S promoter, Meyer *et al.* (1987) produced a new pathway in petunia leading to the accumulation of the brick-red pelargonidin.

The petal colour has also been changed using the *A1* gene under the control of the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) promoter by Benfey *et al.* (1990). They used the EPSPS/A1 construct to transform the RL01 mutant and the mutant line W80, which completely lacks visible anthocyanin pigments. In RL01 a uniform pink-orange colour was produced while in W80 a lighter pink colour was detected.

Besides producing a new flower colour in petunia, the *A1* gene has proven to be an excellent phenotypic marker to follow transgene activity and to analyse the parameters that influence expression of a transgene. Transformation of the *A1* gene led to formation of an even, brick-red colouration in some of the transformants, and flowers with brick-red sectors were also detected, as well as control-like transformants. Further analysis of the variable phenotypes revealed an inverse correlation between the number of integrated *A1* copies and uniformity of pelargonidin pigmentation. Furthermore, it was shown that the decrease in *A1* expression was correlated to the methylation status of the 35S promoter (Linn *et al.*, 1990; Meyer, Heidmann and Niedenhof, 1993). In the majority of cases, uniform, continuous expression of the *A1* gene was found in plants where the transgene was single-copy integrate and its promoter was not methylated. Most white and variegated plants had multiple copies of the *A1* gene, which may cause co-suppression. However, Pröls and Meyer (1992) reported that even the transformants with one integrated copy of the *A1* gene did not show stable expression if the gene was integrated into a region of highly repetitive DNA.

In order to trap transposable elements from petunia, one of the *A1* transformant lines (RL01–17), containing only one copy of the *A1* gene, was chosen for large field tests, performed in 1990. Integration of a transposable element into *A1* should have resulted in either a white or a variegated phenotype (Meyer *et al.*, 1992). Over 30 000 transgenic plants were grown under field conditions and monitored for changes in pigmentation. White and variegated phenotypes were detected during the summer. A single white mutant was detected that bore white flowers throughout the growing season. It was shown to have arisen through a deletion of the *A1* gene. In addition, weakly coloured, white and sectored phenotypes were detected, which correlated

with hypermethylation of the 35S promoter. Environmental factors, such as high temperature and high light intensity during the growth season, reduced the floral pigmentation. Also, variation in pigmentation was dependent on endogenous factors, such as the age of the parental plant from which the seed was derived, or the time at which crosses were made (Meyer *et al.*, 1992). The possibility of instability in the transgene expression must be kept in mind in other applications.

Making use of the homology of previously isolated *dfr* genes, the corresponding gene was isolated from gerbera, and the functionality of the gene was shown by introducing it into the petunia mutant RL01 (Helariutta *et al.*, 1993). The maize *A1* gene was used as a control gene, cloned into the same *Agrobacterium* vector under the control of the 35S promoter. Using the leaf-disc transformation method (Horsch *et al.*, 1985), 15 transformants were obtained harbouring the gerbera *gdfr* construct. Most of them showed intense brick-red colouration and one transformant had flowers with sectors. The intensity of the colour was stronger compared to the *A1* transformants, which also showed brick-red phenotypes. In addition to this, it was noticed that the *A1* transformants showed variable phenotypes with sometimes totally white flowers during the summer season. The transformants with the gerbera *gdfr1* gene instead showed stable colouration, even when grown outdoors. The copy numbers of the introduced genes were studied by Southern hybridization and no differences between the *A1* and *gdfr* transformants were detected – both had clones with 1–5 gene copies in the genome (unpublished results). In gerbera *gdfr1* transformants more intense colouration was detected in transformants with several transgene copies. By analysing the expression levels of the introduced genes by Northern analysis, it was shown that *gdfr* RNA levels were higher than the *A1* transcript levels, and they correlated with the detected phenotypes. Whether this was due to stability of the *gdfr* transcripts or to differences in transcription rates is still to be determined.

Production of novel red and yellow pigmentation in ornamentals

It has been demonstrated that transformation of an appropriate petunia mutant with a *dfr* gene from maize or gerbera leads to the production of pelargonidin, a pigment normally absent from petunia. However, the production of pelargonidin relies on the absence of flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase enzymes. The inability of the DFR enzyme to act on dihydrokaempferol has only been reported for solanaceous plants.

The major anthocyanin produced by chrysanthemum (*Dendranthema morifolium*) flowers has been identified as cyanidin 3-malonylglucoside (Saito *et al.*, 1988). Delphinidin pigments are not produced, presumably due to the absence of a *f3'5'h* gene. Pelargonidin pigments are also missing. However, the lack of pelargonidin is not due to the DFR specificity (Schwinn, Markham and Given, 1994) but due to the absence of plants lacking F3'H activity. Therefore, suppression of *f3'h* gene expression should lead to the production of pelargonidin pigments and novel red flower colours in chrysanthemum.

The colour range of lisianthus (*Eustoma grandiflorum*) flowers is limited to purple, mauve, pink, whites and creams. Petals of purple lisianthus are characterized by delphinidin glycosides accompanied by variable, but generally low, quantities of cyanidin glycosides (Markham and Ofman, 1993). When developing

flower buds were fed with tetcyclasis, a cytochrome *P*450 inhibitor which prevents both F3'H and F3'5'H activities, a flower colour change occurred due to the production of pelargonidin pigment (Nielsen *et al.*, 1994). Reduction of F3'5'H activity via an antisense or co-suppression gene construct should lead to the synthesis of pelargonidin or cyanidin pigments, resulting in the production of novel red-coloured varieties of lisianthus.

