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# Plant Biotechnology of Flavonoids

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## Abstract

Flavonoids, including anthocyanins are ubiquitous compounds constituting about 5-10% of the known secondary metabolites imparting vivid floral, seed and foliage colors in plants ranging from bryophytes to angiosperms. Plants are specialized in synthesizing and accumulating specific combinations of flavonoids out of a pool of about 5000 known flavonoids implying their adaptive functions. The flavonoid pathway has been well characterized in a few select plants. The genetic and molecular analysis revealed that the pathway is governed by a number of loci dispersed across the plant genome and regulated by distinct regulatory gene families in a temporal and spatial manner. With the rapid growth in molecular and biochemical characterization of the genes and their products it has now become possible to precisely elucidate the role of flavonoids in plant survival. Flavonoids have been implicated in diverse functions such as UV-B protection, signal molecules in plant - microbe symbiotic associations, plant defense response, cold stress response, modulators of hormone response and pollen fertility. In addition, the role of flavonoids as very powerful dietary anti-oxidant supplements in human nutrition is increasingly demonstrated. The review highlights certain structural and functional aspects of flavonoids particularly their role in stress response. Further, recent advances in application of biotechnology tools to manipulate flavonoid pathway in different plants has been described. Flavonoid genes as benign and visible reporters of plant origin in transformation experiments appears to be promising. Studies on transgenic plants carrying genetically engineered flavonoid genes leading to the accumulation of flavonoids by sense over-expression or decrease or elimination by anti-sense suppression respectively, have been used to manipulate plants defense response against bacterial and fungal diseases. Flavonoid biotechnology has become a powerful tool to manipulate flower color in horticulture industry. This review critically evaluates various functions of flavonoids and describes specific instances and strategies of biotechnological manipulation to improve plant performance and value addition.

## Abbreviations

***A1***: Anthocyanin-1; ***A2***: Anthocyanin-2; ***B*** booster: ***Bz1***: Bronze-1; ***Bz2***: Bronze-2; ***C1***: Colored-1; ***C1-I***: Color inhibitor; ***C2***-Colored 2; ***del***: delila; ***Lc***: Leaf color; ***P***: pericarp; ***Pl***: plant color; ***R***: Red; ***Vp1***: Viviparous; ***Ans***: Anthocyanidin synthase; ***CHS***: Chalcone synthase; ***CHI*** : Chalcone isomerase; ***CHR***: Chalcone reductase; ***F3'H***: Flavonone-3'-hydroxylase; ***F3H***: Flavonoid-3-hydroxylase; ***FGT***: Flavonoid-3-O-glycosyltransferase; ***GST***: Glutahione-S-transferase; ***IFS***: Isoflavone synthase; ***IOMT***: Isoflavone O-methyl transferase; ***IFOH***: Isoflavone 2'-hydroxylase; ***IFR***: Isoflavone reductase; ***PTS***: Pterocarpan synthase; ***QTL***: Quantitaive Trait loci; ***VAM***: Vesicular arbuscular mycorrhizae.

## 1. Introduction

Flavonoids are a major class of secondary metabolites constituting about 5-10% of the known secondary products in plants ranging from bryophytes to angiosperms. The diversity and complexity of flavonoids and the related compounds produced by an extension of the phenylpropanoid pathway in plants has been extensively investigated. To-date, about 5000 flavonoids are documented in plants and the list is steadily increasing. It is a common observation that plants are specialized in synthesizing and accumulating only a certain combination of flavonoid compounds out of a large pool of known flavonoids among plant genera. Such a

selectivity at the species, genus and family level implies an evolutionary function associated with plant life. Various intermediates and end products of the phenylpropanoid/flavonoid pathway are functionally implicated in a range of biological processes such as defense response, plant microbe interactions, UV-B protection, signal molecules in various transduction pathways and interactions, pollination and male fertility. In addition, the phenylpropanoid pathway that primarily supplies precursors to the flavonoid pathway lead to the production of related compounds such as lignins and aromatic acids that are also implicated in plant defense. Inclusion of flavonoids with such a wide range of attributed functions in the generic class "secondary plant products" is hardly justified. Recent studies are aimed at broadening our understanding of these compounds, unequivocally establishing their known functions and explore the yet unassigned functions of such a diverse group of molecules. Successful elucidation of molecular mechanisms of flavonoid gene expression and regulation in homologous as well as heterologous systems served as a model to investigate the flexible genetic program of constitutive and induced synthesis of secondary metabolites in plants.

Flavanoid biotechnology to day is undoubtedly one of the emerging areas of intense research activity. Application of biotechnology tools to manipulate the flavonoid pathway has been possible, though in a few plants, mainly due to the unique genetic/chemical attributes of the pathway. These include an availability of wealth of mutants, clearly defined structural and regulatory genes, diverse allelic series at many of the loci with clear cut phenotypic effect and well characterized enzymes, precursors and the end products. In addition, the development of specialized techniques of gene isolation such as transposon tagging and reproducible transformation methodologies played a major role in the elucidation of this pathway. Most importantly, the discovery of plant regulatory genes, beginning with the *C1(colored-1)* locus of maize in the late eighties, opened up new avenues for manipulation of the flavonoid pathway. Subsequent identification and isolation of a number of such regulatory genes and elucidation of their role in the control of such pathways led to the genetic engineering of secondary metabolism in several plant species.

Manipulation of secondary metabolism has tremendous potential in agriculture, horticulture and also pharmaceutical industry. Although, there were significant developments in genetics, biochemistry and molecular biology of the pathway, it could not be fully exploited because the regulatory circuits that govern the expression of the component genes of the pathway in cells and whole plants have not been rigorously characterized. Recent developments in utilization of different regulatory genes to control specific but overlapping pathways in cultured cells of corn hold promise for a successful genetic engineering of secondary metabolism (Grotewald *et al.*, 1998).

The present review is an attempt to highlight the instances where biotechnology /genetic engineering strategies were used in directing the pathway to produce novel products with agronomic and commercial importance in select plants. Also, the review focuses on certain properties of flavonoids such as their role in plant defense and their dietary value in human nutrition. Further, brief references are made on descriptions of the fundamental genetics and molecular biology of the pathway in model plants, which may serve, as an aid to understand the system in the context of genetic engineering of the pathway.

## 2. Chemistry, genetics and molecular biology of anthocyanin biosynthesis

Studies on the chemistry of flavonoids began long ago, and by the 70's, the comparative chemistry of flavonoids had been well established. Early studies mainly dealt with structure, distribution, and development of chemical and physical methods of flavonoid separation and characterization. Later, advanced physical methods such as proton-NMR and mass spectral analysis were extensively used to confirm the structural identity of flavonoids. The first intermediate of the pathway, the naringenin chalcone, a C15 unit composed of three planar rings A, C and B (Fig 1) is formed by the condensation of one molecule of 4-coumaroyl CoA with three molecules of malonyl-Co A catalyzed by the key enzyme, chalcone synthase (CHS). While structurally simple derivatives of this basic C15 unit are found in bryophytes and ferns, more specialized and complex forms are found in gymnosperms and angiosperms. Most classes of flavonoids have a similar A ring hydroxylation pattern at 5 and 7 position but differ widely in the B and C ring substitutions. Various reactions, such as hydroxylation, methylation, methoxylation, glycosylation, acylation and oxidation / reductions in the central pyran nucleus and the B ring generally determine the chemical nature and therefore, the structural diversity and the color variation among flavonoids.

