RESEARCH ARTICLE

HOW GENES PAINT FLOWERS AND SEEDS

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ABSTRACT

Colour is one of the most attractive features of flowers determining its capacity to attract insect pollinators. It also plays a major role in its market acceptance. In nature, flowers and seeds of different hues and shades are observed. In the beginning of 19th century, scientists proposed the Blending hypothesis to explain the inheritance of flower and seed colour. During 1866, the Austrian monk Gregor Johann Mendel proved this theory false. Subsequently, the Particulate hypothesis of inheritance accrued wide acceptance (Holton and Cornish, 1995). Pigments contribute to flower and seed colour. However, other factors like co-pigmentation, vacuolar pH and cell shape also influence colour development. The pigments belonging to classes *viz.,* chlorophyll, flavonoid, carotenoid and betalain are responsible for colour development. Of these, flavonoid (mainly anthocyanins) is the most common pigment group contributing to the development of range of colours from red to purple. These are found in vacuoles (Grotewold, 2006). Pigments are the end-products of various biosynthetic pathways. The different intermediate steps in these pathways are catalysed by enzymes; the production of which is governed by genes. Any alteration in the genes encoding the enzymes or regulation of gene expression will result in modification of pigment development leading to various shades and hues of flowers and seeds. For example, genes encoding key enzymes in the branch of the flavonoid biosynthetic pathway produce pigmentation in flower (Mol *et al.,* 1998). Flavonoids are derived from a general phenylpropanoid pathway with aromatic amino acid phenylalanine as the basic substrate. Phenylalanine is catalyzed by the enzyme chalcone synthase (CHS) into chalcone which is a key intermediate in the formation of flavonoids. Chalcone imparts yellow pigmentation and any alteration in the CHS coding gene will affect chalcone production and thereby inhibit yellow colour development. Regulatory genes MYB, bHLH and WD40 are also involved in controlling the expression of the flavonoid biosynthesis genes. In addition, variegated flowers are said to result from insertion or excision of transposons in flavonoid biosynthetic genes or regulatory genes. Such variegated flowers have been observed in petunia, snapdragon, morning glory, azalea and others (Iida *et al.,* 2004). Conventional breeding methods have been extensively used to develop cultivars with flowers varying in both colour and intensity. The cultivated roses were developed by extensive inter-specific hybridization involving yellow-flowered (producing carotenoids) and orange-flowered (producing pelargonidins) wild species. Mutation breeding has played a major role in the development of variable flower and seed coat colour. Introduction of novel genes encoding enzyme activities or transposable elements and inactivation of endogenous genes to modify flower and seed colour have been attempted through genetic engineering. Blue roses were produced by introduction of pansy F3'5'H genes into rose. This resulted in a significant amount of delphinidin derived anthocyanin production and accumulation in petals of the transgenic rose plants. Suppression of CHS gene in petunia through gene silencing approaches resulted in production of white flowers (Tanaka *et al.,* 2009).

INTRODUCTION

Since the experiments of Gregor Mendel in the 19th century, scientists have recognized the potential of plant and flower pigmentation as a tool for elucidating some of the basic principles of genetics and biochemistry. The biosynthesis of flower pigments can be used as a system for studying the temporal and spatial control of gene expression and the compartmentalization of metabolites in a cell. Because of the commercial value of flowers, their pigmentation has also been a subject of applied research for nearly four centuries. Until recently, it was only possible to develop new varieties by

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traditional breeding techniques (i.e. continuous crossing and selection) and, to a lesser extent, by mutation breeding. Today, however, transgenic approaches are also being introduced. The main function of flower pigmentation is to attract pollinators– the colour patterns of flowers make them easily recognizable against their background. However, not all pollinators perceive flower colour in the same way. Red flowers, for example, are visible to hummingbirds but appear colourless to bees (kevan *et al*., 1996). Thus, it can be envisaged that the mutational inactivation of only a few structural genes (thereby changing petal colour) is sufficient to change the type of pollinators, which might result in genetic isolation and, possibly, speciation. Indeed, the wide range of flower colours, patterns and shapes displayed by the genus

Mimulus results from mutations at only a few loci. However, clarification of the role of flower pigmentation genes in the speciation of *Mimulus* will have to await the cloning and molecular analysis of one or more of these loci (Bradshaw., 1995).

Genetic design of flower colour

The first botanist who has crossed two species of plants was the British gardener Thomas Fairchild (1667-1729). He combined two species of carnations, putting the pollen of Dianthus barbatus on the pistil of Dianthus caryophyllus, to create a hybrid form of both. Fairchild was heavily criticised for manipulating God's creation, but the interest to get new kinds of flowers was greater. There are two hypothesis to explained genetic design of flower colour. They are:

1.Blending hypothesis: A long time it was assumed that crossing of flowers with different colours is just like mixing paint on an artist's palette. Thus, a hybrid of a red and a white-flowered plant would have a pink colour. In 1866, the Augustinian monk Gregor Johann Mendel proved this theory false.

2. 'Particulate' hypothesis of inheritance: Mendel (1822-1884) discovered the concept of recessive alleles, one of the insights which were later called the laws of Mendelian inheritance. A genetic allele (or DNA sequence) is recessive, when an individual of a certain species needs two copies of the relevant genes so that a certain genetic trait is expressed. If the individual has only one copy, by the male or the female side of inheritance, the trait is not expressed - in contrast to a dominant allele. Mendel studied the flower colour of peas and found out that the dominant allele is purple and the recessive allele is white. Individuals with both alleles purple (BB) have a purple colour as well as individuals with one allele purple and one allele white (Bb). Only peas with both alleles white (bb) develop white flowers. Following Mendel, Charles Chamberlain Hurst (1870-1947) was the first who studied albinism in orchids. He discovered that actually two genes are responsible for a certain flower colour: *Factor C enabled the formation of colour, while the other factor, R, determined what particular colour would appear.* Each of these genes also exist in an inactive form, c and r. Coloured plants have inherited one or two of the active alleles: CC and RR, Cc and RR, Cc and Rr or CC and Rr. White flowers have either cc or rr. C and R are understood as dominant alleles which determine certain enzymes required by the production of pigments - among them the anthocyanins, which are especially important for flower colour.Since then, much more detailed reasearch into the genetic processes of determining flower colour has been done. At least 35 genes are known to affect flower colour in petunia. Among them are regulatory genes, which influence the timing, distribution, and amount of anthocyanin pigmentation. The gene, a certain region of the chromosome, contains the coding for creating the enzymes which are necessary for the biosynthesis of pigments, for their "biochemical pathway". Thus, the genetic makeup, which is called the genotype, defines the phenotype of a flower, its visible character (Holton and Cornish, 1995).

Factors involved in the determination of flower colour

The colouration of flowers and fruits is due to the accumulation of flavonoids (including anthocyanins), carotenoids and betalains. The first two classes are widespread, whereas betalains are found exclusively in one group of angiosperms, the Caryophyllales (including beetroot and *Amaranthus*), but never in combination with anthocyanins. Anthocyanins are the major flower pigments in higher plants and have been studied extensively. In addition to the accumulation of pigments, however, a range of additional factors determine flower hue, including co-pigmentation, vacuolar pH and cell shape (Stafford, 1994).