Cyanic colour is particularly abundant within the Bromeliaceae family, giving in many plants purple-blue to orange-red flowers. A survey of the anthocyanins in 32 species and two cultivars indicated that cyanidin derivatives were the predominant anthocyanidins in the inflorescences and leaves, in spite of the fact that the pelargonidin-like colours dominate in these tissues (Saito and Harborne, 1983). The reason for this apparent discrepancy came with the discovery of a new glycosidic type of cyanidin (3,5,3'-triglucoside) in 60% of the plants sampled. Three other 3'-glucosides were also found in the Bromeliaceae. All four novel cyanidin glucosides are bright scarlet instead of the deep crimson of other cyanidin glycosides.

A number of different glycosyltransferase genes have been isolated from plants, animals and bacteria. The flavonoid glycosyltransferases 3GT and 3RT share regions of sequence similarity with each other and with glucuronosyltransferases from animals. It is likely that the enzyme catalysing 3'-glucosylation of cyanidin pigments in the Bromeliaceae would also share sequence similarity with other glycosyltransferases. Transformation of plants such as chrysanthemum with a 3'-glucosyltransferase gene might enable the production of flowers with novel scarlet-red colours. This could provide an interesting alternative to the F3'H gene suppression approach outlined previously.

In *Sinningia cardinalis* and *Columnea* flowers a reductase was demonstrated which catalyses the NADPH-dependent reduction of flavanones to flavan-4-ols (Stich and Forkmann, 1988a,b). The latter compounds are precursors for the rare 3-deoxyanthocyanidins. The purified *Dahlia* DFR was shown to catalyse both reactions, the reduction of dihydroflavonols and of flavanones (Fischer *et al.*, 1988), indicating that possibly one and the same enzyme is involved in flavanone and dihydroflavonol reduction. The 3-deoxyanthocyanidins are redder than the corresponding anthocyanidin derivatives. Therefore, a flavanone reductase gene might be used to engineer 3-deoxycyanidin pigments in plants such as chrysanthemum, which would result in the production of novel red colours.

In carnation flowers, the gene *I* controls chalcone isomerase activity. Genotypes with recessive *ii* alleles produce yellow flowers, which contain the chalcone isosalipurposide as the major petal pigment (Forkmann and Dangelmayr, 1980). Yellow carnations are also mutated in gene *a*, which prevents the conversion of dihydroflavonols into anthocyanins. In *iiAA* plants the flowers are orange due to the production of a mixture of chalcones and anthocyanins. Isosalipurposide, the 2'-glucoside of chalcone, is also the major petal pigment of *Paeonia trolloides, Asystasia gangetica* and *Aeschynanthus parvifolius* (Harborne, 1966).

Antisense *chi* experiments have been carried out in petunia in an attempt to produce yellow flowers through the accumulation of chalcone pigments. These experiments have failed to suppress *chi* gene expression, for unknown reasons (De Lange, 1994). However, even if *chi* expression was eliminated in the petals it is unlikely that chalcone would accumulate. This is because chalcone is rapidly isomerized spontane-

ously to the colourless naringenin in aqueous solution (Forkman, 1991). *Chi-A* mutants of petunia produce yellow pollen due to the accumulation of chalcone (van Tunen *et al.*, 1991). However, flavonoids in pollen are deposited in the exine and/or its cavities (Wierman and Vieth, 1983). This hydrophobic environment may stabilize the chalcone molecules and prevent isomerization to the colourless naringenin.

In carnation CHI mutants, chalcone is stabilized by 2'-glucosylation, which prevents isomerization to naringenin. In order to genetically engineer yellow, chalcone-accumulating flowers it would be necessary to suppress CHI activity and anthocyanin production, while also introducing a chalcone 2'-glucosyltransferase, if there is no such endogenous enzyme.

Engineering of blue pigments

The hydroxylation pattern of the anthocyanin B-ring is generally determined by the activity of two different cytochrome *P*450 enzymes – flavonoid 3'-hydroxylase and flavonoid 3'5'-hydroxylase. Flavonoid 3',5'-hydroxylase activity is essential for the production of the blue delphinidin pigments. The introduction of a F3'5'H gene into pelargonidin- or cyanidin-producing plants should divert the biosynthetic pathway towards the production of delphinidin pigments. Provided that the vacuolar pH and co-pigment levels are sufficiently high, violet or blue-violet flowers should result.

F3'5'H activity relies on the transfer of electrons from a *P*450 reductase enzyme. A potential problem may therefore arise if a heterologous F3'5'H enzyme does not interact efficiently with the endogenous *P*450 reductase in a target plant (Forkmann, 1993). However, this is an unlikely scenario, given that the petunia F3'5'H enzymes were shown to be functional in yeast, using the endogenous yeast *P*450 reductase (Holton *et al.*, 1993). Recombinant petunia F3'5'H genes have also proven to be functional in flowers of petunia (Holton *et al.*, 1993), tobacco, carnation and chrysanthemum (unpublished results).

The initial complementation experiments in petunia used a line (Skr4xSw63) which was mutated in each of the flavonoid hydroxylase genes. Due to the substrate specificity of the DFR in petunia, this line produces very little anthocyanin and accumulates dihydrokaempferol (one of the substrates of F3'5'H). The *hf1* and *hf2* mutations could be complemented by transformation of Skr4xSw63 with cDNA clones of *Hf1* or *Hf2* under the control of the constitutive MAC promoter (Holton *et al.*, 1993). Manipulation of the 5'-leader and promoter sequences has enabled the production of transgenic petunia plants accumulating 'wild-type' levels of delphinidin derivatives in the flowers by using either a MAC promoter or a chalcone synthase promoter (unpublished results).

Efficient production of delphinidin derivatives in other, more important, ornamental species may be more difficult. 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) through to delphinidin glycosides and accumulate these anthocyanins in the vacuole.