On the basis of the above, flavonoids are clas-

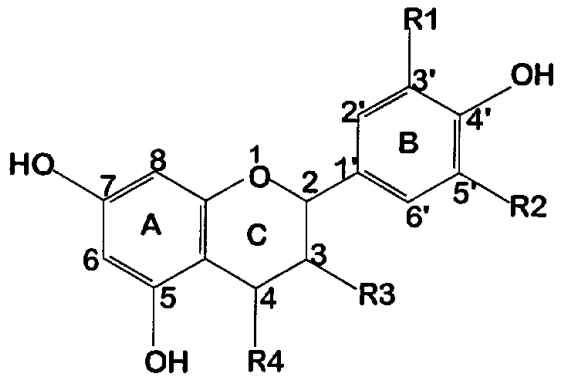


**Fig. 1.** The basic flavonoid molecule. Modification of the 'B' and 'C' give rise to an arrey of flavonoids.

Flavan, R3-H, R4-H, O1=C2, C3=C4
Flavanone, R3-H, R-4 — >=O
Flavone, R3-H, R4->=O, C2=C3
Flavonol, R3->=O, R4->=O, C2=C3
Dihydroflavonol, R3-OH, R4->=O
Leucoanthocyanidin, R3-OH, R4-OH
Anthocyanidin, R3-OH, R4-H, O1=C2, C3=C4
Anthocyanin, R3-OGlc, R4-H, O1=C2, C3=C4
Monohydroxyl flavonoid (e.g., pelargonin), R1-H, R2-H
Dihydroxyl flavonoid (e.g., cyanin), R1-OH, R2-OH
Isoflavone, shift of aryl group (B ring) from C2 to C3 position

sified into 12 groups: chalcones, aurones, flavones, flavonols, flavanones, dihydrochalcones, catechins, flavan-3-4-diols, biflavonoids, isoflavonoids, proanthocyanidins and anthocyanins (red/purple and blue pigments). Of the above groups, anthocyanins and flavonols are ubiquitously found in angiosperms and their chemistry most extensively studied. In addition, plants are known to have an inherent capacity to elaborate a wide range of natural products based on the phenylpropanoid precursors through common biosynthetic enzymes and intermediates. Such molecules include complex lignins-structural polymers commonly found in xylem cell walls, antimicrobial phytoalexins including isoflavonoids and furanocoumarins along with organic acids and their esters. The biosynthesis of such a wide array of biologically important molecules, sharing common regulatory genes and reaction intermediates, offer unique advantages to manipulate the pathway and to produce a range of important compounds.

Advances in genetic analysis of the flavonoid biosynthesis in plants have been made mainly due to the naturally occurring, non-lethal and visually scorable distinct flavonoid mutant phenotypes serving as "ready made" genetic variability. In addition, several transposon induced mutants in plants such as maize and *Antirrhinum* contributed significantly. In parallel, simple and reproducible chemical techniques coupled with well-established genetic methodologies contributed to the identification of the genetic determinants governing the synthesis of specific flavonoid molecules. Such a multidisciplinary approach led to the understanding of the genes, enzymes, precursors and regulation of this pathway in a few select plants. Among cereals, corn (*Zea mays*) anthocyanin biosynthetic pathway has been rigorously analyzed and exploited which, by and large, served as a prototype for other plants.

Genetic-biochemical analysis coupled with molecular investigations on *Zea mays* flavonoid biosynthesis revealed three functionally distinct classes of genes. The Class I genes are structural genes encoding enzymes that drive the pathway. The Class II genes are regulatory genes encoding transcription activator proteins that interact among themselves and with the promoter elements of the structural genes resulting in the up-regulation of the pathway. The class III genes are largely known as modifying genes that alter the intensity and distribution of pigments by not yet clearly defined mechanisms. The effect of most of the genes at the phenotypic level is clearly manifested and easily identified in segregating populations as variations in intensity, shade and distribution of color. The pathway thus, encompasses a complex system of coordinate regulation of expression of dispersed genes in the genome, acting sequentially in a temporal and spatial manner.

Progress in understanding the genetics and molecular biology of flavonoid biosynthesis in maize has been reviewed (Coe *et al.*, 1988; Dooner 1983; Dooner and Robbins 1991; Forkman 1993; Heller and Forkmann 1988; Neuffer *et al.*, 1997). The structural genes encoding the enzymes in maize are *Pal* (phenylalanine ammonia lyase), *C2* (chalcone synthase), *CHI* (chalcone isomerase), *A1* (dihydroflavonol reductase), *A2* (anthocyanidin synthase), *Bz1* (flavonoid 3-O-glycosyltransferase) and *Bz2* (glutathione -S-transferase). The regulatory loci, *B* (booster), *C1* (colored-1), *P* (pericarp), *Pl* (plant color), *R* (red) and *Vp1* (viviparous) encode transactivating factors governing the expression of the pathway genes (Dooner and Robbins 1991; Neuffer *et al.*, 1997). The structural genes, enzymes, biosynthetic steps, and the regulatory features have been clearly elaborated in plants such as *barley* (Kristianten 1984; Jende Strid 1993) *Arabidopsis* (Feinbaum and Ausubel 1988; Feinbaum *et al.*, 1991; Kubasel *et al.*, 1992; Shirley and Goodman 1993;

**Table 1.** Structural Genes Encoding Anthocyanin Biosynthetic Enzymes

| Enzyme | Maize | | Rice | | *Petunia* | | Barley | |
|---|---|---|---|---|---|---|---|---|
| | Locus | Clone | Locus | Clone | Locus | Clone | Locus | Clone |
| **Chalconesynthase** | *c2* | + | *chs* | + | NA | + | – | + |
| (CHS) | *Whp* | + | – | – | – | – | – | – |
| **Chalcone Isomearse** (CHI) | NA | + | NA | – | *Po* | + | – | – |
| **Flavonone 3-hydroxylase** (F3H) | NA | + | NA | – | *An3* | + | *ant*17? *ant*22? | + |
| **Flavonoid 3'-hydroxylase** (F3'H) | *pr* | – | NA | – | *Ht1/Ht2* | + | – | – |
| **Dihydroflavovol Reductase** (DFR) | *a1* | + | *Dfr* | + | *An6* | + | *ant*18 | + |
| **Anthocyanidin Synthase** (ANS) | *a2* | + | *ans* | + | NA | + | *ant1? ant2? ant5?* | – |
| **Flavonoid 3-glucosyltransferase** (FGT) | *Bz1* | + | NA | – | NA | + | – | + |
| **UDP rhamnose Anthocyanidin -3-glucoside** | NA | – | NA | – | *Rt* | + | NA | – |
| **Anthocyanin acyl Transferase** (AAT) | NA | – | NA | – | *Gf* | – | NA | – |
| **Anthocyanin methyl Transferase** (AMT) | NA | – | NA | – | *Mf1/Mf2* | + | NA | – |
| **Glutathione S-transferase** (GST) | *Bz2* | + | NA | – | *An13* | + | NA | + |

Rice data is exclusively from ARR lab.
The information from Barley and Petunia is adapted from Refs. 9 and 24 respectively.

Shirley *et al.*, 1995), *Antirhinum* (Martin *et al.*, 1991; Jackson *et al.*, 1992) and *Petunia* (Cornu *et al.*, 1990; de Vlaming *et al.*, 1984; Gerats and Martin 1992; Van Tunen and Mol 1991; Wiering 1974). Regulatory genes and gene families have been isolated from plants such as maize, *Petunia*, and *Antirrhinum* and the phenomenon of regulation has been extensively reviewed in maize (Coe *et al.*, 1988; Heller and Forkmann 1988; Neuffer *et al.*, 1997) *Petunia* (de Vlaming *et al.*, 1984; Van Tunen and Mol 1991) *Antirrhinum* ( Gerats and Martin 1992; Martin and Gerats 1993) and an overview of all the above plants can be found in (Holton and Cornish 1995). Well-characterized regulatory genes in *Antirrhinum* include *Delila*, *Eluta* and *Rosea* (Martin *et al.*, 1991) and *An1*, *An2*, *An3*, and *An4* in *Petunia* (Beld *et al.*, 1989 ; Gerats *et al.*, 1982b). The available information on structural genes in a few representative plants is given in Table 1.