1. Pigments: Most important factor involved in determination of flower colour and seed coat colour. The biochemistry and enzymology of the pigment pathway is well understood and virtually all the genes that encode the enzymes of biosynthesis have been isolated. In many cases, mutations in such genes result in the accumulation of pathway intermediates, giving new flower or seed colours. However, no species displays all the possible flower colours (Koes *et al.*, 1994). Flavonoids plays a vital role in determination of flower colour and water soluble pigments are based on the amino acid Phenylalanine, which is found in high levels in the breast milk of mammals. There are two different groups of flavonoids are

1a) Anthocyanins: These pigments provide a broad range of colours from orange/red to violet/blue. The specific colour is determined by other pigments, metal ions and the pH value (they change from change from red in acids to blue in bases) (Tanaka, 2008). Anthocyanins are most prominent in the petals of flowers - all the albiflora varieties of orchids are missing them. The colour provided by anthocyanins has several biological functions. One of them is to reflect light waves to the chlorophyll regions of the plants to increase the production of glucose. Furthermore, anthocyanins protect sensible parts of the plant from possibly destructive light effects by absorbing blue-green and UV light. Last but not least the colour of the flower is attracting pollinators. There are probably more than 550 different kinds of anthocyanins. Among them are the brick-red pelargonidin, the red cyanidin and the blue delphinidin pigments.

In a complex process of biosynthesis more than five enzymes are needed to produce the water soluble anthocyanins in the vacuoles of the cell. *Any even minor disruption in any of the mechanism of these enzymes by either genetic or environmental factors would halt anthocyanin production. The name is derived from the Greek word (anthos is "flower" and kyanos is "blue").*

1b) Flavones and Flavonols: These flavonoids are called co-pigments, because they are colourless for the human eye, but can influence the colour of anthocyanins. The difference between both is that Flavonols have an additional hydroxyl in their molecular structure. *As they absorb UV, which insects recognize, they give colour and patterns to flowers to attract insects* (Tanaka, 2008,). Flavones and Flavonols can be found in most white petals. There are no white pigments with plants, but white flowers reflect all visible light and are therefore white. *Non-coloured flavonoids provide 'depth' to many white or cream flowers* (Grotewold, 2006).

2. Co-pigmentation

The colour of anthocyanins is influenced by the presence of metal ions and co-pigments such as flavonols and flavones.

Table 1. Classification of pigments

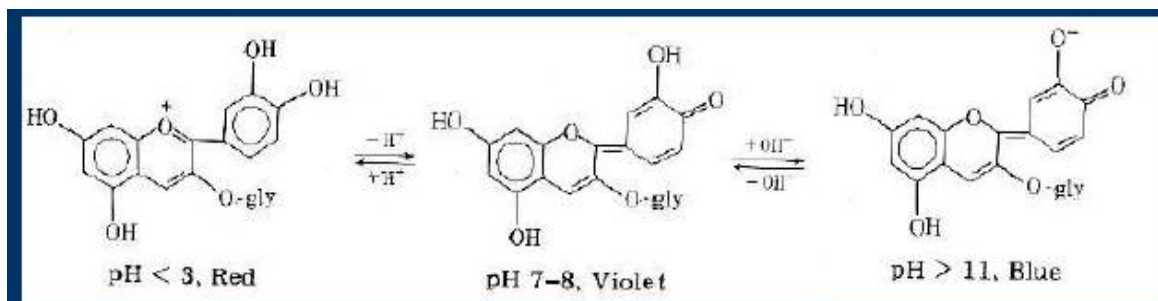| Pigment group	Type	Source	Colour
Phycobilins	Phycocyanin, Phycoerythrin	Cyanobacteria, Rhodobacteria	Bluish red
Chlorophylls	Chlorophyll a, b	All photosynthetic plants	Green
Carotenoids	Carotenes, Xanthophylls	Plants, Bacteria and some crustaceans	Yellow, orange, red
Flavonoids	Chalcones, Flavonols, Anthocyanins	Gymnosperms and Angiosperms	Cream, pale yellow, pink red, blue, black
Betalains	Betaxanthins, Betacyanins	Caryophyllales, cactus and some fungi	Yellow and red

Fig. 1. Effect of vacuolar pH on flower colour

Copigmentation involves the formation of stacked complexes that cause a blue shift in the anthocyanin absorption spectrum. Flavonols are yellow or colourless flavonoids and, like anthocyanins, are derived from dihydroflavonols, via flavonol synthase (FLS, a 2-oxoglutarate-dependent oxygenase) activity. In petunia, the *Fl* locus encodes FLS. The enzyme influences flower colour in different ways: by co-pigmentation and by substrate competition. Competition between FLS and DFR for common dihydroflavonol substrates can shift the flavonol : anthocyanin ratio (Halton *et al.*, 1993). Flavonols impart an ivory colour to 'white' flowers, but insects recognize flavonol-producing flowers much better by virtue of their strong UV-absorption. Recently, evidence has also been presented for a role for acylation in intramolecular co-pigmentation (Figueiredo, 1996).

3. Vacuolar pH: Aging flowers of many plant species display 'blueing' of the colour, and this correlates with an increase of the pH in the vacuole. Both environmental and genetic factors appear to be involved in the control of vacuolar pH. In petunia, seven loci have been defined (*ph1–ph7*) that, when mutated, cause blueing of the flower. These mutations do not alter the anthocyanin composition, but do increase the pH of petal extracts. One of these loci, *ph6*, was isolated by transposon tagging using the maize element *Ac*. The recent cloning of *anthocyanin1* (*an1*), which encodes one of the regulators of anthocyanin biosynthesis, revealed that *an1* and *ph6* are alleles of the same locus (C. Spelt, unpublished). Two other regulators of the anthocyanin pathway, *an2* and *an11*, also display a pH effect when mutated. This indicates that one or more of their subordinate genes should contribute to the control of pH and raises the question as to whether a genetic relationship exists between regulatory *an* and *ph* loci. Epistatis studies suggest that *ph4* and *an1* operate in the same pH pathway; *ph3* appears to participate in a second pathway. Determining the mechanism of pH control will require the cloning of *ph* loci and their functional analysis (Houwelingen, 1998).

4. Cell shape: The shape of the cells that accumulate anthocyanin pigments influences their optical properties and thereby the colour that is perceived. Cells of the inner epidermis of wild-type *Antirrhinum majus* petals are conical,

which confers the properties of higher light absorption and velvet sheen. The fainter colour of *mixta* mutants results from a flattening of these epidermal cells. The homology of *mixta* with genes encoding *Myb*-domain proteins suggests that the MIXTA protein controls cell shape by regulating gene expression. Mutation of a homologous gene in petunia (*mybPh1*) results in a similar phenotype. In petunia *shrivelled up* (*shp*) mutants, a large fraction of the cells in the petal epidermis have a collapsed 'flat tyre' appearance, which results in a drastic change of the flower colour. Neither the *mixta* nor *shp* downstream target genes have been identified, and therefore the molecular mechanism underlying these cell-shape changes remains to be resolved (Noda, 1994).

Flavonoid biosynthesis

There are three major routes for the production of secondary metabolites in plants: the shikimate pathway through which flavonoids and anthocyanins are produced, isoprenoid pathway leading to the production of alkaloids, steroids, terpenoids, carotenoids etc., and the polyketide pathway for the synthesis of aromatic compounds. Of the three pathways, shikimate pathway is the major defense pathway in plants by which the phenyl propanoids and flavonoids are synthesized forming the bulk of metabolites.