If a cyanidin-producing plant is transformed with a F3'5'H gene, expression of the *f3'5'h* gene must be sufficient to effectively compete for substrate with the endogenous genes producing F3'H, FLS and DFR activity. In petunia, when all of these enzyme

activities are present the only anthocyanins produced are delphinidin derivatives, as well as the flavonols kaempferol and quercetin. If F3'H activity is absent, then only delphinidin derivatives and kaempferol are produced. Pelargonidin is not produced because of the substrate specificity of DFR. Similarly, myricetin is not produced in large amounts due to the substrate specificity of FLS. If the endogenous DFR enzyme of a target species has a preference for dihydrokaempferol or dihydroquercetin over dihydromyricetin, it may be difficult to produce high levels of delphinidin.

Efficient production of delphinidin is the first requirement for obtaining blue flowers. It is essential that the delphinidin is produced in flowers that have a sufficiently high vacuolar pH and co-pigment level. Stewart, Norris and Asen (1975) determined that the pH of petal extracts from a number of different blue-violet or blue-flowered species ranged from 5.1 to 7.5. A gene (*Ph6*) that modifies flower colour by affecting the pH of the corolla has been cloned by Chuck *et al.* (1993) from petunia using maize transposable element (*Activator*). Production of sufficient levels of co-pigments is very important to create blue flowers in some species, whereas in other species polyacylation of the anthocyanins can lead to efficient intramolecular co-pigmentation (Goto and Kondo, 1991). No anthocyanin acyltransferase genes have yet been isolated.

Geissmann and Mehlquist (1947) 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, have demonstrated that the anthocyanins are glucosides acylated with malic acid (Terahara *et al.*, 1986).

In the presence of dominant alleles of the gene *R* (cyanidin type), part of dihydrokaempferol is hydroxylated in the 3'-position to dihydroquercetin, and both quercetin and kaempferol derivatives are formed in flowers (Stich, Eidenberger and Wurst, 1992). However, at the anthocyanin level, pelargonidin derivatives are nearly completely replaced by cyanidin derivatives in flowers of genotypes with dominant *R* alleles (Stich *et al.*, 1992). This pattern clearly corresponds to the substrate specificity of FLS and DFR, which both use dihydroflavonols as substrates. While for FLS no difference in the rate of the conversion of dihydrokaempferol and dihydroquercetin to the respective flavonols was observed, the DFR enzyme, being involved in anthocyanin synthesis, was found to reduce dihydroquercetin significantly faster (four times) than dihydrokaempferol. This means that FLS and DFR compete for dihydroquercetin as a substrate, whereas dihydrokaempferol is preferentially converted to kaempferol. Thus, relatively high amounts of kaempferol derivatives are formed even in plants containing F3'H activity.

Stich *et al.* (1992) showed by feeding experiments that carnation petals contain enzymes necessary for the conversion of dihydromyricetin into delphinidin derivatives. Administration of dihydroquercetin and dihydromyricetin initiated, in the petals of the red and orange strains, the formation of cyanidin and delphinidin derivatives, respectively, in addition to the pelargonidin derivatives which are 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. Carnations, which naturally accumulate pelargonidin pigments, have been transformed with a petunia *f3'5'h* gene. Flowers from the transgenic plants accumulate a mixture of

pelargonidin and delphinidin pigments, and the colour of the flowers was shifted towards blue, as expected (unpublished results). Experiments aimed at increasing the efficiency of delphinidin production in carnation are currently under way.

Rose pigments in species and cultivars have been studied extensively. Yokoi (1974) analysed the anthocyanin pigments contained in the flowers of 670 cultivars and eight 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. Most of the so-called 'blue' roses, which are in reality lilac or purple, contained only cyanidin 3,5-diglucoside, together with large amounts of flavonols. 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. 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 (Holton and Tanaka, 1994).

The pH of the outer epidermal cells has been determined in a range of rose varieties. The range of pH was 3.56 to 5.38 (Biolley and Jay, 1993) – purple- and lilac-coloured roses had the higher pH values. If such purple or lilac cultivars could be transformed with a *f3'5'h* gene and were able to efficiently synthesize delphinidin, the pH of the flowers is probably high enough to produce violet or blue-violet colours.

MODIFICATION OF GENES REGULATING THE FLAVONOID PATHWAY

Many regulatory genes are known to affect flower pigmentation, either by regulating directly the expression of structural genes or having secondary effects on flower colour by modulation of pH or concentration of anthocyanins in the vacuole (van Tunen and Mol, 1991). The genes involved in regulation of the anthocyanin pathway have been best characterized in maize, petunia and snapdragon, as reviewed by Dooner, Robbins and Jorgensen (1991), van Tunen and Mol (1991) and Martin and Gerats (1993). The regulatory proteins, with homology to the mammalian *myc* and *myb* transcription factors, are highly conserved, both structurally and functionally. Differences in pigmentation patterns in various species have evolved through changes in the expression of the conserved regulators (Goodrich, Carpenter and Coen, 1992). The set of target genes varies in the species studied so far. In maize, the regulatory genes act on the whole anthocyanin pathway, while in snapdragon and petunia only the late part of the pathway is regulated by these factors. The division between the early and late parts varies as well, being either before F3H (*Antirrhinum*) or after F3H (petunia) (Quattrocchio *et al.*, 1993). Isolation of the regulatory genes has now allowed the alteration of flower pigmentation and creation of novel pigmentation patterns at a more general level. In addition, production of high concentrations of anthocyanin pigments can lead to the appearance of black colours in flowers of pansy, cornflower and tulip. Similarly, in maize the recessive intensifier, *in*, results in such a greatly increased quantity of pigment that the kernels of both *Pr* (cyanidin-producing) and *pr* (pelargonidin-producing) strains appear black (Reddy and Peterson, 1978). Therefore, by increasing production of anthocyanins in ornamental species, novel black or very dark colours might be engineered.