Rice, the most important food crop of the world, with a small genome equipped with well-established transformation systems and molecular maps, considerably lags behind in the exploitation of flavonoid pathway. Much of the information available on purple/red pigmentation in rice deals with phenotypic descriptions, the inheritance of specific loci governing the pigmentation pattern and their chromosomal map position (Kinoshita and Maekawa 1986; Kinoshita and Takahashi 1991; Ramaih and Rao 1953; Reddy 1996; Reddy *et al.*, 1994, 1995, 1997, 1998). Broadly, the anthocyanin gene pigment system and its phenotypic variation in rice

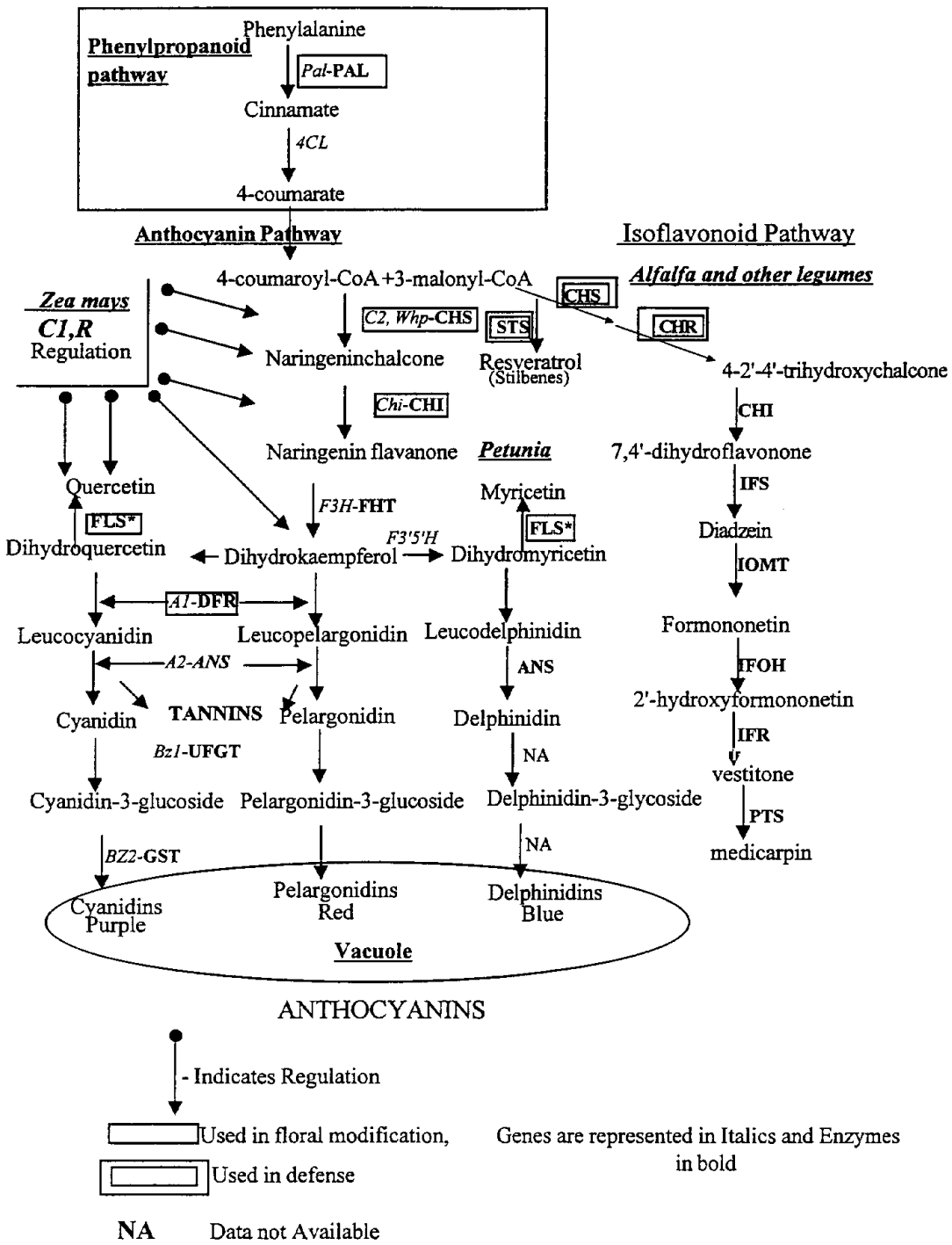


**Fig. 2.** Generalised Flavonoid Pathway

consists of structural genes, the *C* (Chromogen), A (Activator) and the *Rc* and *Rd* (determining the brown pericarp). The regulatory genes *P* (purple) and *Pl* (purple leaf), each with a number of alleles, govern the distribution of purple pigments in various plant organs. Apart from the above-mentioned genes, rice genome is enriched with a class of dominant inhibitor alleles that eliminate color expression in diverse tissues. Detailed phenotypic effects of these inhibitor alleles in rice are described (Reddy 1996). Studies on biochemical and molecular aspects of the pathway in rice have started recently in our lab with the objective of engineering rice for an improved stress resistance. Table 1 includes the status of rice structural genes isolated and characterized so far (Reddy 1996; Reddy *et al.*, 1994, 1995, 1997, 1998,).

The maize example deserves special mention as it served as a model system in plants. The regulatory genes of maize encode trancription-activating factors that share sequence homology with *Myb* and *Myc* class of protooncogenes. For instance, the *R* locus encodes a protein with characteristic acidic N-terminal domain sharing homology with transcrip-

tion activators and a basic C-terminus that is homologous with helix-loop-helix motif of *Myc* family of protooncogenes (Ludwig *et al.*, 1989). The *C1* gene encodes a protein having the basic N-terminus with 40% homology to *Myb* protooncogenes and an acidic C-terminus with features of transcription activator (Paz-Ares *et al.*, 1986, 1987, 1990). The protein products of the regulatory genes of *Zea mays* with their transcription activation domains interact among themselves and with the promoters of the structural genes and thus lead to the regulation of gene expression. A simplified version of the hierarchy of genetic control of the pathway along with the target genes in maize, *Petunia* and alfalfa is depicted in Fig. 2.

In maize, the transcription of the structural genes *C2*, *Chi*, *F3H*, *A1*, *A2*, *Bz1* and *Bz2* is regulated by *R* and *C1* genes in various plant tissues (Bodeau and Walbot 1992 ; Deboo *et al.*, 1995; Dooner 1983). Interestingly, the *P* locus governs *C2*, *Chi* and *A1* in pericarp tissue (Grotewald *et al.*, 1994) and in cultured maize cells (Grotewald *et al.*, 1998) indicating a selectivity at the level of gene regulation. At the pigment level, while the *C1/R* family of genes control the production of anthocyanins, 3-hydroxyflavonoids, proanthocyanidins and flavonol glycosides, the *P* locus controls 3-deoxyflavonoids and 3-deoxyanthocyanidins, *C*-glycosylflavones, and phlobaphanes. Thus, different regulatory genes in maize have specific target genes. Similarly, in snapdragon, the regulatory mutants of *delila* and *eluta* show distinct patterns of *Chs* and *Chi* mRNA accumulation from those of *F3H* and *Dfr* (Jackson *et al.*, 1992; Martin *et al.*, 1991), while the regulatory mutants *an1*, *an2* and *an11* affect the anthocyanin levels in *Petunia* floral color (Quattrocchio *et al.*, 1993). Though, there appears to be significant differences in the regulation of anthocyanin biosynthesis in different plants such as maize, *Petunia* and snapdragon, there exists several striking similarities in plants which constitutes the basis for genetic engineering. Thus, the *Myb* sequences which recognize, with high fidelity, the target motifs in promoter elements and activate expression of genes of a pathway provide critical leverage points for manipulating product concentration, both in homologous and heterologous systems. Particularly, the *Myb* genes are powerful tools in the context of metabolic engineering of a range of plant species for the activation of distinct sets of flavonoid genes in a given cell, tissue, organ and whole plant (Grotewald *et al.*, 1998). Recent studies revealed that the *myb* sequences might respond to different environmental stimuli such as light, salt stress, or plant hormones such as GA and ABA(Gubler *et al.*, 1995; Hattori *et al.*, 1992; Urao *et al.*, 1993). Most of the genes involved in the synthesis of anthocyanins/flavonoids have been identified, cloned and characterized from a number of plant species (reviewed in Holton and Cornish 1995). In maize, the genes of this pathway are cloned using a variety of molecular strategies ranging from the classical cDNA cloning to transposon tagging (Cone *et al.*, 1986; Grotewald *et al.*, 1991a; Ludwig *et al.*, 1989; Mc Laughlin and Walbot 1987; Menssen *et al.*, 1990; O'Reilly *et al.*, 1985; Paz-Ares *et al.*, 1986; Sommer *et al.*, 1987; Theres *et al.*, 1987; Wienand *et al.*, 1986). Some of the genes involved in the pathway are organized into multigene families, each encoding a single enzyme as in the case of *Chs* family which has at least 6-10 copies in *Petunia*, French bean and soybean, and 2-3 copies in maize and rice respectively (Reddy *et al.*, 1997 and the references there in). The function of multiple forms of CHS however, is still not clear. It is presumed that the presence of such multiple forms reflects the requirement for an increased enzymatic plasticity that may be necessary to perform diverse functions like response to elicitors, infection, and wounding and developmental cue (Dixon *et al.*,1992). Besides, some enzymes with related functions are encoded by duplicate genes as in the case of CHS and STS (stilbene synthase), where CHS is involved in the synthesis of naringenin leading to the formation of flavonoids and anthocyanins, while STS is involved in the synthesis of resveratrol a stilben which is closely related to flavonoids and shares common precursors (Tropf *et al.*, 1994). Such rigorously characterized genes governing well-defined control mechanisms for flavonoid biosynthesis and distribution can be used as powerful tools in metabolic engineering.