Structure and Function of Flavonoids

Anthocyanins are the brightly colored compounds belonging to the general class of flavonoids. Chemically, anthocyanins are classified as water-soluble glycosides, which arc the derivatives of polyhydroxyl and polymethoxyl compounds of 2-phenylbenzopyrylium (flavylium cation). They are derived from a flavonoid molecule consisting of a typical A-ring benzoyl and B-ring hydroxycinnamoyl system composed of three planar rings A. C and B. While the B ring of the flavonoid skeleton originates from the phenylpropanoid pathway, the A ring is derived from acetyl-malonyl pathway. On the basis of their structural diversity, and the oxidation level of the central pyran nucleus, flavonoids are broadly classified into 12 groups Chalcones, aurones, flavones, flavonols, flavanones, dihydrochalcones, catechins, flavan-3-4-

diols, biflavonoids, iso-flavonoids, proanthocyanidins and anthocyanins (red/purple and blue pigments). The flavonoid biosynthetic route has two component pathways, the phenylpropanoid pathway and the flavonoid pathway. The first step in the flavonoid pathway is the deamination of phenylalanine to transcinnamic acid by phenylalanine ammonialyase (PAL). PAL activity links primary metabolism with the phenylpropanoid pathway, the beginning of secondary metabolic pathway. Cinnamate, thus formed is hydroxylated by Cinnamate-4-hydroxylase to form 4-coumarate which is further transformed to 4-coumaroyl Co-A by 4-coumarate Co- A ligase. The above steps constitute the phenylpropanoid pathway, and all the subsequent steps belong to the flavonoid pathway.

The first step in the flavonoid pathway is the condensation of the three molecules of malonyl-Co A and 4-coumarate Co-A to form the first C/5 chalcone intermediate (4,2',4", and 6' - tetrahydroxy chalcone) catalyzed by chalcone syntheses (CHS). Isomerization of this product would lead to the formation of naringenin (2S - flavonone) catalysed by chalcone isomerase There are two types of chalcone isomerases found in nature, one catalysing the cyclization of 6'-hydroxychalcone to 5-hydroxyflavonone and the other isomerising both 6'-hydroxy and 6'-deoxychalcone to 5-hydroxy and 5-deoxyflavanones. Iso-flavonoid formation are catalysed by 2-hydroxy-isoflavone synthase, a mixed cytochrome P450 monooxygenase that is involved in the oxidative rearrangement of the flavanone. Although leguminaceae members are specialized in producing these groups of compounds this iso-flavonoid branch pathway is completely missing in cereals. Flow chart of Flavonoid biosynthesis:
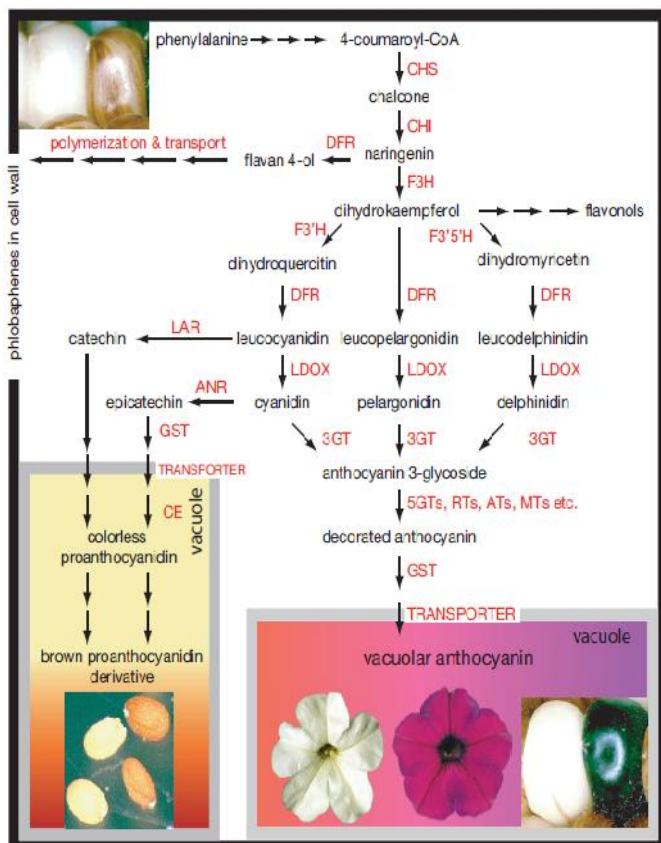

Fig. 2. flow chart of flavonoid biosynthesis

The hydroxyflavonols are formed from flavanones via hydroxylation at the 3 position catalyzed by the flavanone 3-hydroxylase. These dihydroflavonols are the biosynthetic intermediates in the formation of flavonols, catechins, leucoanthocyanidins, proanthocyanidins and anthocyanidins. Dihydroflavonols are converted to flavan 2,3-trans-3, 4- cis-diols generally called as leucoanthocyanidins, by the enzyme dihydroflavonol reductas (DFR). Leucoanthocyanidns are the colorless active precursors for the synthesis of catechins, proanthocyanidins and anthocyanins. While catechins are synthesized from leucoanthocyanidins by the action of flavan 3,4-cis-diol reductase, the proanthocyanidins are formed by the condensation of catechins and leucoanthocyanidins. Polymers of leucoanthocyanidins are also found in many plants. The exact chemical steps from leucoanthocyanins to anthocyanidins are unknown. Genetic analysis revealed the conversion of leucoanthocyanidin to the corresponding colored anthocyanidin catalysed by the enzyme anthocyanidin synthase, which belongs to a class of plant dioxygenases. This enzyme has been characterized from only a few plant species. The next obligatory step is the glycosylation at the 3' position of anthocyanidin aglycone to form the anthocyanin glucoside, namely, cyanidin-3-glucoside. This 3-O-glycosylation of anthocyanins and flavanols is catalyzed by flanonol-3-glucosyl transferase (FGT). The function of GST in anthocyanin pathway was recently uncovered by Marrs *et al,* (1995). Cyanidin-3-glucoside is the substrate for Glutathione-S-Transferase (GST) which tags the anthocyanins with glutathione and thus mediates its movement into vacuoles. The glutathione conjugates of anthocyanins transiently serve as transport intermediates. Malonylated anthocyanins are found as the final products in vacuole, which might have been formed as a part of the pathway that removes the glutathione tag in the vacuole.

Pro-anthocyanidins in rice seed

Flavonoid compounds, such as anthocyanins, flavonols and proanthocyanidins, are major secondary metabolites in plants, and are red, purple and brown in color. Pro-anthocyanidins (colorless polymers which are oxidized on desiccation to produce the darkcolored pigments of most seed coats) that are known as condensed tannins constitute a major subgroup of flavonoids in plants. Most rice plants have green leaves and white seeds; however, there are some varieties with purple leaves, purple seeds, red seeds or brown seeds. Purple and red seeds contain anthocyanins and proanthocyanidins, respectively. The genetics of anthocyanin accumulation in rice has been studied for more than half a century. Coloration by anthocyanins, as well as coloration by compounds other than anthocyanins, is largely regulated by structural genes and their regulatory genes. Kinoshita (1995) summarized that there are 26 genes involved in anthocyanin coloration, 10 genes involved in the inhibition of anthocyanin coloration, and 15 genes involved in coloration by compounds other than anthocyanins, such as proanthocyanidins. These genes were identified by segregation analysis. Several genes involved in anthocyanin coloration of rice have been extensively studied. Two genes, C and A, are involved in the formation of anthocyanin pigments. The organ-specific regulatory element Pl has also been identified (Nagao and Takahashi, 1963). C and A comprise a multiple allelic series of genes: six alleles have

been found at the C locus and four at the A locus. They are ranked in order of dominance (Nagao *et al.*, 1962). Red coloration in rice grains is determined by the complementary effect of two genes, Rc and Rd, and each of these genes is inherited monogenetically. Nagao *et al.* (1957) described how the Rc gene is responsible for the accumulation of pigments in the pericarp of brown-colored grains. Furthermore, red rice grains require the Rd gene to increase the content of the pigment. Therefore, the Rc and Rd genes are involved in the red pigmentation in rice grains, Rc and rd are involved in the brown pigmentation in rice grains, and either rc and rd or rc and Rd produce ordinary white rice grains. Rc is mapped onto chromosome 7 and is responsible for the production of the pigment in so-called brown rice, which has dark-brown irregular speckles on a reddish-brown background. Rd by itself does not produce any pigment and mapped onto chromosome 1.

