Novel coloured flowers
Jos Mol*,†, Edwina Cornish‡§, John Mason‡ and Ronald Koes**

The floricultural industry has focused its attention primarily on the development of novel coloured and longer living cut flowers. The basis for this was laid down some years ago through the isolation of ‘blue’ genes and ethylene biosynthesis genes. Recently, a novel ‘blue’ gene has been discovered and yellow pigments were produced in petunias by addition of a new branch to the phenylpropanoid pathway. More insight was obtained into the sequestration of anthocyanin pigments into storage vacuoles. Significant progress has been achieved in the commercialisation of genetically modified flower varieties.

Addresses
*Department of Molecular Genetics, Vrije Universiteit, De Boelelaan 1087, 1081HV, Amsterdam, The Netherlands
†e-mail: mol@bio.vu.nl
‡e-mail: koes@bio.vu.nl
§Florigene Ltd, 16 Gipps Street, Collingwood, Melbourne, Victoria 3066, Australia
‡e-mail: ecornish@florigene.com.au

Current Opinion in Biotechnology 1999, 10:198–201
http://biomednet.com/elecref/0958166901000198
© Elsevier Science Ltd ISSN 0958-1669

Abbreviations
An Anthocyanin
CYTb E cytochrome b
CYTP450 cytochrome P450
DFR dihydroflavonol 4-reductase
DiF Differential F
F3,5′H flavonoid 3,5′-hydroxylase
GST glutathione S-transferase

Introduction
The past years have witnessed a tremendous increase in our understanding of the molecular basis of flower development. Much has been learned about how the various meristems of the plant develop and differentiate and how the floral meristem generates a flower with its complex array of whorls. This knowledge has precipitated a number of reviews on diverse aspects of flowering [1–7].

Much of the progress reported in this area is based on the analysis of loss-of-function mutants and gain-of-function transgenic lines. Because of the pleiotropic effects of many of the genes involved, the engineering of only a single novel phenotype may be complicated in many cases. Given the progress that has been made in understanding the molecular basis of petal colouration [5,8], it is not surprising that one of the first commercial applications of gene technology within the flower industry has been the development of novel coloured cut-flowers. This review will summarise the recent achievements in this area.

Anthocyanin flower colour
It is unusual to find the full spectrum of colours within conventionally bred flower species. For example, the orange pigment pelargonidin is not found in petunias. This is because the dihydroflavonol 4-reductase (DFR) enzyme from petunia is unable to convert dihydrokaempferol into the substrate for pelargonidin, kaempferol. An orange petunia was, however, produced more than a decade ago and represents the first product of successful manipulation of flower colour by gene technology. This was achieved by producing the maize DFR enzyme, which is able to convert dihydrokaempferol, in a white petunia variety accumulating this substrate. This experiment was designed in the first place to ‘trap’ transposable elements. Many of the transformants, however, suffered from epigenetic instability (i.e. unstable expression due to variation in promoter methylation) of the introduced Dfr transgenes, which resulted in similar phenotypes as would have resulted from transposon visitation [9]. Furthermore, epigenetic instability of the Dfr transgene would bar commercial applications. In spite of all this, researchers at Novartis succeeded in obtaining uniformly-coloured orange petunias by introgression of the maize Dfr gene into the multiflora petunia plant type [10]. Similar results were obtained by Elomaa et al. [11] by introducing a gerbera Dfr gene in the same petunia background. The authors suggest that this transgene is more ‘compatible’ with the petunia genome than the maize Dfr gene, firstly, because of its dicotyledonous origin (which makes the genes more similar in terms of sequence homology) and, secondly, because it has fewer methylation sites.