## 3. Flavonoid biotechnology in the improvement of biotic and abiotic stress response

Flavonoids have a key role in stress response mechanisms in plants. The stringently adaptive recruitment of flavonoids in plant defense against bacterial, fungal and viral diseases including pests is beginning to gain importance. Increasing evidence indicates that the flavonoids show anti-fungal properties in diverse plants such as potato (French and Towers 1992), cereals (Dillon *et al.*, 1997), date palm (Ziouti *et al.*, 1992) and rose (Treutter and Feucht 1990). In addition, exogenous application of quercetin lead to a decrease in the lesions caused by *Chenopodium*, the causal agent of tomato ring

spot disease (Malhotra *et al.*, 1996). Sakuranetin, a methylether derivative of naringenin was also implicated in the resistance of rice plants against blast infection (Dillon *et al.*, 1997; Kodama *et al.*, 1992). Maysin, a C-glycosylflavone was demonstrated to confer resistance of maize plants to corn earworm *Helicoverpa zea* (Byrne *et al.*, 1996a; MC Mullen and Simcox 1995). The QTL (Quantitative Trait Loci) controlling maysin production in maize silks was identified as the *P* locus, which constitutes the first detailed report of the application of QTL to a well-documented pathway (Byrne *et al.*, 1996b). *P* locus is involved in the production of 3-deoxyflavonoids, 3-deoxyanthocyanins, flavan-4-ols, and phlobaphanes (Grotewald *et al.*, 1994,1998; Menssen *et al.*, 1990; Styles and Ceska 1977). Polymerised anthocyanins and tannins, which include proanthocyanidins are also reported to exhibit antimicrobial activity (reviewed in Scalbert 1991). Further, antifungal properties of flavonoids have been demonstrated *in vitro* against many fungal pathogens. For instance, kaempferol diacyl rhamnoside shows growth inhibition of *Staphylococcus* aureus (Bloor 1995) . Antibacterial nature of naringenin was demonstrated *in vitro* against a major rice pathogen, *Xanthomonas oryzae* (Padmavathi *et al.*, 1997).

The role of iso-flavonoids, as a class of phytoalexins (low molecular weight anti-microbial compounds) produced due to attempted infections, was demonstrated in many *Leguminosae* members including *Medicago sativa* (Dalkin *et al.*, 1990), soybean (Zacharius and Kalan 1989) and bean (Gnanmanickam and Patil 1977). Recent evidences indicate that monocots are also equipped with flavonoid-related phytoalexin chemical arsenal like hydroxycinnamic acids in rye, *Sorghum* and rice (Niemeyer 1988; Snyder and Nicholson 1990; Snyder *et al.*, 1991). In *Sorghum*, 3-deoxyanthocyanidins and flavonoids such as luteolinidin, apigenin and caffeic acid ester of arbinosyl-5-o-apigenin (Tenkouano *et al.*, 1993), apigeninidin acyl ester (Hipskind *et al.*, 1990), and methyl ether of luteolinidin (Lo *et al.*, 1996) are proven as phytoalexins. Rice phytoalexins that are synthesized through the isoprenoid pathway, another secondary metabolic pathway, include oryzalexins (Kodama *et al.*, 1992) and momilactones. These are shown to inhibit *in vitro* the growth of the fungal pathogen *Magnoporthe griesae* (Cartwright *et al.*, 1981). Thus, the isoflavonoid pathway (see Fig. 2) involved in the synthesis of phytoalexins seems to lead to an array of phytoalexins with proven antibacterial properties. It can be inferred that plants have evolved the strategy of biosynthesis and accumulation of specific sets of flavonoids in response to attempted pathogen invasion as an integral part of the native defense mechanisms. This innate capacity in plants can be further enhanced using genetic engineering and transformation approaches.

Recent evidences indicate that biotechnology tools are being successfully applied in several plant species to alter the genetic makeup and produce novel flavonoids or flavonoid-related compounds. For example, the introduction of grapevine stilbene synthase (*Sts*) gene into tobacco led to the production of a resveratrol and consequently an increased resistance to the bacterial pathogen *Botrytis cinerea* (Hain *et al.*, 1993). This probably represents the first example of metabolic engineering strategy for improved disease resistance using the stilbene biosynthetic pathway. Subsequently, tomato and potato *sts* transgenics are reported to exhibit an improved resistance against the fungal pathogen, *Phytophthora infestans* (Stahl 1994; Thomzik 1995). In addition, preliminary results from the analysis of the *sts*-rice transgenics also showed an improved resistance against *Magnoporthe grisea* (Strak-Lorenzen *et al.*, 1997). The above examples represent a crop improvement strategy of introduction of novel genes into crop plants that lack them and thereby enabling them to synthesize new flavonoids which might be advantageous to the plant. With the above successful examples of gene introduction and manipulation of pathways in agriculturally important plants, a wide range of gene-tailored plants with altered metabolic profiles is expected to occupy the market in the next few years.

Most of the genetic engineering exercises used constitutive promoters to drive the flavonoid pathway genes in transgenic plants. However, inducible or stress responsive promoter elements will be more useful. For instance, the promoter of *IFR* (isoflavone reductase) gene that responds to a more specific set of signals like elicitor/infection is an ideal candidate in engineering fungal resistance (Oomen *et al.*, 1994).

Antisense strategies provide avenues for modifying the endogenous levels of the flavonoid intermediates in target cells or whole plants. In theory, such strategies genetically block the pathway at a given step, resulting in the selective accumulation of precursors. For instance, in a transgenic plant, the increased accumulation of precursor A can be achieved by an overexpression of the respective gene and the anti-sense down regulation of the succeeding gene so that the precursor A cannot be further converted. As a specific example, the over-expression of *chs* and antisense suppression of *F3H*

in a transgenic plant, should lead to the accumulation of naringenin. Such transgenic plants are useful in addressing important questions about the consequences of alterations of metabolic flux and the role of the elevated/suppressed levels of secondary metabolites in plants to confer resistance to biotic and abiotic factors. This strategy of altering the metabolic flux is demonstrated in transgenic tobacco plants exhibiting *Pal* sense suppression, which show decreased levels of phenylpropanoids and an increased disease susceptibility towards *Cercospora nicotianae* (Maher *et al.*, 1994). Different strategies of altering the metabolic flux in plants through genetic engineering of secondary metabolic pathways are under progress in many labs including ours. Generation of a series of transgenic rice lines carrying sense and antisense transgenes of flavonoid pathway controlling defined steps is underway (Madhuri *et al.*, 1998). Infact, results from the preliminary evaluation of the *chs*-rice transgenics indicated an improved resistance against *Magnoporthe griesae* (Madhuri *et al.*, 1997). With the exciting advances in agri-genomics, combinatorial plant biochemistry and biotechnology, such transgenics with engineered pathways would prove to be extremely useful in generating crop plants with improved defense. It can be argued that present day crop plants would have lost important metabolic pathways of defense, such as the flavonoid pathway, completely or in part due to crop breeding exercises or natural selection. Thus, the introgression into crop plants of genes representing such lost genetic functions governing the flavonoid pathway or its modification through genetic engineering amounts to replacing such lost steps and enhancing the plant survival under adverse conditions.