a) Schematic representation of the loci involved in pro-anthocyanidin synthesis b) Phenotypes and genotypes of Rc and Rd
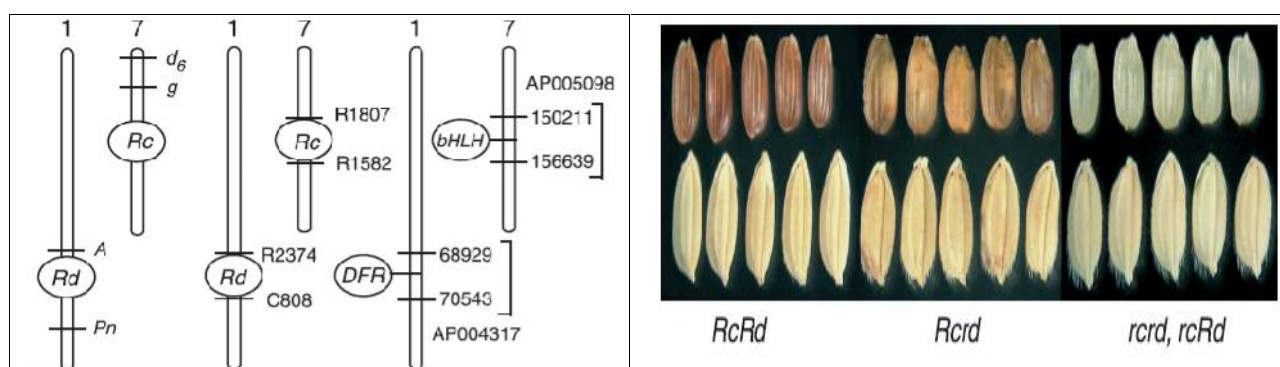
expression patterns of the regulatory genes. Genetic control of pigmentation is two types. They are:

a) **Transcriptional regulation of pigmentation**: Genes contain regulatory region and coding region. The final concentrations of anthocyanins/flavonoids in plant cells are not only determined by expression levels of enzymes involved in flavonoid biosynthetic pathway, it is now demonstrated very clearly that some regulatory genes are also involved in controlling the transcription level of the flavonoid biosynthesis genes in some plants examined including maize, snapdragon, *Petunia*, *Arabidopsis* and tomato. In general, these regulatory genes in the flavonoid biosynthesis pathway are specific transcription factors. These DNA binding proteins interact with promoter regions of the target genes and regulate the initiation rate of mRNA synthesis. These regulatory genes relevant to flavonoid biosynthesis can be divided into 2 classes: one is MYB transcription factors, another is basic-Helix-Loop-Helix (bHLH) transcription factors (Mol *et al.*, 1998). An additional third class of WD40 proteins may also be important and universal, although the mechanism is not known. In various plant species, the tissue-specific expression pattern of the

Fig. 3. a) Schematic representation of the loci involved in pro-anthocyanidin synthesis b) Phenotypes and genotypes of Rc and Rd

Table 2. Genes involved in flavonoid biosynthesis in rice

Pathway	Chromosome	Gene Locus	Gene by molecular cloning	Reference
Anthocyanins (leaf colour)	1	A	DFR	Furukawa, 2007
	6	C	MYB	Saitoh *et al.,* 2004
	4	Pl	bHLH	Sakamoto *et al.,* 2001
Proanthocyanidins(grain colour)	7	Rc	bHLH	Furukawa, 2007 Sweeney *et al.*, 2006
	1	Rd	DFR	Furukawa, 2007

Table 3. Regulatory genes of the flavonoid pigmentation pathway in maize, Antirrhinum majus and petunia

Pathway	Gene family	Maize	*A. majus*	Petunia
Anthocyanins	MYB-1	c1 and pl	myb308	an2,mybPh3
	bHLH-1	r, b, sn, lc	delila	jaf13
	bHLH-2	Unidentified	Unidentified	an1
	WD40	Unidentified	Unidentified	an11
Common flavonoids	MYB-2	Unidentified	myb305	Unidentified
	MYB-2	Unidentified	myb340	Unidentified
Phlobaphenes	MYB-3	p	Unidentified	Unidentified

Genetic control of pigmentation

Co-pigmentation, cell-shape and vacuolar pH can at most change flower colour, whereas pigmentation patterns are established by cell-specific accumulation of anthocyanins. The activity of the anthocyanin biosynthesis genes is largely regulated at the transcriptional level and consequently the pigmentation patterns must be largely specified by the

structural genes in the flavonoid biosynthetic pathway is controlled by the combination of regulatory genes from these two classes of transcription factors. Some transcription factors have been expressed ectopically in various transgenic plant species such as *Petunia*, tobacco and tomato, indicating the functional conservation of these regulatory genes among different plant species. Moreover, the final concentration and class of flavonoids in transgenic plants are determined by the

following parameters: the binding affinity of the transcription factor to the promoter regions of the target genes, the ability to cooperate with endogenous transcription factors, and the functionality of the endogenous transcription factors (Schijlen *et al.* 2004). The most characterized regulatory genes affecting anthocyanin production are the maize leaf colour (*Lc*) gene which belongs to *R* gene family and the maize colourless (*C1*) gene which belongs to MYB-type transcription factor. The predicted proteins of the *R* genes (*R, Sn, B* and *Lc*) contain a bHLH motif, and for this reason, maize *Lc* gene is classified as bHLH-type transcription factors. When the expression vector containing the constitutive CaMV 35S promoter and the maize *Lc* gene was transformed into tobacco and *Arabidopsis*, production of anthocyanins was increased in floral tissues from both plants. Although the maize MYB-type gene *C1* alone had no effect on anthocyanin production in *Arabidopsis*, expression of both *C1* and *Lc* genes resulted in tissues that normally did not produce anthocyanins (Lloyd *et al.* 1992). Transformation of maize *Lc* gene into tomato enhanced anthocyanin production in all vegetative tissues that normally did not produce anthocyanins (Goldsbrough *et al.* 1996). Similarly, by transforming maize *Lc* gene into Petunia, it was found that both vegetative and floral tissues in transgenic plants had increased pigmentation (Bradley *et al.*, 1998). The appearance of purple leaves in transgenic Petunia plants, due to accumulation of anthocyanins activated by maize *Lc* regulatory gene, may create a novel ornamental plant of commercial value. However, same approach of transforming maize *Lc* gene into rose and carnation, no significant increase or even decrease in anthocyanin production was detected in these transgenic plants (Tanaka *et al.*, 2005).

b) Transposon approach: Variegation in either flowers or leaves often attracts attention from consumers and thus variegated plants can create high value in the ornamental market. Variegated flowers have been observed in natural populations including *Petunia*, snapdragon, morning glory, azalea and others, and some of this variegation is caused by transposable elements. Insertion or excision of transposons in flavonoid biosynthetic genes or regulatory genes produces a mosaic or variegated phenotype whose pattern is dependent on the frequency and timing. In general, insertion of a flavonoid biosynthetic gene or regulatory gene results in white sectors of a colored background, and excision of such a transposon from a particular gene often leads to produce colored sectors on a white background; the sizes of sectors are dependent on the timing of excision: early excision produces large sectors, and late excision produces tiny sectors. Variegated flowers have been studied in various plant species, including *Petunia* and morning glory (Iida *et al.*, 2004).