By expressing the genes regulating the structural genes of the phenylpropanoid

pathway, it has been possible to enhance anthocyanin production in tobacco and *Arabidopsis* in normally acyanic organs (Lloyd, Walbot and Davis, 1992). Expression of the maize *C1* (the *myb* homologue) under the control of the 35S promoter alone did not have an effect on the phenotype of transformed tobacco or *Arabidopsis*. A transgenic tobacco line expressing the maize *R* (the *myc* homologue) showed instead intense red pigmentation in the corolla, tube and collar of the flower, as well as in the anther filaments, which normally are only light pink. In *Arabidopsis*, all *R* transformants produced more anthocyanin than normally, but without change in the pigmentation pattern. By crossing an *R*-expressing *Arabidopsis* transformant with a *C1*-expressing one, some of the progeny produced anthocyanins in root, petal and stamen tissues, which are normally acyanic (Lloyd, Walbot and Davis, 1992). In this way, totally new pigmentation patterns were produced. In a similar way, regulatory genes could be placed under the control of tissue- or cell-specific promoters, and thereby anthocyanin synthesis could be turned on in normally acyanic tissues (van Tunen and Mol, 1991).

In petunia, several regulatory genes affecting the flavonoid pathway have been characterized. Quattrocchio *et al.* (1993) concluded that products of *an1*, *an2* and *an11* genes control the transcription of the *dfrA* and *rt* genes in petunia. In addition, *an4* was shown to be regulatory gene controlling the expression of flavonoid biosynthetic genes in anthers. Transformation of two petunia lines V26 (*an4*) and W115 (*an2* and *an4*) with the maize *R* gene under the control of the 35S promoter activated petunia flavonoid genes in both lines. About 80% of the transformed calli developed red or purple cells, but none of them formed shoots. Some green shoots derived from these calli turned purple at early stages of growth, but did not form roots or grow further. Transformants that could be regenerated showed low or no accumulation of *R* mRNA and, therefore, it was not possible to test whether the *R* gene could have restored flower pigmentation in these mutants. Transient expression analysis using particle bombardment into leaf or floral tissue, instead, showed that *C1* and *R* genes activated *dfrA* promoter *in trans* in leaves and also in petal limbs of the three mutants. These experiments indicate that it is possible to activate floral pigmentation genes in tissues where they are normally silent.

Expression of the *Antirrhinum* anthocyanin regulatory gene, *del*, under the 35S promoter in petunia (V26), instead, disrupted anthocyanin biosynthesis (Robbins and Harbord, 1994). The corolla pigmentation was reduced and stress-induced leaf pigmentation enhanced in primary transformants. Reduction in corolla pigmentation was even more pronounced when *del* was transformed to V26/W115 background. The phenotypes of the transformants resembled the *Ph6* mutation in petunia that affects the vacuolar pH. However, it is not clear how the DEL protein interferes with the regulation of the anthocyanin pathway (Robbins and Harbord, 1994).

Summary and future prospects

Flower colour modification using molecular methods has now become reality. To date, the most successful applications have been in suppressing flavonoid biosynthesis at certain steps of the pathway, either by using the antisense or sense approach. In this way, pale or acyanic varieties have been produced in several species.

In the cases studied so far, the degree of suppression varies in different transformants

and has resulted in the formation of new phenotypes with variably reduced pigmentation. This is not necessarily bad from a breeder's point of view, since a range of new colour shades can be produced from an élite genotype. However, what is more puzzling, is that the antisense (or sense) effect is not seen in every transformant. The mere presence of the antisense RNA does not guarantee the phenotypic effect (van Blokland, 1994). Obviously, additional factors influence the suppression. In chrysanthemum, an antisense effect was found in only 3 out of 83 transformants; while in gerbera, although only a few transformants were obtained, half of them showed changed phenotype. The sense approach was comparable to the antisense approach in chrysanthemum. Also, in rose it has not been possible to obtain fully white transformants using the sense approach. This could be due to properties of the gene construct used, or the chromosomal position of the integration in the transformants studied so far. It is also possible that some members of the *chs* gene family are still expressed and cause the residual pigmentation.

Although quite a few successful examples exist of suppression of gene expression at certain steps in the flavonoid pathway, it has not been possible to suppress chalcone isomerase genes using antisense methods. However, as already mentioned, suppression at this step might not result in accumulation of yellow chalcones, as they are spontaneously isomerized to colourless flavanones (Forkmann, 1991). De Lange (1994) transformed petunia plants with various partial and full-length *chi* antisense constructs. Suppression of expression of the endogenous *chi* gene was not detected in any of the transformants, despite the presence of large amounts of antisense RNAs. By crossing a plant containing the *chi* transgene with different antisense transformants, transcriptional suppression of the *chi* transgenes was detected in some of the progeny. It was concluded that different modes of suppression of the same *chi* transgene may indicate that the genomic position of the antisense genes, or the sense gene, could be involved in the interaction. The resident *chi* could be situated in a genomic region that prevents it from being silenced (De Lange, 1994).