Changes in flavonoid biosynthesis in plants under a variety of abiotic stress conditions have been documented for long, though there are only a few instances of unambiguous evidence of their role in abiotic stress resistance. Flavonoids with their light absorbing properties are implicated in protecting plants from the damaging UV-B radiation. The potential consequences of the increased UV-B radiation on plant life globally in general and tropics in particular, have been reviewed (Caldwell and Flint 1994). Owing to their accumulation in the upper epidermal cells, flavonoids effectively protect the sensitive inner cells from the damaging effects of UV-B by absorbing the radiation (Caldwell *et al.*, 1983) and this epidermal absorption presumably serves to protect DNA and organelles such as chloroplasts. Flavonoids can effectively protect maize plants from the UV-B damage as measured by the decrease in the formation of cyclobutane pyrimidine dimers (CPD) (Stapleton and Walbot 1994.). Further, the genes of the anthocyanin pathway are differentially induced in response to UV-B light (Taylor and Briggs 1990). There seems to be a differential accumulation of classes of flavonoids among UV susceptible and tolerant varieties of rice indicating their role as UV-B protectants rather than as simple UV-B screens (Ken Markham, New Zealand, personal communication).

Low temperatures have been reported to have significant impact on the synthesis of anthocyanins in plants. The levels of anthocyanins, and transcripts of structural genes namely, *Pal*, *C2*, *4CL*, *Chi*, and *Bz1* including the regulatory genes *R* and *C1* show a dramatic increase during cold acclimation, analogous to the well characterized *Cor* (cold regulated) genes (Christie *et al.*, 1994). Low temperatures also are shown to enhance the anthocyanin levels in *Sorghum* (Shieijo *et al.*, 1993) and *Arabidopsis* (Levya *et al.*, 1995) . Cold stress and light seem to act in a synergistic fashion leading to the accumulation of anthocyanin pathway gene-specific transcripts such as *Pal* and *Chs*. These observations shed light on the impact of the environmental stress factors on anthocyanin biosynthesis. It is not yet known if environmental stress alters the same or distinct signal transduction pathways in activating the anthocyanin production. There is evidence, however, that the stress-mediated changes in flavonoid pools in *Arabidopsis* involve different signal transduction pathway (Knight *et al.*, 1996).

Successful application of biotechnology tools to dramatically alter the levels of stress responsive flavonoids in cell cultures or plants requires a thorough understanding of processes associated with their mobilization into vacuoles. Genes that control such process are beginning to be uncovered in a few plants. In maize, one of the anthocyanin pathway genes, namely *Bz2* (*bronze2*) encoding GST is shown to control the mobilization of anthocyanin (Marrs *et al.*, 1995). The overexpression of such genes and manipulation of intracellular distribution of flavonoids in transgenic plants will have the added advantage of stable accumulation and sequestration, thus offering protection from the possible adverse effects of free bio-active flavonoids, particularly when their accumulation reaches toxic levels. An integrated approach of metabolic engineering using the information on flavonoid biosynthetic pathway, the phenomenon of regulatory specificity and the intracellular accumulation was demonstrated in maize BMS cell lines (Grotewald *et al.*, 1998). The cell lines expressing the anthocyanin regulatory genes, *R* and *C1* produced anthocyanins, while cell lines expressing only the *P*

genes showed the accumulation of 3-deoxyflavonoids. These elegant experiments revealed the intracellular trafficking where the cells expressing *R/C1* genes accumulate the anthocyanins in anthocyanoplasts that are coalesced from endoplasmic reticulum. On the contrary, cells expressing the *P* gene showed large diffuse olive-green bodies containing mainly 3-deoxyanthocyanins (Grotewald *et al.*, 1998).

## 4. Flavonoids as signal molecules in plant - microbe symbiosis: A potential area for biotechnology application

Flavonoids are rapidly emerging as key players in certain beneficial plant-microbe symbiotic associations. A flavone, luteolin released from *alfalfa* roots, induces the expression of *nod A, B* and *C* genes of *Rhizobium meliloti* (Peters *et al.*, 1986). Flavonoids released by the roots of host plants activate the inducible *nod* genes in a *nod D* gene-dependent process (Firman *et al.*, 1986; Peters *et al.*, 1986, 1988; Maxwell *et al.*, 1989). Anthocyanidins such as delphinidin, malvidin and petunidin also induce transcription of *nod* genes of *Rhizobium leguminosarum* (*biovar Phaseoli*) (Hungria *et al.*, 1991). The inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarun* biovar *viciae* (R.I.viciae) results in the release of flavones, flavanones and chalcones, all known for their *nod* gene activation property (Recourt *et al.*, 1991; 1992). Genistein, glycitein and diadzein with malonyl glucose and acetyl substitutions present in soybean seed extracts induce specific *nod* genes (Smit 1992). Particularly, the roots of *Vicia* contain four 3-O-glycosides of kaempfereol formed as a result of *de-novo* flavonoid biosynthesis during interaction with the microbe. Glycosides of flavonoids are reported to induce the *Vir* genes of *Agrobacterium*, though not strongly as the other well-known phenolics do (Clarke *et al.*, 1992).

Flavonoids are also involved in VAM-plant (Vesicular Arbuscular Mycorrhizae) symbiosis. Quercetin, an abundantly occurring flavonol in many plants, greatly stimulates hyphal development of the VAM fungus, *Gigaspora margarita* (Becard *et al.*, 1995). Isoflavonoids including diadzein and coumestrol accumulate during *Rhizobium meliloti* colonisation of *Medicago sativa* (Dakora 1995). During colonisation of the fungus, *Glomus versiformae* in *Medicago truncatula* roots, expression of genes belonging to flavonoid and isoflavonoid biosynthesis is altered leading to the accumulation of specific flavonoid molecules with the concomitant repression of iso-flavonoids (Harrison and Dixon 1993).

Although restricted to a single plant family, the involvement of a wide variety of flavonoids like chalcones, flavones, dihydroflavones, flavanones and isoflavonoids, flavonols and anthocyanidins in plant - microbe interaction is a note worthy observation. However, the accumulation of flavonoids in such symbiotic associations does not seem to be a universal phenomenon, because no such role of flavonoids was found in *mycorrhizal* associations in maize (Becard *et al.*, 1995). There are several routes for application of biotechnology to improve such beneficial interactions. Development of transgenic plants with root specific accumulation of nod gene-inducing flavonoids in leguminous plants is one such strategy to increase nodulation and thereby the nitrogen fixation and productivity. At the same time, antisense suppression of succeeding genes in the pathway, in the same cells, would prevent further conversion of signal molecules into other flavonoid end products of no particular value in such a context. For instance, blocking the conversion of dihydroflavonol to leucoanthocyanidin by antisense repression of *Dfr* (encoding dihydroflavonol-4-reductase) in transgenics is expected to result in accumulation of flavanones, the well-known *nod* inducers. When the nitrogen fixation capability is eventually transferred to non-leguminous plants, such as cereals, manipulation of flavonoid biosynthesis towards improved plant-microbe interactions will be a powerful tool to exploit in crop improvement.