In higher plants, transposons can be classified into 3 groups: the *Ac/Ds* superfamily, the *En/Spm* superfamily, and the *Mu* family. In Japanese morning glory, two mutants carrying variegated flowers were caused by integration of *En/Spm* transposable elements into the *dfr* or *chi* gene; another mutant in the common morning glory bearing variegated flowers was caused by insertion of *Tip100*, belonging to the *Ac/Ds* family, into the *chi* gene. Insertions of a transposable element *dTdic1*, belonging to the *Ac/Ds* superfamily, in both *chi* and *dfr* genes were found in carnation cultivars bearing variegated flowers. To evaluate the potential of using transposons as molecular

tools in producing variegated flowers and to ensure the effectiveness of changing color patterns, a new strategy of employing transposons and regulatory genes was developed. In brief, the *Arabidopsis* transposon *Tag1* (3.3 kb) was inserted between the CaMV 35S promoter and the maize *R* gene of the plant expression vector pAL69, and the resulting expression vector was transformed into tobacco via *Agrobacterium*-mediated method. The transposon *Tag1* belongs to the *Ac* family and is an autonomous element active in *Arabidopsis*, tobacco and rice. Once the expression cassette is integrated into host plant genome, the regulatory *R* gene can be actively transcribed only when *Tag1* is excised, as a result, up-regulating the anthocyanin biosynthetic genes and accumulating pigments can be observed. Half of the transgenic tobacco plants had observable variegated flower patterns; moreover, each line had a different pattern (Liu *et al.*, 2001).

Approaches to modify pigmentation

Different approaches involved in modification of pigmentation are:

1.Conventional breeding: Conventional breeding through hybridization helps in modification of pigments in flavonoid biosynthesis. It leads to development of new colour in hybrids. **Studies on inter specific hybridization in *Dianthus plumarius*:** The mating or crossing of two plants or lines of dissimilar genotype is known as hybridization. In plants, crossing is done by placing pollen grains from one genotype, the male parent, on to the stigma of flowers of the other genotype, the female parent. Yellow flowers are rare in the genus and found in only one wild species, *D. knappii*. **Analysis of the flower pigments:** Yellow flower colour of *D. knappii* resulted from flavone and flavonol glycosides. Yellow carnations were due to chalcones. Thus, the F1 hybrids with *D. knappii* were yellow because they contained the same pigments as *D. knappii* but the hybrids with the carnations were pink due to their ability to convert chalcones through dihydroflavones and then to anthocyanins.

2.Polyploidy: The somatic chromosome number of any species, whether diploid or polyploidy, is designated as 2n, and the chromosome number of gametes is denoted as n. An individual carrying the gametic chromosome number, n, is known as haploid. A monoploid, on the other hand, has the basic chromosome number, x. In a diploid species, n=x; one x constitutes a genome or chromosome complement. The different chromosomes of a single genome are distinct from each other in morphology and or gene content and homology; members of a single genome do not show a tendency of pairing with each other. Thus a diploid species has two, a triploid has 3 and a tetraploid has 4 genomes and so on. In euploids, the chromosome number is an exact multiple of the basic or genomic number. Euploidy is more commonly known as polyploidy. When all the genomes present in a polyploidy species are identical, it is known as autopolyploid and the situation is termed as autopolyploidy. In the case of allopolyploids, two or more distinct genomes are present. Euploids may have 3 (triploid), 4(tetraploid), 5 (pentaploid), or more genomes making up their somatic chromosome number. In case of autopolyploidy, they are known as autotriploid, autotertaploid, autopentaploid, and so on, while in the case of

allopolyploidy they are termed as allotriploid, allotetraploid, allopentaploid, etc. Polyploidy induced through *in vitro* colchicine treatment Increasing the relative concentration of the major metabolite quercetin-3-sophoroside and decreasing the relative concentration of the minor metabolite quercetin-3,7-diglucoside.

3. Mutational breeding: The term mutation was coined by Hugo Devries in 1900 for the first time and the word is derived from the latin word 'MUTARE' means to change. Mutation is the sudden heritable change other than the Mendelian segregation and gene recombination in an organism. Mutation may be the result of a change in a gene, a change in chromosome that involves several genes or a change in plasmagene. Mutations produced by changes in the base sequence of genes are known as gene or point mutations some mutations may be produced by changes in chromosome structure or even in chromosome number they are termed as chromosomal mutation. There are three types of mutations based on genetic basis of heritable change: 1. Gene mutations: These are produced by change in the base sequence of genes. The change may be due to base substitutions, deletion or addition. 2. Chromosomal mutation: These arise due to change in chromosome number that may leads to polyploidy or aneuploidy or change in chromosome structure that result in deletions duplication, inversion and translocation. 3. Cytoplasmic or plasmagene mutation: These are due to change in the base sequence of plasma genes. The plasma genes are present in mitochondria or chloroplast. Here the mutant character occurrs in buds or somatic tissues which are used for propagation in clonal crops. Many different genes are involved in controlling the synthesis of the pigments. In a multi-step process, if a single enzyme is not present and particular step in the synthetic pathway will not happen. It leads to accumulation of intermediates, results in development of new colour. It is a generally accepted idea that ionizing radiation induces mutations randomly throughout the genome. The ability to efficiently induce mutations for a particular phenotypic trait, i.e., to control of the direction of mutations, would be a major breakthrough. However, no effective methods have been established to control the direction of mutations in practical plant breeding. Nagatomi *et al.* (1997) examined the mutagenic effects of ion beams in chrysanthemum and reported that flower color mutants were obtained with higher frequency when cultured petals rather than cultured leaves were irradiated.

The advantage of mutation breeding lies in its ability to improve one or a few desirable traits without altering the remaining characteristics. Ion beams are better for this purpose than low-linear energy transfer (LET) radiation because energy deposition is highly localized along the path of ion particles. In fact, ion beams induce mutations with lower doses than did low-LET radiation. The mutation frequency in *Arabidopsis* using carbon ions was 20-fold higher per unit dose than that using electron beams (Shikazono *et al.* 2005). Therefore, it anticipated that consecutive irradiation with ion beams would improve desirable traits in a stepwise manner. Three cultivars of fragrant cyclamen were created by crossing cyclamen cultivars (*Cyclamen persicum*) with the scented cyclamen species *C. purpurascens* (Ishizaka 2008).

Effect of mutation on seed coat colour in groundnut

Healthy and dry seeds of groundnut having uniform size and equilibrated to moisture level of 7% were packed in small polyethene bags and irradiated to Co_{60} at three different doses viz. 10 kR, 15 kR and 20 kR in the gamma chamber. Critical screening was done though the M1, M2 and M3 generation for seed coat colour. The M3 progeny derived from irradiated seeds segregated the character seed coat colour. The frequency of plants carrying different seed coat colour was highest in 15 kR dose of gamma rays treated seeds. The mutant seed coat phenotype and their number of plants in M3 generation are highest as 09. The mutant's seed coat colour varied from off white to dark pink off white seed coat is found in only four plants of M3 generation. In the present study 15 kR dose of gamma rays induced a highest frequency of seed coat colour mutants (Satpute and Suradkar, 2011). Liao and Lie (2004) reported about 28.8% protein present in black seed coat groundnut cultivar.

Genetic engineering

Flower colour is predominantly due to three types of pigment: flavonoids, carotenoids and betalains. Betalains are the least abundant of the three and contribute to various hues of ivory, yellow, orange, red and violet. Carotenoids are C-40 tetraterpenoids that are lipid soluble and are located in the plastid and contribute to the majority of yellow hues in a number of flowers (Forkmann, 1991). Carotenoids, along with red or magenta anthocyanins, also contribute to the orange/red, bronze and brown colours seen in flowers such as roses and chrysanthemums. The flavonoids are the most common of the three types of pigment and contribute to a range of colours from yellow to red to blue. They are water-soluble compounds and occur in a wide range of plants. The flavonoid molecules which make the major contribution to flower colour are the anthocyanins which are all O-glycosides (Stafford, 1990) and are usually localized in the vacuoles of petal epidermal cells. The modification of flower colour via genetic engineering has generally focused on metabolic engineering of the flavonoid pathway. Flavonoid molecules are secondary metabolites of the phenylpropanoid pathway. The flavonoid pathway leading to the first coloured anthocyanins, anthocyanidin 3-O-glucosides, is generally conserved among plant species and is well established. Genes encoding flavonoid pathway enzymes have been cloned from many plants, including floricultural crops, and can be easily extracted from public DNA data bases. The first coloured anthocyanins, anthocyanidin 3-O-glucosides can be further modified with sugars, aliphatic acids, aromatic acids and methyl groups. There are both species- and variety-specific differences in the extent of modification and the types of glycosyl and acyl groups attached to the anthocyanidin core molecule. However, the final visible colour of a flower is generally a combination of a number of factors including the type of anthocyanin accumulating, modifications to the anthocyanidin molecule, co-pigmentation and vacuolar pH. Each of these factors is regulated by a number of genes, many of which have now been cloned and characterised.