In order to alter the flower colour in a more predictable and efficient way, the fundamental work done in petunia in order to reveal the basic mechanisms under both sense and antisense inhibition is of greatest importance. Van der Krol *et al.* (1990c) have studied the promoter and sequence requirements for the antisense effect in petunia. In most cases of colour modification the constitutive 35S promoter has been used, but inhibition can also be obtained using homologous promoters, since an excess of antisense RNA is not necessary for efficient suppression. Several studies have been made to retrieve strong antisense fragments from weak ones (van der Krol *et al.*, 1990c; de Lange *et al.*, 1993). In the case of chalcone synthase in petunia, a quarter-length RNA complementary to the 3' end of *chs* mRNA was able to affect flower pigmentation. However, an antisense gene corresponding to the 5' end had no effect on the phenotype (van der Krol *et al.*, 1990c). De Lange *et al.* (1993) have developed a system to study antisense inhibition in protoplasts, using the *uidA* marker gene in order to improve the effectiveness of the antisense inhibition and, on the other hand, selectively suppress members of the gene family. Maraña and Gerats (1994) reported that chimeric constructions containing a *uidA* marker gene under the control of a *dfrA* or *dfrC* promoter created a block in anthocyanin biosynthesis when introduced into petunia. In half of the transformants a dramatic reduction of *dfrA* expression and GUS activity were detected, and novel pigmentation patterns were

observed. Using this system it is possible to monitor visually the native gene suppression, and histochemically the suppression of the marker gene (Maraña and Gerats, 1994).

It is now possible to affect the expression of single structural genes at various steps of the pathway, also including genes affecting co-pigmentation and further modification steps of the anthocyanin molecule. Still, production of pelargonidin pigmentation in petunia is the only example of bringing totally new colours into a pre-existing gene pool. In theory, all genes that should be needed for production of blue colours into the most important commercial species have been cloned, and transformation methods for them have been developed. Testing of the possibilities of colour modification in these plants is currently underway.

Cloning of regulatory genes whose gene products are needed for the expression of the structural flavonoid genes will, without any doubt, lead to flower-colour modification at a more general level. This type of modification may create more diversity in pigmentation patterns and also in colour shades.

Besides producing new phenotypes, flower-colour modification has turned out to be an excellent system to monitor visually the variations in expression and regulation of transgenes, and also to study the fundamental processes involved in the interactions between a transgene and the endogenous genes. This information is valuable also in the modification of other important pathways in plants. Moreover, the factors influencing the stability of the altered phenotype need to be examined when commercial-scale propagation is required.

Acknowledgements

We gratefully acknowledge Dr Neal Courtney-Gutterson, Dr Karen Nielsen and Dr Kevin Davies for providing us with information on their work. We also wish to thank Dr Teemu Teeri for his help with the figures and for valuable comments on the manuscript. Yrjö Helariutta and Mika Kotilainen are also thanked for critical reading of the manuscript and for their comments.

References

AN, G., WATSON, B.D., STACHEL, S., GORDON, M.P. AND NESTER, E.W. (1985). New cloning vehicles for transformation of higher plants. *EMBO Journal* **4**, 277–284.

BELD, M., MARTIN, C., HUITS, H., STUITJE, A.R. AND GERATS, A.G.M. (1989). Flavonoid synthesis in *Petunia hybrida*: partial characterization of dihydroflavonol-4-reductase genes. *Plant Molecular Biology* **13**, 491–502.

BENFEY, P.N., TAKATSUJI, H., REN, L., SHAH, D.M. AND CHUA, N.-H. (1990). Sequence requirements of the 5-enolpyruvylshikimate-3-phosphate synthase 5'-upstream region for tissue-specific expression in flowers and seedlings. *The Plant Cell* **2**, 849–856.

BIOLLEY, J.P. AND JAY, M. (1993). Anthocyanins in modern roses: chemical and colorimetric features in relation to the colour range. *Journal of Experimental Botany* **44**, 1725–1734.

BOASE, M.R. AND SMITH, J.L. (1994). Expression of the GUS gene in 11 *Pelargonium* cultivars transformed by *Agrobacterium tumefaciens. 4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

BOLTON, G.W., NESTER, E.W. AND GORDON, M.P. (1986). Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* **232**, 983–985.

BRITSCH, L., RUHNAU-BRITSCH, B. AND FORKMANN, G. (1992). Molecular cloning, sequence analysis and *in vitro* expression of flavanone 3-β-hydroxylase from *Petunia hybrida*.

Journal of Biological Chemistry **267**, 5380–5387.

BRITSCH, L., DEDIO, J., SAEDLER, H. AND FORKMANN, G. (1993). Molecular characterization of flavanone 3-β-hydroxylases. Consensus sequence, comparison with related enzymes and the role of conserved histidine residues. *European Journal of Biochemistry* **217**, 745–754.

BRUGLIERA, F., HOLTON, T.A., STEVENSON, T.W., FARCY, E., LU, C.-Y. AND CORNISH, E.C. (1994). Isolation and characterization of a cDNA clone corresponding to the *Rt* locus of *Petunia hybrida*. *The Plant Journal* **5**, 81–92.

CHRISTOU, P. (1992). Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal* **2**, 275–281.

CHUCK, G., ROBBINS, T., NIJJAR, C., RALSTON, E., COURTNEY-GUTTERSON, N. AND DOONER, H.K. (1993). Tagging and cloning of a petunia color gene with the maize transposable element *Activator*. *The Plant Cell* **5**, 371–378.

COURTNEY-GUTTERSON, N., NAPOLI, C., LEMIEUX, C., MORGAN, A., FIROOZABADY, E. AND ROBINSON, K.E.P. (1994). Modification of flower color in florist's chrysanthemum: Production of a white-flowering variety through molecular genetics. *Bio/Technology* **12**, 268–271.

DAVIES, K.E., BRADLEY, J.M., SCHWINN, K.E., MARKHAM, K.R. AND PODIVINSKY, E. (1993). Flavonoid biosynthesis in flower petals of five lines of lisianthus (*Eustoma grandiflorum* Grise.). *Plant Science* **95**, 67–77.