## 5. Flavonoids and the biotechnology of flower color

With the rapidly increasing information on genetics and biosynthesis of flower color pigments, essentially the flavonoids and anthocyanins, flower-breeding research has become a scientifically stimulating and commercially rewarding experience. An understanding of the exact contribution of flavonoids and anthocyanins in imparting a particular color to a flower or foliage offers a powerful route to produce exotic variation in colors, both for aesthetics and commerce. Structurally diverse flavonoids and anthocyanins primarily determine the color of flowers and fruits. For instance, it is known that anthocyanins such as delphinidins with three hydroxyl groups in the B-ring impart a bluish or mauve color, while cyanidins with two hydroxyl groups are purple/red, and pelargonidins with one hydroxyl group are pink/scarlet/orange. The final

color of the flower is largely determined, however, by the chemical nature of anthocyanins, their acylation and methylation status, the pH of the vacuole (in which the pigments are normally located), presence of metal ions, and the extent of co-occurrence of other flavonoids.

The production of novel brick red flowers in *Petunia* transgenics carrying maize DFR gene represents the first successful application of biotechnology tools to floral color manipulation and molecular flower breeding (Meyer *et al.*, 1987). Petunias lack the typical dihydroflavonol-4-reductase (DFR) to convert dihydrokaempferol into pelargonidin, and the maize transgene, *Dfr*, exactly provides such a new function and consequently the brighter red phenotype in transgenic *Petunia*. Following this, there are several reports of generation of novel floral colors by genetic engineering of the anthocyanin pathway. This was achieved by different strategies of genetic engineering such as complementing the missing biochemical step(s), suppression of the gene activity by antisense inhibition, down-regulation of gene activity by sense suppression. All the above strategies have been successfully applied to modify floral color in a directed fashion in *Petunia*.

Significant advances are made in understanding the phenomenon of sense suppression and the production of variegated *Petunias* (Jorgensen 1994, 1995; Napoli *et al.*, 1990). *chs* antisense expression in transgenic *Petunia* dramatically affects the floral pigmentation resulting in variegated or sectored phenotype (Van der Krol *et al.*, 1988). The pigmentation pattern of the sense suppressed transgenic plants is different from that of the anti-sense plants, thus adding to the range of floral patterns that can be obtained. Modification of flower color via transgenics was also demonstrated in ornamentals, such as *Chrysanthemum*, *Cyclamen*, *Pelargonium*, *Lisianthus*, and *Gerbera* (Courtney-Gutterson *et al.*, 1994; Davies *et al.*, 1997; Deroles 1998; Elomma *et al.*, 1993; 1996). In all the above examples the *Chs* was the target gene for genetic manipulation of flower color. Introduction of the *alfalfa* CHR cDNA under the control of 35S *CaMV* promoter into *Petunia* resulted in the production of 6'-deoxychalcones (which are otherwise absent in *Petunia*) thus, changing the flower color from white to pale yellow, and deep purple to pale purple (Davies *et al.*, 1997) - a successful example of diversion of the pathway by altered precursor channeling to produce novel color phenotypes. Genetic crosses between such transgenics, for e.g., yellow flowered chalcone-producing plants and wild type plants gave rise to progeny with brightly colored flowers accumulating increased levels of chalcone. Another example of such a diversion of the flavonoid pathway is in *Lisianthus*, where the transgenics carrying the *Antirrhinum FLS* gene (encoding flavone synthase) exhibit novel flower phenotype (Elomma *et al.*, 1993). Interestingly, the intensity of flower colors in *Petunia* (RL101) transgenics carrying *dfr* transgene depends on the origin of the transferred cDNA. *Petunia* flowers with the maize *A1* transgene are pale red while flowers carrying the *Gerbera gdfr* are bright, although, both sequences encode dihydroflavonol-4-reductase (Elomma *et al.*, 1996). Also, there exists an inverse relationship between the copy number and color of *Petunias* where plants with multiple integration of transgene show instability of anthocyanin pigmentation unlike the single copy-carrying transgenics (Linn *et al.*, 1990). It is worth noting here that the gene transfer by particle delivery normally leads to the multiple integration of transgenes while *Agrobacterium* mediated gene transfer leads to single or few copy integrations.

In roses, shades of red and purple colored flowers are largely due to the accumulation of the cyanidin and pelargonidin derivatives. On the other hand, delphinidin-accumulating roses are not found in nature. The enigmatic blue roses, however, fascinated the poets, rose breeders and genetic engineers for long. Established blue roses like 'Sterling silver' 'blue moon' or 'cardinal de Richelieu' accumulate only cyanidin-3, 5-di glucoside along with large amounts of flavonoids (Holton and Tanaka 1994). Production of blue roses requires development of transgenics carrying flavonoid 3',5'-hydroxylase (F3'5'H) gene to produce delphinidin glucosides that may impart blue color, given the appropriate pH and co-pigmentation (Griesbach 1996). Florigene has developed the Moondust™, a blue colored transgenic carnation producing delphinidin pigments is in the process of commercialization. Transgenic Surfinia™ carrying rose flavonol synthase gene showed bluer color than the host due to the increased amounts of flavonols and decreased amount of anthocyanins (Tanaka *et al.*, 1998) compared to the untransformed . Surfinia™ is a trade name for a *Petunia* variety with long flowering period and relatively disease resistant and reported to have limited blue-violet variants. Genetic engineering of roses for blue flowers also requires the manipulation of vacuolar pH and co-pigmentation levels. The intensity of anthocyanin pigmentation in *Petunia* is controlled by multiple independent Mendelian genes, *Hf* and *Mf* (Chuck *et al.*, 1993), while the vacuolar pH is governed by independent co-dominant pH genes like *Ph1*, *Ph2*, *Ph3*, *Ph4*,

*Ph5* and *Ph6* influencing the floral color by modifying the pH (de vlaming *et al.*, 1984; Chuck *et al.*, 1993). *Ph1* and *Ph2* determine the genetic differences affecting the color, while other genes show pleiotropic effects. Recessive genotypes for any of the above pH genes are expected to increase the pH from 5.5 to 6.0 resulting into the flowers of bluer hue. Infact, high vacuolar pH reduces the amount of cyanidin derivatives but not delphinidin derivatives and thus affect floral color (Gerats and Martin 1992). On the other hand in *Petunia*, the color intensity is affected by *In1* and *In2* loci (Gerats *et al.*, 1982a), and also a dominant fading factor of floral color *Fa* (de Vlaming *et al.*, 1982) . With the understanding of such multiple factors in the development and preservation of color, production of a flower with the color of our choice is no more a distant dream. Infact, with the availability of rapidly developing methodologies for genetic transformation of rose, commercial production of blue roses and other such novel flowers would soon be a reality.

## 6. Genetic engineering of male sterility by regulated flavonoid production in pollen

The UV-B absorbing flavonoids and brightly colored anthocyanins act as signals and honey guides attracting bees, insects and birds for pollination. The role of flavonoids in pollination has been reviewed extensively (Harborne 1993). The association of flavonoids with pollen fertility in several plants is an important function of flavonoids. The involvement of flavonoids in pollination was first uncovered in maize by a series of elegant genetic experiments utilizing the naturally occurring mutants at the duplicate loci, the *c2* and the *whp* (*white pollen*) both encoding the chalcone synthase. While the former is expressed in seed and other plant parts, the latter is expressed in reproductive organs such as anthers, pollen, and stigma. The maize plants deficient in CHS activity produce white sterile pollen in contrast to wild type yellow pollen (Coe *et al.*, 1981). The fertile pollen contains abundant amounts of flavonols (Coe *et al.*, 1981; Davies *et al.*, 1993; Koes *et al.*, 1989; Pollak *et al.*, 1993; Ylstra 1995). Both maize and *Petunia* show a phenomenon of conditional male fertility, where pollen deficient in chalcone synthase are not functional on similar CHS deficient stigmas, but are normally functional on wild type stigmas (Coe *et al.*, 1981; Mo *et al.*, 1992; Pollak *et al.*, 1993; Ylstra 1995; Ylstra *et al.*, 1994). Similarly, *Petunia* transgenics for *chs*, produce infertile pollen due to the elimination of CHS activity by co-suppression or anti-sense suppression are also male sterile (Taylor and Jorgensen 1992; Van der Meer *et al.*, 1992; Ylstra *et al.*, 1994). The fertility function can be chemically restored by spraying the infertile pollen with low concentrations of a flavonol, kaempferol.