Traditional breeding of the top selling cut-flower species rose, chrysanthemum and carnation, has produced a wide array of colours. They lack the genetic capacity, however, to produce the delphinidin-derived pigments found in most ‘blue’ flowers. This has been one of the major limitations to breeding varieties in these colour ranges. In 1993, Holton et al. [12] reported the isolation of two petunia genes, Hf1 and Hf2. These encode membrane-bound cytochrome P450 (CYTP450) enzymes (flavonoid 3,5′-hydroxylase [F3,5′H]) capable of 3,5′-hydroxylation of the anthocyanin B-ring, which leads to a ‘blueing’ of flower colour. To isolate F3,5′H clones, the authors employed a PCR strategy using the conserved CYTP450 heme-binding domain. The activity of candidate clones was verified by expression in yeast and assaying for F3,5′H activity and by genetic complementation of mutant petunia lines. To display full activity, CYTP450s have to associate with electron donors such as NADPH:CYTP450 reductases, which catalyse the transfer of electrons from NADPH to the heme group of the CYTP450 protein. That these reductases are rather unspecific is illustrated by the observation that the yeast reductase can substitute for the petunia one in activating the petunia F3,5′H enzyme.
Recently, a novel gene was identified in petunia that encodes a cytochrome \( b_5 \) (CRYt\( b_5 \)) [13•]. Initially this gene was named Differential F (DiffF), as it was identified in a collection of cDNAs whose corresponding mRNAs are down-regulated in petunia flowers in a mutation in the regulatory Anthocyanin (An)-1 gene [14]. DiffF encodes a protein of 149 amino acids which represents a new class of CYT\( b_5 \) proteins. Targeted transposon \( dPkl1 \) inactivation of DiffF resulted in decreased levels of F3\',5'H activity and reduced accumulation of 5'-hydroxylated anthocyanins in the petal. The CYT\( b_5 \) encoded by DiffF seems rather specific because it neither affects 3'-hydroxylation of anthocyanins by F3\',5'H flavonoid 3'-hydroxylase nor does it influence the activity of cinnamate 4-hydroxylase, a distinct CYTP450 operating in the general phenylpropanoid pathway.

Several \textit{in vitro} studies have indicated that CYTP450 activity can be modulated by CYT\( b_5 \) and in some cases CYT\( b_5 \) acts to significantly enhance activity [15,16]. This can involve the donation of a second electron, in addition to that donated by NADPH:CYTP450 reductase, to the CYTP450; however, sometimes apo CYT\( b_5 \) works as well [16]. The mechanism by which this occurs is still poorly understood although there has been recent progress towards developing a kinetic model [17]. In petunia, it appears that the co-action of specific CYTP450 and CYT\( b_5 \) activities in conjunction with an unspecific NADPH:CYTP450 reductase is required to achieve maximal production of 3',5'-hydroxylated anthocyanins. Although there is no evidence to date as to whether this relationship exists in other plant species, it is possible that Diff-F may be useful in boosting F3',5'H activity in transgenic plants.

\textbf{Anthocyanin sequestration}

Anthocyanins are toxic substances that could be harmful to the cell. To cope with this potential problem, anthocyanins are stabilised and detoxified by transport to the vacuole. Recent evidence indicates that glutathionation and active transport of the conjugate by a glutathione S–X pump play a crucial role in this process.

Marrs \textit{et al}. [18] showed that the Bz2 gene of maize encodes a type III glutathione S-transferase (GST). Bz2 mutants lacking this activity accumulate anthocyanins in the cytosol, conferring a tan-bronze phenotype. The petunia gene An9 encodes a type I GST that, although it belongs to a different class of GSTs, functions in a similar way to BZ2 [19•].

Evidence that glutathionation is not the last step in anthocyanin pigmentation comes from phenotypic analysis of mutable alleles of anthocyanin genes. Mutatable alleles of the regulatory genes \textit{An1}, \textit{An2} and \textit{An11} yield coloured revertant cells immediately adjacent to unpigmented cells. In contrast, mutable alleles of \textit{An9}, Bz2 and those of structural anthocyanin genes cause a diffusion zone of coloured anthocyanin intermediates around revertant cells, indicative of cell-to-cell transport of anthocyanin intermediates. If glutathionation of anthocyanins was the last step controlled by \textit{An1}, \textit{An2} and \textit{An11}, one would expect to find halos for revertant mutations of those regulatory genes (which is not the case). Thus, glutathionation is not the last step controlled by the regulatory genes. It is conceivable that a multifunctional tonoplast ATP-Binding Cassette (ABC)-transporter, such as recently identified in Arabidopsis (AtMRP2), fulfills this last step (i.e. transport of anthocyanin–GST conjugates) [20•].

The anthocyanin regulatory genes \textit{An1}, \textit{An2} and \textit{An11} also affect the pH of petal extracts. A similar effect is observed for the \textit{Ph} genes (\textit{Ph1}–\textit{Ph7}). In this case, a shift in colour towards blue is observed while anthocyanin content is unaffected. The \textit{Ph} genes seem to affect vacuolar pH and this may also be the case for \textit{An1}, \textit{An2} and \textit{An11}. For some of the \textit{Ph} genes, an epistatic relationship with a regulatory anthocyanin gene has been established [5]. Whether vacuolar proton pumps are affected in \textit{an} and \textit{ph} mutants remains to be investigated.