DEBLAERE, R., BYTEBIER, B., DE GREVE, H., DEBOECK, R., SCHELL, J., VAN MONTAGU, M. AND LEEMANS, J. (1985). Efficient octopine Ti plasmid-derived vectors for *Agro-bacterium*-mediated gene transfer to plants. *Nucleic Acids Research* **13**, 4777–4788.

DE LANGE, P. (1994). Gene suppression in *Petunia hybrida* by antisense genes. Unpublished Thesis, Free University of Amsterdam.

DE LANGE, P., DE BOER, G.-J., MOL, J.N.M. AND KOOTER, J.M. (1993). Conditional inhibition of β-glucuronidase expression by antisense gene fragments in petunia protoplasts. *Plant Molecular Biology* **23**, 45–55.

DEROLES, S.C., LEDGER, S.E., MILLER, R.M., DAVIES, K.M. AND GIVEN, N.K. (1993). Transformation in *Eustoma grandiflorum* (Lisianthus). In *Biotechnology in Agriculture and Forestry*, Vol. 22, *Plant Protoplasts and Genetic Engineering III* (Y.P.S. Bajaj, Ed.), pp. 202–212. Springer- Verlag, Berlin.

DOONER, H.K., ROBBINS, T.P. AND JORGENSEN, R.A. (1991). Genetic and developmental control of anthocyanin biosynthesis. *Annual Review of Genetics* **25**, 173–199.

ELOMAA, P., HONKANEN, J., PUSKA, R., SEPPÄNEN, P., HELARIUTTA, Y., MEHTO, M., KOTILAINEN, M., NEVALAINEN, L. AND TEERI, T.H. (1993). *Agrobacterium*-mediated transfer of antisense chalcone synthase cDNA to *Gerbera hybrida* inhibits flower pigmentation. *Bio/Technology* **11**, 508–511.

FISCHER, D., STICH, K., BRITSCH, L. AND GRISEBACH, H. (1988). Purification and characterization of (+)dihydroflavonol (3-hydroxyflavanone) 4-reductase from flowers of *Dahlia variabilis*. *Archives of Biochemistry and Biophysics* **264**, 40–47.

FORKMANN, G. (1991). Flavonoids as flower pigments: the formation of the natural spectrum and its extension by genetic engineering. *Plant Breeding* **106**, 1–26.

FORKMANN, G. (1993). Control of pigmentation in natural and transgenic plants. *Current Opinion in Biotechnology* **4**, 159–165.

FORKMANN, G. AND DANGELMAYR, B. (1980). Genetic control of chalcone isomerase activity in flowers of *Dianthus caryophyllus*. *Biochemical Genetics* **18**, 519–527.

GEISSMAN, T.A. AND MEHLQUIST, G.A.L. (1947). Inheritance in the carnation, *Dianthus caryophyllus*. IV. The chemistry of flower colour variation, I. *Genetics* **32**, 410–433.

GOODRICH, J., CARPENTER, R. AND COEN, E.S. (1992). A common gene regulates pigmentation pattern in diverse plant species. *Cell* **68**, 955–964.

GOTO, T. AND KONDO, T. (1991). Structure and molecular stacking of anthocyanins – flower color variation. *Angewandte Chemie International* Edition in English **30**, 17–33.

HAMILTON, A.J., LYCETT, G.W. AND GRIERSON, D. (1990). Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* **346**, 284–287.

HARBORNE, J.B. (1966). Comparative biochemistry of flavonoids – I. Distribution of chalcone and aurone pigments in plants. *Phytochemistry* **5**, 111–115.

HELARIUTTA, Y., ELOMAA, P., KOTILAINEN, M., SEPPÄNEN, P. AND TEERI, T.H. (1993). Cloning

of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of *dfr* expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). *Plant Molecular Biology* **22**, 183–193.

HOLTON, T.A. AND TANAKA, Y. (1994). Blue roses – a pigment of our imagination? *Trends in Biotechnology* **12**, 40–42.

HOLTON, T.A., BRUGLIERA, F. AND TANAKA, Y. (1993). Cloning and expression of flavonol synthase from *Petunia hybrida*. *The Plant Journal* **4**, 1003–1010.

HOLTON, T.A., BRUGLIERA, F., LESTER, D.R., TANAKA, Y., HYLAND, C.D., MENTING, J.G.T., LU, C.-Y., FARCY, E., STEVENSON, T.W. AND CORNISH, E.C. (1993). Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* **366**, 276–279.

HOOYKAAS, P.J.J. (1989). Transformation of plant cells via *Agrobacterium*. *Plant Molecular Biology* **13**, 327–336.

HOOYKAAS-VAN SLOGTEREN, G.M.S., HOOYKAAS, P.J.J. AND SCHILPEROORT, R.A. (1984). Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. *Nature* **311**, 763–764.

HORSCH, R.B., FRY, J.E., HOFFMANN, N.L., EICHHOLTZ, D., ROGERS, S.G. AND FRALEY, R.T. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229–1231.

HUTCHINSON, J.F., KAUL, V., MAHESWARAN, G., MORAN, J.R., GRAHAN, M.W. AND RICHARDS, D. (1992). Genetic improvement of floricultural crops using biotechnology. *Australian Journal of Botany* **40**, 765–87.

KLEIN, T.M., WOLF, E.D., WU, R. AND SANFORD, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**, 70–73.

KOES, R.E., QUATTROCCHIO, F. AND MOL, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays* **16**, 123–132.