Modification of anthocyanins

Anthocyanins determines flower colour through biochemical modifications.

Over-expressing or silencing the structural gene

Modification of pigment in flavonoid biosynthesis through genetic engineering by suppression of genes like Chalcone synthase (CHS), Chalcone isomerase (CHI), Flavanone hydroxylase/ Flavonoid-3 -hydroxylase (F3H)/ Flavonoid-3 ,5 -hydroxylase (F3 5 H), Dihydroflavonol-4-reductase (DFR), Anthocyanidin synthase (ANS) and Flavonoid 3-*O*-*glucosyltransferase* (FGT).
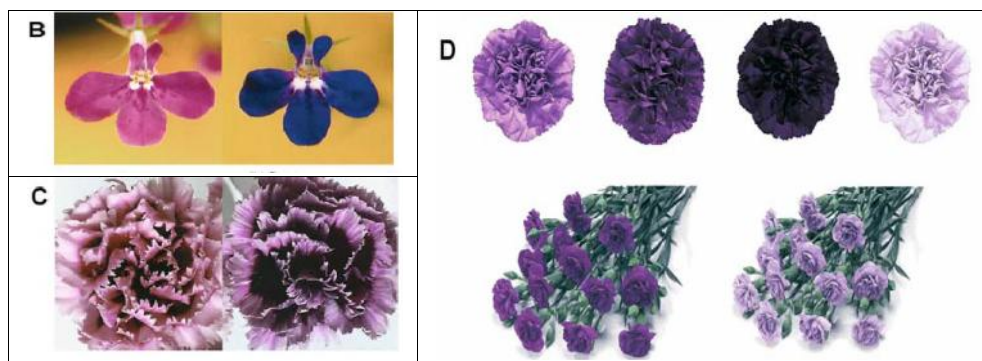
Generating white flowers by gene suppression

Down regulation of an anthocyanin biosynthesis structural gene has been achieved in many plant species. Since hybridization and mutational breeding also readily lead to development of white flower varieties, some of these results are model experiments only as transformation technology is generally more expensive than traditional breeding. Nevertheless, molecular breeding of a white variety can be commercially viable as only the flower colour is modified presumably without sacrificing any other desirable characteristics. In particular, when the hosts are sterile or the resultant transgenic plants have novel colouration patterns, genetic engineering can complement traditional breeding. It should be possible to obtain white flowers from anthocyanin producing flowers by down-regulating the expression of one of many structural or regulatory genes in the pathway. Successful reduction of anthocyanin biosynthesis has been reported in gerbera, chrysanthemum, rose, carnation. More recently, Nishihara *et al*. (2003) transformed a blue gentian (Gentian triflora) using an antisense gentian CHS gene and successfully obtained transgenic gentian plants whose flower colour varied from white to pale blue. Hokko Chemical Industry Co. Ltd (Japan) has also generated transgenic cyclamen with down-regulated levels of CHS. The flower colours obtained were white, red, pink and a mixture of red and white.

deleterious side effects. However, as Zuker *et al*. (2002) unexpectedly found when they down regulated carnation F3'H, anthocyanin levels were not the only change observed in the transgenic carnations produced. The carnations were also more fragrant due to an increase in methylbenzoate, which may be considered a more positive outcome when commercialising these flowers. Jorgensen *et al*. (2002) discovered that co-suppression of F3'5'H or DFR in petunia resulted in female infertility, presumably due to the accumulation of dihydroflavonols in the seed coat.

Generating blue flowers

Most blue flowers contain aromatically acylated delphinidin derivatives. Rose, chrysanthemum and carnation make up over 50% of the world cut flower market but only accumulate pelargonidin and cyanidin derivatives that are not modified with aromatic acyl groups. Thus they have become targets for attempts at engineering the synthesis of delphinidin derivatives with the hope of eventually generating blue flowers. The absorbance of anthocyanin shifts towards longer wavelengths (blue) by about 10 nm with each hydroxylation of the B ring and by 4 nm following an aromatic acylation. Transformation of a pink Lobelia erinus with a lisianthus F3¢5¢H gene under the control of a CaMV35S promoter produced blue coloured flowers. One of the transgenic plants is shown in Figure B. Lobelia appears to be a useful model plant for the study of colour modification as it is easy to transform and flowers as little as 3-4 months after co-cultivation of tissue with Agrobacterium carrying binary transformation vectors (Kanno *et al*., 2003). Expression of a petunia F3'5'H in a carnation line that accumulated cyanidin-based pigments resulted in very low levels of delphinidin production and no dramatic effect on flower colour. It appears that the introduced petunia F3'5'H could not efficiently compete with the endogenous carnation F3'H and DFR enzymes. However, when a petunia cytochrome

(B) Colour modification of Lobelia erinus, Left: the host, right; a transgenic lobelia expressing lisianthus F3'5'H (C) Carnation cultivar Exquisite accumulates predominantly cyanidin-based pigments, transgenic Exquisite flower expressing petunia F3'5'H (D) Transgenic carnation expressing F3'5'H and petunia DFR genes.

The CHS gene is the most common target for down-regulation of anthocyanin biosynthesis. However, since blockage of CHS can result in flavonoid-free transgenic plants and flavonoids have been found to play an important role in UV protection, general plant defense and signaling (Winkel-Shirley, 2002), down regulation of the CHS gene may not represent an ideal strategy to develop white varieties. Indeed, we have observed that plants whose CHS gene is suppressed are more sensitive to a range of stresses. Down regulation of other genes in the pathway, such as DFR or F3'H, may be a more viable alternative to generating white flowered varieties without

b5 gene along with the petunia F3'5'H gene were expressed in the same carnation line the result was a dramatic improvement in the level of delphinidin production and a shift in the flower colour from a variegated pink and red to variegated mauve and purple (Figure C). Florigene Ltd. and Suntory Ltd. have successfully developed a range of transgenic violet carnations by introduction of a F3'5'H gene together with a petunia DFR gene into a DFR-deficient white carnation. The petals of the engineered carnations contain predominantly delphinidin that native carnations do not produce. Such a bluish hue in the

transgenic flowers has never been achieved by traditional breeding of carnation (Figure 2D).

Generating red to orange flowers

Petunia DFR is unable to reduce dihydrokaempferol and so petunia flowers rarely contain pelargonidin-type anthocyanins and therefore do not produce orange to brick-red colours. Transgenic brick-red petunias accumulating pelargonidin-type anthocyanins have been obtained by expression of DFR genes from heterologous species such as maize, gerbera and rose in a mutant petunia line that accumulated dihydrokaempferol (deficient in F3'5'H, F3'H and FLS). Identification of a similar dihydrokaempferol accumulating line in commercially important species (or cultivars) can be difficult. Mizutani *et al.* (2003) were able to engineer a red petunia line that normally accumulates cyanidin-based pigments to produce pelargonidin-based pigments (orange) by down regulation of the F3'H gene and expression of a rose DFR gene. Many important floricultural species including cyclamen, delphinium, iris, gentian and Cymbidium are presumed not to accumulate pelargonidin due to the substrate specificity of their endogenous DFRs. Similar strategies could therefore be employed to generate orange coloured flowers in these species. Ueyama *et al.* (2002) used a two-step transformation process to produce dark pink flowers from a normally blue flowered torenia. Initially the F3'5'H gene was down regulated so that a pink flowered torenia was obtained. Further transformation of this torenia with a CaMV35S promoter driving a torenia F3'H gene and different selection marker resulted in dark pink torenia flowers.