Interestingly, flavonoids do not seem to be universally required for fertility in plants. Role of flavonoids in pollen viability in potato is not clear, even though the enzymes of CHS, F3H, and FLS and the total flavonoid content is altered during pollen and pistil development (Sommer and Saedler 1986). Transposon induced mutations of the single copy *chs* gene did not affect the fertility in parsley (Sommer and Saedler 1986). Similarly, *Arabidopsis* mutants, with impaired CHS enzyme and protein did not affect male fertility (Shirley *et al.*, 1995; Ylstra 1995). More importantly, even a null mutation in the first enzyme of the pathway did not effect the male fertility in *Arabidopsis* (Burbulis *et al.*, 1996). Indeed, several independent flavonoid mutants of *Arabidopsis* that are self-fertile were identified to have no effect in fertility. Of many such *Arabidopsis* mutants, the *tt4* (*transparent testa -4*) allele carrying a mutation that disrupts the CHS transcript in both seedlings and flowers, has no effect on fertility. Such mutants were found to have no flavonoids, and yet are fertile. Similarly, flavonoids do not seem to be associated with the pollen fertility in *Antirrhinum* as transposon insertion mutants at *chs* locus are fertile (Sommer and Saedler 1986). Owing to the limited information on the role of flavonoids and male sterility, the association of flavonoids with pollen fertility deserves a more focussed investigation in a wide range of plant species particularly, crops. Not withstanding these limitations, the role of flavonoids in pollination/ fertilization process has direct relevance in the production of hybrid plants by genetic engineering in plants such as maize and *Petunia*. It is theoretically possible to knockout the flavonoids by antisense suppression in maize and thus generate male sterile plants. Thus, genetic manipulation of plant reproduction through flavonoid pathway engineering has immense potential in hybrid industry.

Use of anthocyanin genes to maintain male sterile lines was recently demonstrated in an European Patent No. WO 95/34634. The genome of the male-sterile parent plant contains one of the anthocyanin regulatory gene, while the maintainer line has the other regulatory gene which when crossed with male sterile plant would produce purple/ red color. Thus, male sterile plant has a homozygous male sterility locus and at least one regulatory locus which by itself is not capable of producing the

color, unless crossed with the second parent which has the restorer genes and an anthocyanin regulatory marker gene capable of complementing the earlier locus and produce color. In essence the maintainer lines would have color while the male sterile lines do not. The seeds obtained from such a cross can be identified on the basis of color where the absence of color indicates male sterility.

## 7. Flavonoids as dietary antioxidants and pharmacological agents: Biotechnology applications

Role of plant flavonoids as anticarcinogenic, antiproliferative and antiviral agents is becoming increasingly evident. For instance quercetin could spontaneously regress the growth of metastatic tumors and prevent colon cancer in rats (Okada *et al.*, 1990). Flavonoids, like 3-hydroxyflavone, 3'4'-dihydroxyflavone, 2'3'-dihydroflavone, apigenin, and luteolin inhibited the proliferation of tumor cells (Deschner *et al.*, 1991). Chinese and Japanese traditional medicine used roots of *Scutellaria baicalensis* rich in 5,7,2',5'-tetrahydroxy-6,8-dimethoxyflavone for the treatment of atherosclerosis, hyperlipideamia, inflammation and skin diseases.

Flavonoids are known for their radical scavenging activity (Hosokawa *et al.*, 1990a) with implications as inhibitors of tumor growth. Quercetin inhibits colon cancer cells, gastric cancer cells (Hosokawa *et al.*, 1990a), human squamous sarcoma cells (Kandaswami *et al.*, 1993), and multidrug resistant human breast cancer cells (Scambia *et al.*, 1991) by interfering with cell cycle events. In addition, several groups investigated antiviral properties of flavonoids. Quercetin, hesperetin, and catechin were studied on infectivity and replication of HSV-1, poliovirus type-1, parainfluenza type 3 and respiratory synctial virus (RSV) as seen by viral plaque assay (Kaul *et al.*, 1985). Considerable information is now available on the role of flavonoids in many beneficial human functions, and presently it is a very important area of research aimed at the improvement of human health.

Recent evidences indicate that flavonoids have antioxidant activity with the potential being higher than vitamin C, *B*-carotene and vitamin E on a molar basis (Canada *et al.*, 1990; Myara *et al.*, 1993). This is due to the ability of flavonoids to scavenge harmful reactive oxygen intermediates (ROIs) such as $O_2^-$, $H_2O_2$, $OH^-$ and lipid peroxyl radicals (Jovanovic *et al.*, 1994) . Thus, flavonoids have both radical scavenging and radical protecting effects against lipid peroxidation (Bors *et al.*, 1990). In general, antioxidant activity is determined by the structural arrangements of the hydroxyl groups on the anthocyanin molecule. Flavonoids with greatest antioxidant activity are those with ortho 3, '4'-dihydroxy group in B ring represented by compounds such as catechin, luteolin and quercetin. Glycosylation, acylation, methylation of any of the hydroxyl groups would substantially decrease the antioxidant activity.

Consumption of plant based diet rich in flavonoids can prevent the development and progression of chronic conditions associated with extensive neovascular diseases including malignant tumors (Fotsis *et al.*, 1997). It is believed that flavonoids found in broccoli, reduce platelet activity in blood and helps to prevent blood clots. Resveratrol a stilbene found in grapes (50 to 100 ug /g) is reported to have cancer chemopreventive activity by inhibiting the events associated with tumor initiation, progression and carcinogenesis in a dose dependent manner (Jang *et al.*, 1997). Natural food sources like grapes, blueberry, green tea, citrus plants, peanuts and vegetables like broccoli are rich in flavonoids. For instance, sweet orange contains hespiridin and narirutin, while sour orange contains naringenin with neoeriocitrin (7-neohesperidoside of eriodictyol). Other phenylpropanoids found in citrus fruits are caffeic acid, hydroxycinnamic acids, p-coumaric, caffeic and ferullic acids (Rice-Evans *et al.*, 1997). Isovitexin, a C-glycosyl flavonoid, is abundantly present in rice hull and young barley leaves, is shown to have pharmacologically active antioxidant properties (Ramaratnam *et al.*, 1989; Shibamoto *et al.*, 1994).

The major flavonoid constituents in wine are catechin, epicatechin, cyanidin, malvidin-3-glucoside, rutin, quercetin, myricetin and the related compound resveratrol constituting about 350mg/*l* in red wine, while catechin, epicatechin and traces of gallic acid constitutes 60mg/*l* in white wine (Rice-Evans *et al.*, 1997). In view of the proposed antioxidant activities of the above-mentioned phenolic and flavonoid compounds it is hypothesised that these compounds confer the expected antioxidant property to red wine. The antioxidant properties of red wine is mainly due to the PCO (proanthocyanidolic oligomers) the most potent antioxidants known to man, which are very effective in attacking superoxides.

In addition to red grapes the best source of related phyto chemicals are reported in tea leaf. Phenols and polyphenols found in green tea are powerful antioxidants promoting the production of anticancer enzymes. Thea flavins found in black and oolong tea are shown to exhibit antioxidant properties

against lipid peroxidation in erythrocyte membranes and microsomes. Thus, green tea consumption is associated with lowered risk of cardiovascular diseases. Flavonoids found in tea help in treatment of multiple sclerosis (MS), linked to the breakdown of blood Brain Barrier (BBB) and also help in scavenging the oxygen free radicals (Timstout 1996).

Market is flooded with capsules formulated from the concentrates of various plant extracts, rich in flavonoids with catchy trade names like Inforce™ and Defender™. Pycnogenol is a registered trade name of a nutrient discovered by a French Family, composed mainly of flavonoids, bioflavonoids, oligomers and organic acids and dimeric proanthocyanidins. It is claimed to be active against diseases like heart disease, cancer, arthritis and is effective in strengthening the capillaries, nourishing the skin, balancing histamine production, and other diseases that are linked to the action of free radicals. The grape seed extract found in Defender™ or Inforce™ is reported to stay about 24 times longer than vitamin C, making the most powerful free radical scavengers known to date. PhytoLife™ called the World's Most Complete Antioxidant Superfood, is a broad-spectrum antioxidant - made from 15 different plants mainly composed of antioxidant flavonoids.