KOES, R.E., SPELT, C.E. AND MOL, J.N.M. (1989). The chalcone synthase multigene family of *Petunia hybrida* (V30): differential, light-regulated expression during flower development and UV light induction. *Plant Molecular Biology* **12**, 213–225.

KROON, J., SOUER, E., DE GRAAFF, A., XUE, Y., MOL, J. AND KOES, R. (1994). Cloning and structural analysis of the anthocyanin pigmentation locus *Rt* of *Petunia hybrida*: characterization of insertion sequences in two mutant alleles. *The Plant Journal* **5**, 69–80.

LEDGER, S.E., DEROLES, S.C. AND GIVEN, N.K. (1991). Regeneration and agrobacterium-mediated transformation of chrysanthemum. *Plant Cell Reports* **10**, 195–199.

LINN, F., HEIDMANN, I., SAEDLER, H. AND MEYER, P. (1990). Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: Role of numbers of integrated gene copies and state of methylation. *Molecular and General Genetics* **222**, 329–336.

LLOYD, A.M., WALBOT, V. AND DAVIS, R.W. (1992). *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. *Science* **258**, 1773–1775.

LU, C.-Y., NUGENT, G., WARDLEY-RICHARDSON, T., CHANDLER, S.F., YOUNG, R. AND DALLING, M.J. (1991). *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). *Bio/Technology* **9**, 864–868.

MARAÑA, C. AND GERATS, T. (1994). Inhibition of flower pigmentation in petunia by GUS gene expression under the control of the *dfrA* promoter. *4th International Congress of Plant Molecular Biology, Amsterdam*. (Abstracts).

MARKHAM, K. AND OFMAN, D.J. (1993). Lisianthus flavonoid pigments and factors influencing their expression in flower colour. *Phytochemistry* **34**, 679–685.

MARTIN, C. AND GERATS, T. (1993). The control of flower coloration. *The Molecular Biology of Flowering* (B.R. Jordan, Ed.), pp. 219–255. CAB International, Wallingford, UK.

MEYER, P., HEIDMANN, I. AND NIEDENHOF, I. (1993). Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. *The Plant Journal* **4**, 89–100.

MEYER, P., HEIDMANN, I., FORKMANN, G. AND SAEDLER, H. (1987). A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* **330**, 677–678.

MEYER, P., LINN, F., HEIDMANN, I., MEYER, Z.A.H., NIEDENHOF, I. AND SAEDLER, H. (1992). Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. *Molecular and General Genetics* **231**, 345–352.

MO, Y., NAGEL, C. AND TAYLOR, L.P. (1992). Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proceedings of the National Academy of Sciences, USA* **89**, 7213–7217.

MOL, J.N.M., STUITJE, A.R. AND VAN DER KROL, A. (1989). Genetic manipulation of floral pigmentation genes. *Plant Molecular Biology* **13**, 287–294.

MULLEN, J., BLOWERS, A., ADAM, G., VAN ECK, J., FRARY, A. AND EARLE, E. (1994). Transformation of plant cells with yeast artificial chromosomes. *4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

NAPOLI, C., LEMIEUX, C. AND JORGENSEN, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *The Plant Cell* **2**, 279–289.

NIELSEN, K., SCHWINN, K., DAVIES, K., DEROLES, S., MANSON, D., MILLER, R., PODIVINSKY, E. AND MARKHAM, K. (1994). Flower colour modification in lisianthus (*Eustoma grandiflorum*). *4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

PRÖLS, F. AND MEYER, P. (1992). The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. *The Plant Journal* **2**, 465–475.

QUATTROCCHIO, F., WING, J.F., LEPPEN, H.T.C., MOL, J.N.M. AND KOES, R.E. (1993). Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct set of target genes. *The Plant Cell* **5**, 1497–1512.

REDDY, A.R. AND PETERSON, P.A. (1978). The action of the intensifier (*in*) gene in flavonoid production in aleurone tissue of maize. *Canadian Journal of Genetics and Cytology* **20**, 337–347.

REIF, H.J., NIESBACH, U., DEUMLING, B. AND SAEDLER, H. (1985). Cloning and analysis of two genes for chalcone synthase from *Petunia hybrida*. *Molecular and General Genetics* **199**, 208–215.

ROBBINS, T. AND HARBORD, R. (1994). A regulatory gene from *Antirrhinum* disrupts anthocyanin biosynthesis in *Petunia hybrida*. *4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

SAITO, N. AND HARBORNE, J.B. (1983). A cyanidin glycoside giving scarlet coloration in plants of the *Bromeliaceae*. *Phytochemistry* **22**(8), 1735–1740.

SAITO, N., TOKI, K., HONDA, T. AND KAWASE, K. (1988). Cyanidin 3-malonylglucuronylglucoside in *Bellis* and cyanidin 3-malonylglucoside in *Dendranthema*. *Phytochemistry* **27**, 2963–2966.

SCHWINN, K.E., MARKHAM, K.R. AND GIVEN, N.K. (1994). Floral flavonoids and the potential for pelargonidin biosynthesis in commercial chrysanthemum cultivars. *Phytochemistry* **35**,. 145–150.

SMITH, C.J.S., WATSON, C.F., RAY, J., BIRD, C.R., MORRIS, P.C., SCHUCH, W. AND GRIERSON, D. (1988). Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334**, 724–726.

STEVENSON, T.W. (1991). Molecular biology of flavonoid pigment biosynthesis in flowers. In *Molecular Approaches to Crop Improvement* (E.S. Dennis and D.J. Llewellyn, Eds), pp. 127–148. Springer Verlag, Wien.

STEWART, R.N., NORRIS, K.H. AND ASEN, S. (1975). Microspectrophotometric measurment of pH and pH effect on color of petal epidermal cells. *Phytochemistry* **14**, 937–942.