\textbf{Mauve carnations}

Introduction and expression of F3',5'H and \textit{Dfr} genes into a white carnation variety that accumulated dihydrokaempferol resulted in the production of delphinidin...
(T Holton, personal communication). The transgenic carnations varied in intensity of colour depending on the level of expression of the petunia genes. In general, the colour of the flowers was a pale violet/mauve. Two separate effects were reported. In acyanic backgrounds, stable 6′-desoxychalcones accumulate and yellow colouration of the flowers was observed. In cyanic backgrounds, however, paler flower colours were observed due to the competition of chalcone reductase and chalcone flavanone isomerase for the common 6′-hydroxychalcone substrates. To our knowledge, this approach has not been applied yet in commercially important cut flower species.

**Commercialisation of genetically modified flower varieties**

Genetically modified flowers are now commercially available in many parts of the world. The first market introduction occurred in Australia in October 1996 when Florigene launched the mauve Moondust™ carnation. Since then, this variety has been commercially produced and sold in Japan, USA and Europe.

A second novel coloured carnation variety, the Moonshadow™ carnation, was launched onto the Australian market during the first quarter of 1998 and the necessary regulatory approvals have been secured for larger scale production and marketing elsewhere in the world.

Regulatory approvals are also in place for Florigene to market carnation varieties with enhanced vase life (produced by antisensing an ethylene biosynthesis gene [aco]) in the USA and Australia.

**Conclusions**

It is now possible to genetically transform most of the important cut flower species [22] and it is probable that over the next decade this technology will be used increasingly in the development of new commercial varieties. The initial focus has been on manipulation of flower colour and the extension of vase life, two traits that are clearly recognised by consumers. In the future, new flower varieties may also incorporate genes conferring resistance to disease and insect pests. For example, *Fusarium oxysporum f. sp. dianthii* is a major problem for carnation growers in most regions of the world, particularly where plants are grown in soil. It may be possible to improve the resistance of carnation varieties to the disease through the introduction of transgenes encoding hydrolytic enzymes such as chitinase and glucanase, which, in other crops, have conferred improved resistance to related species of Fusarium [23].

Consumer concern over the introduction of genetically modified organisms remains an issue in the successful commercialisation of all transgenic crops, although a recent poll conducted in Britain showed that consumers are more...
comfortable with the application of gene technology to flower crops than to food crops [24].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
** of outstanding interest


A novel petunia gene (DIF) is required for 3′,5′-hydroxylation of anthocyanins and purple flower colours. It encodes a cytochrome b5 and is exclusively expressed in the flower. Inactivation of DIF by targeted transposon mutagenesis led to reduced flavonoid 3′,5′-hydroxylase (F3′,5′H) activity and lower levels of delphinidin derivatives. No further phenotypic effects were observed. The authors speculate that DIF may be helpful to increase F3′,5′H activity in transgenic ornamental plants, which is considered the critical step in the generation of blue flower colours. This paper provides the first in vivo evidence for the regulation of the activity of a specific cytochrome P450 by a cytochrome b5.


The maize Bz2 and petunia An9 loci encode functionally equivalent glutathione S-transferases (GSTs). Interestingly, they have evolved independently from distinct types of GSTs, but each is regulated by the conserved transcriptional activators of the anthocyanin biosynthetic genes. The authors speculate that an anthocyanin-specific tonoplast pump may exist in plants or that regulators of the anthocyanin pathway increase the expression of a constitutively expressed more general pump.

20. Lu YP, Li ZS, Drozdowicz YM, Hortonsteiner S, Martinioa E, Rea PA: AtMRP2, an Arabidopsis ATP-binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: functional comparisons with AtMRP1. Plant Cell 1998, 10:267-282. Molecular cloning, physical mapping, and heterologous expression of a gene, AtMRP2, from Arabidopsis thaliana that encodes a multispecific ABC transporter is described. The capacity of AtMRP1 for transport of glutathionylated cadin-3-glucoside is in the range of that for Bn-NCC-1, a chlorophyll catabolite and glutathionated metolachlor. The authors mention that unconjugated cadin-3-glucoside is not transported by AtMRP2. It remains to be established whether transporters of this type are involved in the sequestration of anthocyanins in the vacuoles of flower petals.


In this paper, a new strategy was employed to produce 6′-hydroxylchalcones in petunia. By introducing a chalcone reductase cDNA from Medicago sativa under the control of a 35S promoter into acyanic and cyanic petunia varieties, flower colour was changed from either white to pale yellow or deep purple to pale purple, respectively.