Transgenic plants of food crops with enhanced potential for production of flavonoid antioxidants will improve dietary value. For instance, enrichment of rice endosperm with flavonoids should enormously improve the antioxidant activity of rice meal. Similarly, over production of flavonoid antioxidants in foliage of crop plants may have dietary value for ruminants. However, the utility of such antioxidant food crops would depend on the ability to express flavonoid genes in a tissue specific manner. Also, the color acceptability by consumers and their taste preference are two most important areas of concern of such commercial production of genetically engineered flavonoid rich crops.

## 8. Anthocyanin genes as reporters in transformation

Owing to the visible, benign, dispensable and cell autonomous pigmentation phenotype, the anthocyanin genes are used extensively as reporters in transformation experiments in many cell types of maize and other plants. Also, such experiments were useful in understanding the phenomenon of gene regulation, through transient assay systems. For instance, introduced anthocyanin transgenes could complement genotypes deficient/impaired in such a function in particle bombardment experiments (Goff *et al.*, 1990; Klein *et al.*, 1988) or electroporation (Bodeau and Walbot 1992) . Use of anthocyanin genes as novel visible markers in transformation into maize aleurone was first shown by using *Lc* (Leaf color) a member of *R* gene family (Ludwig *et al.*, 1990). In the subsequent experiments it was shown that *Lc*, along with *C1* from maize under the control of 35S promoter could activate anthocyanin pathway in heterologous plants such as *Arabidopsis* and *Nicotiana* (Lloyd *et al.*, 1992). Also, *R* and *C1* were used in the production of purple color in maize transformation experiments (Bowen *et al.*, 1992). In addition, *Lc* was used to activate the anthocyanin pathway in tomato (Goldsbrough *et al.*, 1996) and *Petunia* (Bradley *et al.*, 1998; Quattrocchio *et al.*, 1993). Further, the maize *Lc* can complement *ttg* mutation in *Arabidopsis* (Quattrocchio *et al.*, 1993). A stable sugarcane transgenic line expressing purple phenotype was generated with *R* and *B* serving as a non-destructible marker in transformation experiments (Bower *et al.*, 1996). Ectopic expression of *Del* (from *Antirrhinum*) also lead to the increased anthocyanin expression in tomato and tobacco (Mooney *et al.*, 1995). Ectopic expression of *C1/R* or *P* are sufficient to produce anthocyains or 3-deoxy flavonoids in Black Mexican Sweet maize cell lines. This actually forms a viable strategy to regulate the production of secondary metabolites in cell cultures (Grotewald *et al.*, 1998). Experiments in our lab demonstrated the trans-activation of anthocyanin pathway in the calli of a rice line Tp309 by the maize *R*, *C1* and *C2* transgenes under the control of constitutive promoters like ubiquitin and *E35S* (Madhuri *et al.*, 1998) . Furthur, we show that *C2* transgene under the control of ubiquitin is sufficient to restore purple pigmentation in Tp309 (Madhuri *et al.*, 1997). Trans-activation of anthocyanin pathway has tremendous potential. For instance, transgenic plants carrying anthocyanin regulatory genes under the control of an inducible promoter responding to various environmental stimuli would serve as a benign color indicator of environmental perturbations.

## 9. Signal transduction pathways involved in the activation of anthocyanin genes

Considerable data is accumulated in defining the photoreceptors involved in the light mediated activation of flavonoid genes. However, to date the information on exact signal transduction events is still fragmentary. There are two distinct phytoch-

rome signal transduction pathways coupled to the transcription activation of the anthocyanin genes where cGMP is the key player inducing chalcone synthase (CHS) gene expression during photoresponsive anthocyanin biosynthesis (Batschauer *et al.*, 1991; Bowler *et al.*, 1994; Neuhas *et al.*, 1993). Phytochrome is involved in the expression, photoperception and regulation of transcription of anthocyanin genes in mustard (Bowler *et al.*, 1994b), tomato (Batschauer *et al.*, 1991; Bowler *et al.*, 1994) and parsley (Batschauer *et al.*, 1991; Caldwell *et al.*, 1983). In *Arabidopsis*, however, the UV-A/blue and UV-B light signal transduction pathways are distinct though both involve $Ca^{2+}$ as an intermediate ( Batschawer *et al.*, 1996). In addition to the light mediated signal transduction events, other signal molecules like ABA also play a role in triggering anthocyanin biosynthetic genes. For instance, plant hormone ABA (well characterized signal molecule in dehydration or salt stress) induces the expression of At*MYB2*, a *Myb* class of protein in *Arabidopsis*, which is also responsive to salt, and dehydration stress (Shinozaki *et al.*, 1992). Further, the activation of drought responsive genes At*MYB2* and its interaction with the promoter elements of an ABA responsive dehydration gene *rd22* gene was speculated (Iwasaki *et al.*, 1995). The dehydration and ABA inducible expression of *rd22* has two putative responsive sequences, one for *Myc* and the other for *Myb* factors indicating that both (similar to the anthocyanin regulatory genes *R* and *C1*) function as transcription activators in *rd22* gene expression (Abe *et al.*, 1997). Since the coordinated action of *Myb* and *Myc* genes is responsible for the transcription activation of dehydration responsive *rd22* gene, such a response may also require the expression of anthocyanin genes, *C1* and *R* which are also reported to share sequence similarities with *Myb* and *Myc* respectively. Thus, the well-known signal molecule ABA can trigger anthocyanin production in maize cells by interacting with *C1* promoter sequence acting synergistically with light. Also, there may be multiple signal transduction cascades such as light, ABA and other factors like cold and hormones concentration, which may induce the gene expression. *Vp1* over expression and exogenous application of ABA can independently cause transcription activation of reporter genes driven by the *C1* promoter (Gubler *et al.*, 1995). Detailed molecular analysis of the *C1* gene showed that this regulatory gene has separate cis-acting elements for light activation, abscisic acid-responsiveness and vivipary (Kao *et al.*, 1996). Thus, identification and characterization of the signal molecules involved in the cross talk between anthocyanin biosynthesis and stress response mechanisms have tremendous potential in exploiting the pathway in crop improvement.

The exploitation of flavonoid pathway through the application of biotechnology tools, though feasible, is fraught with several impediments largely arising out of lack of adequate information on molecular and cellular phenomenon associated with various aspects of gene expression and regulation in transgenics. Such phenomena range from the simple biochemical process of feed back /product inhibition to recently uncovered regulatory processes such as co-supression, gene silencing, biochemical pleiotropy, and developmental expression. Superimposed on the above are the environmental variables that discriminate between cellular and whole plant systems and the inconsistencies in behavior of modified pathways in transgenics in lab, factory and land situations. Also, cellular competence to synthesize and accumulate flavonoids is another critical factor in adapting the modified pathways to tissue/cell culture production systems. Not withstanding the above limitations, flavonoid biotechnology is amenable for studies and exploitation in a variety of commercial applications in diverse plant species. In fact, a well understood and exploited flavonoid pathway would serve as a model to be adapted in manipulation of other secondary metabolic pathways in plants. With the dissection of regulatory gene families in maize that specifically control the distinct but overlapping pathways *in vitro*, the exploitation of the pathway in production of target compound is indeed become a reality (Grotewald *et al.*, 1998). Some of the immediate target traits for biotechnological modification of the pathway are mechanisms of intracellular mobilization and trafficking of flavonoids across the cell, tissue specificity of synthesis and accumulation, delinking or co-regulation of the pathway and manipulating appropriate cellular pH for generating designer phenotypes of flowers, and bringing the pathway under the control of specific inducers, both biotic and abiotic factors including chemicals. Rapid developments in genetic engineering of the flavonoid pathway and its expression *in vitro* and *in vivo* hold great promise for commercial exploitation.

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