Efforts to generate yellow flowers

Chalcones and aurones contribute to the yellow colours observed in some flowers. The most common chalcone, THC, is yellow but is spontaneously isomerized to naringenin in vitro and rapidly isomerized in vivo by CHI. In yellow flowers of carnation, peony and periwinkle, THC accumulates as a 2'-glucoside (isosalipurposide). Accumulation of THC 2'-glucoside is attributed to a deficiency in CHI activity. A recent study (Itoh *et al.*, 2002) showed that both CHI and DFR genes are disrupted by a transposon in several of the carnation cultivars that bear yellow flowers variegated with white flecks and sectors. Pale yellow cyclamen has been also shown to be deficient in CHI activity. Therefore it appears that a lack of CHI acitivity and presence of a UDPglucose: THC 2'-glucosyltransferase (C2'GT) activity are required for the accumulation of the yellow coloured isosalipurposide. Carnation genes encoding C2'GT activity have recently been isolated (Okuhara *et al.*, 2004). Therefore genetic engineering of isosalipurposide in flowers should now be possible. Aurones are bright yellow flavonoids and therefore provide yet another tempting target for the genetic engineer. Aurones are found in yellow flowers of distantly related species including snapdragon, dahlia, limonium, zinnia and morning glory. The biosynthesis of aurones was, until recently, one of the last unsolved mysteries of flavonoid biosynthesis. Aurone synthase, more specifically aureusidin synthase (AS), was purified from yellow snapdragon petals and the cDNA encoding the enzyme was cloned.

Generating long life flowers

The post-harvest life of flowers is influenced primarily by nutrition, microbial colonization and ethylene; a common plant hormone associated with, amongst other responses, senescence. The most popular cut flowers on the global market are rose, carnation and chrysanthemum. Of these endogenous ethylene production triggers flower senescence in carnation alone. Petal drop in rose can, in some varieties, be promoted by exposure to exogenous ethylene typically, associated with transport and storage of flowers and fruit. All cut flowers are susceptible, in varying degrees, to microbial growth in vase water leading to blockage of vascular tissue preventing movement of water in the stem (xylem) and thus wilting typically leading to reduced vase-life. Such microbes are typically associated with flowers in production and clean practice through all stages of the post harvest treatment, including preparation of vase water, can control the problem. Lack of nutrients, primarily sugars, can also promote senescence. Again such deficiencies can be ameliorated through application of nutrient additives to vase water. A number of different but related strategies have been used to engineer prolonged vase-life in carnations without the need for chemical treatment. The first involved down regulation of ethylene production in carnation flowers via posttranscriptional floral-specific gene silencing of a gene encoding ACC Oxidase (ACO) (Savin *et al.*, 1995) or ACC Synthase (ACS) (Florigene Ltd., Figure E) the enzymes catalyzing the two penultimate steps in ethylene biosynthesis.

E) Long-life carnation with down regulated petal ACC synthase (Florigene Ltd.). Left) The native carnations senesced after 2 weeks of harvest Right) The transgenic carnations have comparable vase-life to STS treated ones (center)

Conclusion

Colour is one of the most attractive features of flowers determining its capacity to attract insect pollinators. It also plays a major role in its market acceptance. Pigments contribute to flower and seed colour. However, other factors like co-pigmentation, vacuolar pH and cell shape also influence colour development. The pigments belonging to classes *viz.,* chlorophyll, flavonoid, carotenoid and betalain are responsible for colour development. Of these, flavonoid (mainly anthocyanins) is the most common pigment group contributing to the development of range of colours from red to purple. Pigments are the end-products of various biosynthetic pathways. The different intermediate steps in these pathways are catalysed by enzymes; the production of which is governed by genes. Any alteration in the genes encoding the enzymes or regulation of gene expression, will result in modification of pigment development leading to various shades and hues of

flowers and seeds. Regulatory genes MYB, bHLH and WD40 are also involved in controlling the expression of the flavonoid biosynthesis genes. In addition, variegated flowers are said to result from insertion or excision of transposons in flavonoid biosynthetic genes or regulatory genes. Such variegated flowers have been observed in petunia, snapdragon, morning glory, azalea and others. Conventional breeding methods have been extensively used to develop cultivars with flowers varying in both colour and intensity. The cultivated roses were developed by extensive inter-specific hybridization involving yellow-flowered (producing carotenoids) and orange-flowered (producing pelargonidins) wild species. Mutation breeding has played a major role in the development of variable flower and seed coat colour. Introduction of novel genes encoding enzyme activities or transposable elements and inactivation of endogenous genes to modify flower and seed colour have been attempted through genetic engineering. Blue roses were produced by introduction of pansy F3'5'H genes into rose. This resulted in a significant amount of delphinidin derived anthocyanin production and accumulation in petals of the transgenic rose plants. Suppression of CHS gene in petunia through gene silencing approaches resulted in production of white flowers.

Future line of work

Although the factors that determine flower hue are well known, many issues related to the mechanism of flower colouration are still unresolved and require further investigation. We know some but not all the factors that control anthocyanin gene regulation. For example, specific activators of *chs* genes in petunia and *A. majus* have not yet been identified by mutation. This might be because of redundancy in gene function or, alternatively, because the mutations are lethal. For genes acting later in the pathway, such factors have been identified (MYB, bHLH and WD40 proteins), but in these cases their mechanism of action is poorly understood. The MYB and bHLH factors probably act as transcription factors, whereas the function of the cytosolic WD40 protein encoded by the *an11* locus is unknown. Some biosynthesis genes that act in overlapping pathways show dual control by a MYB factor and a combination of a different MYB protein and a bHLH protein. On top of this, duplication of *myb* genes might allow specialization of individual copies for either induction or maintenance of flavonoid production. How this system of fine tuning by redundancy and specialization is regulated is a subject for further study. The regulatory loci *an1*, *an2* and *an11* not only control anthocyanin biosynthesis in petunia, but also vacuolar pH. It is not known what kind of proteins *ph* genes encode, although these might be components of proton-translocating pumps, metabolic pathways that produce or sequester protons or regulators of these. Cloning of the relevant genes would be a first step towards resolving this issue. Little is known about the transcriptional regulation of *myb* and *bHLH* genes involved in anthocyanin pigmentation. *Viviparous 1* (*vp1*) mutants of maize have colourless kernels and show precocious germination. Recent data indicate that VP1 is a transcriptional activator of *c1*. Regulators of *bHLH* genes have not been identified. Fundamental research in the field of flower pigmentation also has applied aspects. Production of anthocyanins in vegetative tissues by forced expression of *r* and *c1* genes is feasible in plant species with the maize-type R and C1 control. In all other cases, only a colour enhancement

may be seen in anthocyanin-producing cell types. About a decade ago, anthocyanin-pathway engineering led to the creation of the 'orange' petunia variety. A delphinidinproducing carnation has been generated by the biotechnology company Florigene. Once the controlled manipulation of copigmentation and vacuolar pH is achieved, the dream of creating a blue rose may finally come true.

REFERENCES

Bradley, J. M., Davies, K. M., Deroles, S. C., Bloor, S. J., and Lewis, D. H. 1998. The maize *Lc* regulatory gene up-regulates the flavonoid biosynthetic pathway of Petunia. *Plant J.* 13: 381-392.