STICH, K. AND FORKMANN, G. (1988a). Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*. *Phytochemistry* **27**, 785–789.

STICH, K. AND FORKMANN, G. (1988b). Studies on columnidin biosynthesis with flower extracts from *Columnea hybrida*. *Zeitschrift für Naturforschung* **43c**, 311–314.

STICH, K., EIDENBERGER, T. AND WURST, F. (1992). Flavonol synthase activity and the regulation of flavonol and anthocyanin biosynthesis during flower development in *Dianthus caryophyllus* L. (carnation). *Zeitschrift für Naturforschung* **47c**, 553–560.

STICH, K., EIDENBERGER, T., WURST, F. AND FORKMANN, G. (1992). Enzymatic conversion of dihydroflavonols to flavan-3,4-diols using flower extracts of *Dianthus caryophyllus* L. (carnation). *Planta* **187**, 103–108.

TERAHARA, N., YAMAGUCHI, M.-A., TAKEDA, K., HARBORNE, J.B. AND SELF, R. (1986).

Anthocyanins acylated with malic acid in *Dianthus caryophyllus* and *D. deltoides. Phytochemistry* **25**, 1715–1717.

THOMPSON, J.A., FRAME, B.R., DRAYTON, P.R., BAGNALL, S.V., JIAO, S., LEWNAU, C.J., BULLOCK, W.P., WILSON, H.M., DUNWELL, J.M. AND WANG, K. (1994). Production of fertile transgenic maize plants by silicon carbide fibre-mediated transformation. *4th International Congress of Plant Molecular Biology, Amsterday.* (Abstracts).

VAN BLOKLAND, R. (1994). Trans-inactivation of flower pigmentation genes in *Petunia hybrida.* Unpublished Thesis, Free University of Amsterdam.

VAN DER KROL, A.R., MOL, J.N.M. AND STUITJE, A.R. (1988). Antisense genes in plants: an overview. *Gene* **72**, 45–50.

VAN DER KROL, A.R., LENTING, P.E., VEENSTRA, J., VAN DER MEER, I.M., KOES, R.E., GERATS, A.G.M., MOL, J.N.M. AND STUITJE, A.R. (1988). An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature* **333**, 866–869.

VAN DER KROL, A.R., MUR, L.A., BELD, M., MOL, J.N.M. AND STUITJE, A.R. (1990a). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell* **2**, 291–299.

VAN DER KROL, A.R., MUR, L.A., DE LANGE, P., GERATS, A.G.M., MOL, J.N.M. AND STUITJE, A.R. (1990b). Antisense chalcone synthase genes in petunia: visualization of variable transgene expression. *Molecular and General Genetics* **220**, 204–212.

VAN DER KROL, A.R., MUR, L.A., DE LANGE, P., MOL, J.N.M. AND STUITJE, A.R. (1990c). Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. *Plant Molecular Biology* **14**, 457–466.

VAN TUNEN, A.J. AND MOL, J.N.M. (1991). Control of flavonoid synthesis and manipulation of flower colour. In *Developmental Regulation of Plant Gene Expression* (D. Grierson, Ed.), pp. 94–130. Blackie & Son Ltd. Glasgow and London.

VAN TUNEN, A.J., KOES, R.E., SPELT, C.E., VAN DER KROL, A.R., STUITJE, A.R. AND MOL, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*; coordinate, light-regulated and differential expression of flavonoid genes. *EMBO Journal* **7**, 1257–1263.

VAN TUNEN, A.J., MUR, L.A., RECOURT, K., GERATS, A.G.M. AND MOL, J.N.M. (1991). Regulation and manipulation of flavonoid gene expression in anthers of petunia: the molecular basis of the *Po* mutation. *The Plant Cell* **3**, 39–48.

VAN WORDRAGEN, M.F., DE JONG, J., HUITEMA, H.B.M. AND DONS, H.J.M. (1991). Genetic transformation of chrysanthemum using wild type *Agrobacterium* strains; strain and cultivar specificity. *Plant Cell Reports* **9**, 505–508.

VAN WORDRAGEN, M.F., BONNEMA, G., VERKERK, R., HONTELEZ, J., PEYTAVI, R., GRIMSLEY, N. AND ZABEL, P. (1994). YAC-transfer to plant cell. *4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

WEISS, D., VAN DER LUIT, A.H., KROON, J.T.M., MOL, J.N.M. AND KOOTER, J.M. (1993). The petunia homologue of the *Antirrhinum majus candi* and *Zea mays A2* flavonoid genes; homology to flavanone 3-hydroxylase and ethylene-forming enzyme. *Plant Molecular Biology* **22**, 893–897.

WIERMAN, R. AND VIETH, K. (1983). Outer pollen wall, an important accumulation site for flavonoids. *Protoplasma* **118**, 230–233.

WILMINK, A., VAN DE VEN, B.C.E. AND DONS, H.J.M. (1994). A transformation procedure for tulip. *4th International Congress of Plant Molecular Biology, Amsterdam.* (Abstracts).

WINANS, S.C. (1992). Two-way chemical signaling in *Agrobacterium*–plant interactions. *Microbiological Reviews* **56**, 12–13.

YOKOI, M. (1974). Colour and pigment distribution in ornamental plants. V. Anthocyanin distribution in rose cultivars. *Technical Bulletin of the Faculty of Horticulture, Chiba University* **22**, 13–24.

ZAMBRYSKI, P. (1988). Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annual Review of Genetics* **22**, 1–30.

ZAMBRYSKI, P., JOOS, H., GENETELLO, C., LEEMANS, J., VAN MONTAGU, M. AND SCHELL, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO Journal* **2**, 2143–2150.