Bradshaw, H. D. 1995. Genetic mapping of floral traits associated with reproductive isolation in monkey flowers (*Mimulus*). *Nature* 3: 762–765.

Figueiredo, P. 1996. New aspects of anthocyanin complexation. Intramolecular copigmentation as a means for colour loss? *Phytochemistry* 41: 301–308.

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

Goldsbrough, A. P., Tong, Y., and Yoder, J. I. 1996. *Lc* as a non-destructive visual reporter and transposition excision marker gene for tomato. *Plant J.* 9: 927-933.

Grotewold, E. 2006. The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57: 761–780.

Holton, T. A. and Cornish E. C. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell.* 7: 1071-1076.

Holton, T. A., Brugliera, F., and Tanaka, Y. 1993. Cloning and expression of flavonol synthase from *Petunia hybrida*, *Plant J.* 4: 1003–1010.

Houwelingen, V. A. 1998. Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida*, *Plant J.* 13: 39–50.

Iida, S., Morita, Y., Choi, J. D., Park, K. I. and Hoshino, A. 2004. Genetics and epigenetics in flower pigmentation associated with transposable elements in morning glories. *Adv. Biophys.* 39: 141-159.

Ishizaka, H. 2008. Interspecific hybridization by embryo rescue in the genus *Cyclamen*. *Plant Biotech.* 25: 511–519.

Itoh, Y., Higeta, D., Suzuki, A., Yoshida, H., and Ozeki, Y. 2002. Excision of transposable elements from the chalcone Isomerase and dihydroflavonol 4-reductase genes may contribute to the variegation of the yellow-flowered carnation (*Dianthus caryophyllus*). *Plant Cell Physiol.* 43: 578–585.

Jorgensen, R. A., Que, Q., and Napoli, C. A. 2002. Maternally controlled ovule abortion results from cosuppression of dihydroflavonol-4-reductase or flavonoid-3'5'-hydroxylase genes in *Petunia hybrida*. *Funct. Plant Biol.* 29: 1501–1506.

Kanno, Y., Noda, N., Kazuma, K., Tsugawa, H., and Suzuki, M. 2003. Transformation of *Lobelia erinus*. (in Japanese). *In: The Abstract of 21st Annual Meeting of Jap. Soc. Plant Cell Mol. Biol.* (p. 121).

Kevan, P., Giurfa, M., and Chittka, L. 1996. Why are there so many and so few white flowers? *Trends Plant Sci.* 1: 280–284.

Kinoshita, T. 1995. Report of committee on gene symbolization, nomenclature and linkage groups. *Rice Genet. Newsl.* 12: 9–153.

Koes, R. E., Quattrocchio, F., and Mol, J. N. M. 1994. The flavonoid biosynthetic pathway in plants: function and evolution, *BioEssays* 16: 123–132.

Liao, B., and Lei, Y. 2004. A black seed coat groundnut cultivar Zhoghua 9 released in China. *Int. Arachis Newsl.* 20: 20-21.

Liu, D., Galli, M., and Crawford, N. M. 2001. Engineering variegated floral patterns in tobacco plants using the Arabidopsis transposable element *Tag1. Plant and Cell Physiol.* 42: 419-423.

Lloyd, A. M., Walbot, V., and Davis, R. W. 1992. *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1. Sci.* 258: 1773-1775.

Mizutani, M., Tsuda, S., Suzuki, K., Nakamura, N., Fukui, Y., Kusumi, T., and Tanaka, Y. 2003. Evaluation of post transcriptional gene silencing methods using flower color as the indicator. *Plant Cell Physiol.* 44: 122-125.

Mol, J., Grotewold, E. and Koes, R. 1998. How genes paint flowers and seeds: A review. *Trends Plant Sci.* 3: 212-217.

Nagao, S., and Takahashi, M. 1963. Trial construction of twelve linkage groups in Japanese rice. *J. Fac. Agr., Hokkaido Univ.* 53: 76–131.

Nagao, S., Takahashi, M., and Kinoshita, T. 1962. Genetic studies on rice plant, XXVI. *J. Fac. Agr., Hokkaido Univ.* 52: 20–50.

Nagao, S., Takahashi, M., and Miyamoto, T. 1957. Genetic studies on rice plant, XXI. Biochemical studies on red rice pigmentation. *Jap. J. Genet.* 32: 124–128.

Nagatomi, S., Tanaka, A., Watanabe, H., and Tano, S. 1997. Chrysanthemum mutants regenerated from in vitro explants irradiated with 12C5+ ion beam. *Institute of Radiation Breeding, Technical News* No. 60.

Nishihara, M., Nakatsuka, T., Mishiba, K., Kikuchi, A., and Yamamura, S. 2003. Flower color modification by suppression of chalcone synthase gene in gentian. *Plant Cell Physiol.* 44: 159

Noda, K. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor, *Nat.* 369: 661–664.

Okuhara, H., Ishiguro, K., Hirose, C., Gao, M., Togami, J., Nakamura, N., Ono, E., Ochiai, M., Fukui, Y., Yanaguchi, M., and Tanaka, Y. 2004. Molecular cloning and functional expression of teterahydroxychalcone 2'-glucosyltransferase genes. *Plant Cell Physiol.* 45: 133-135.

Satpute, R. A., and Suradkar, S. W. 2011. Effect of mutation on seed coat colour in groundnut (*Arachis hypogaea L.*) *J. Exp. Sci.* 2: 24-25.

Savin, K. W., Baudinette, S. C., Graham, M. W., Michael, M. Z., Nugent, G. D., Lu, C., Chandler, S. F., and Cornish, E. C. 1995. Antisense ACC Oxidase RNA delays carnation petal senescence. *Hort. Sci.* 30: 970–972.

Schijlen, E. G., Ric deVos, C. H., van Tunen, A. J., and Bovy, A. G. 2004. Modification of flavonoid biosynthesis in crop plants. *Phytochemistry* 65: 2631-2648.

Shikazono, N., Suzuki, C., Kitamura, S., Watanabe, H., Tano, S., and Tanaka, A. 2005. Analysis of mutations induced by carbon ions in *Arabidopsis thaliana. J. Exp. Bot.* 56: 587–596.

Stafford, H. A. 1990. Flavonoid Metabolism. The anthocyanins. In: Harborne JB (ed) The Flavonoids – Advances in Research Since 1986 (pp. 1–22). Chapman and Hall, London.

Stafford, H. A. 1994. Anthocyanins and betalains: evolution of the mutually exclusive pathways, *Plant Sci.* 101: 91–98.

Tanaka, Y., Brugliera, F. and Chandler, S. 2009. Recent progress of flower colour modification by biotechnology. *Int. J. Mol. Sci.* 10: 5350-5369.

Tanaka, Y., Katsumoto, Y., Brugliera, F., and Mason, J. 2005. Genetic engineering in floriculture. *Plant Cell,* 80: 1-24.

Tanaka, Y., Sasaki, N., and Ohmiya, A. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54: 733-749.

Ueyama, Y., Suzuki, K., Mizutani, M., Fukui, Y., Miyazaki, K., Ohkawa, H., Kusumi, T., and Jez, J. M., 2000. Structure and mechanism of the evolutionary unique enzyme chalcone isomerase. *Nat. Struct. Biol.* 7: 786–791.

Winkel-Shirley, B. 2002. Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.* 5: 218–223.

Zuker, A., Tzfira, T., Ben-meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkmann, G., Martens, S., Nata-Sharir, I., Weiss, D. and Vainstein, A. 2002. Modification of flower colour and fragrance by antisense suppression of the flavanone 3- hydroxylase gene. *Mol. Breed.* 9: 33–41.

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