2. LITERATURE REVIEW
2. Literature Review

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. The genetic/chemical attributes of the flavonoid gene pigment system, particularly in maize offered unique advantages to study gene expression, regulation, and the resulting phenotypic diversity observed in higher plants. Flavonoid biotechnology, today, is one of the active areas of research, contributed to the spectacular progress in advancing the knowledge of secondary biosynthetic pathways in general.

The present literature survey deals with the general features of the flavonoid/anthocyanin pathway which includes the structural aspects of the compounds, genetics, molecular biology and genetic engineering of this pathway in plants. In addition, the physiological relevance of the products of this pathway is described with emphasis on the role of these compounds in protecting plants under adverse conditions, such as UV-B stress, and pathogen attack. Further, recent advances in manipulation and genetic engineering of flavonoid pathway with reference to floral color modification, reporters in plant transformation, and in development of new strategies for improved defense response in plants are mentioned.

2.1 Structure and Function of Flavonoids

2.1.1 Structural diversity

Higher plants exhibit remarkable variation in flower, fruit and foliage color largely due to the underlying diversity of flavonoids. The chemical identity of flavonoids/anthocyanins and the consequent phenotypic expression has been worked out initially using standard organic chemical procedures, later followed by the high resolution physical and analytical procedures such as various forms of chromatography,
spectroscopy, including proton NMR and mass spectroscopy. Of the thousands of structurally different flavonoids produced in higher plants, a given plant species normally accumulates a definite combination of a few of these molecules.

Anthocyanins are the brightly colored compounds belonging to the general class of flavonoids. Chemically, anthocyanins are classified as water-soluble glycosides, which are 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 benzoyle and B-ring hydroxycinnamoyl system composed of three planar rings A, C and B as shown in (Fig 2.1.1). While the B ring of the flavonoid skeleton (Fig. 2.1.1) originates from the phenylpropanoid pathway, the A ring is derived from acetyl-malonyl pathway. The precursors of the flavonoid pathway are Malonyl-CoA (obtained by the condensation of acetyl-CoA and carbondioxide catalysed by acetyl CoA carboxylase), and 4-coumaroyl CoA (obtained from Shikimate/phenylpropanoid pathway). Most classes of flavonoids derived from this C15 precursor show similar A ring hydroxylation pattern at 5 and 7 position but differ widely in the B and C ring substitutions. Hydroxylation, methylation, methoxylcylation, acylation and oxidation / reductions in the C and B ring produce a bewildering array of flavonoids. The occurrence and characterization of such structurally diverse flavonoids among wide range of plant genera have been recorded and reviewed extensively (Harborne, 1993).

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).

Different classes of the above group of compounds are specialised to perform distinct chemical, physical and biological properties. For instance, anthocyanins are brightly colored and relatively stable but not physiologically very active, whereas flavonoids, are not so bright in appearance but are involved in several physiologically
Fig 2.1.1. The basic flavonoid molecule. Modification of the 'C' and 'B' give rise to an array of flavonoids.

Flavanone, R3-H, R4−=O
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
Flavone, R3-H, R4=O, C2=C3
Flavan, R3-H, R4-H, O1=C2, C3=C4
Isoflavanone, shift of aryl group (B ring) from C2 to C3 position
Flavonol, R3=O, R4=O, C2=C3
Monohydroxyflavonoid (e.g., pelargonin), R1-H, R2-H
Dihydroxyflavonoid (e.g., cyanin), R1-OH, R2-H
Trihydroxyflavonoid (e.g., delphinin), R1-OH, R2-OH
significant functions. In addition, plants are known to synthesize a range of natural products, from phenylpropanoid/flavonoid pathway precursors and intermediates that are structurally related and share common biosynthetic enzymes. Such compounds include complex lignin’s - structural polymers predominantly found in xylem cell walls, anti-microbial phytoalexins including isoflavonoids and furanocoumarins, organic acids and their esters (Harbome 1993).

The extensive structural diversity of flavonoids found in plants suggests that they have definite functions in plants. Originally, the main function of flavonoids in plants was hypothesized to be their role in protection from UV-B damage. Later, a range of functions have been attributed to flavonoids - mostly on the basis of experimental evidence from several plant genera. In few cases, however, circumstantial evidences were the basis for assigning new functions. Such attributed functions include, fundamental biological processes, such as, attracting birds and insects for pollination, (the color factor), modulation of hormone responses and free radical scavenging. Most importantly flavonoids are reported to have a role in plants defense mechanism against diseases (viruses, bacteria, fungi) and pests. Further, flavonoids were also implicated in pollen viability and fertility and as signal molecules in various transduction pathways including those associated with abiotic stress responses.

2.1.2 Flavonoid biosynthesis

The biosynthetic pathway leading to the production of flavonoids and anthocyanins have been described in detail in many plants. The biochemical steps and the responsible enzymes have been identified, characterized and reviewed (For. e.g. Heller and Forkmann. 1988). To date, the biochemistry of flavonoids in rice has not been rigorously analyzed. Among cereals, unlike rice only maize flavonoid biochemistry is known. The flavonoid biosynthetic route has two component pathways, the phenylpropanoid pathway and the flavonoid pathway. A simplistic version of the pathway is shown in Fig.2.1.2. The first step in the flavonoid pathway is the deamination of phenylalanine to transcinnamic acid by phenylalanine ammonia lyase (PAL). PAL activity links primary metabolism with the phenylpropanoid pathway, the beginning of secondary metabolic pathway. Cinnamate, thus formed is hydroxylated
Fig. 2.1.2. Schematic representation of the flavonoid biosynthetic pathway

Genes are represented in italics; Enzymes in Capitals; dotted lines and T represent regulation.
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.

Chemico-genetic analysis followed by molecular investigations in various plant species lead to the elucidation of the individual biosynthetic steps of the flavonoid pathway, as shown in Fig.2.1.2. Evidences came predominantly form maize, Petunia, Antirrhinnum and Matthiola. However, only the maize pathway is explained in some detail in the present context.

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 synthases (CHS) (Fig.2.1.1.1). 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, the precursors for iso-flavonoids. Iso-flavonoid formation are catalysed by 2-hydroxy-iso flavone synthase, a mixed cytochrome P450 monooxygenase that is involved in the oxidative rearrangement of the flavanonone with an aryl shifts from the position 2 to 3. Although leguminaceae members are specialized in producing these group of compounds this iso-flavonoid branch pathway is completely missing in cereals.

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). Leucoanthocyanidins are the colorless active precursors for the synthesis of catechins, proanthocyanidins and anthocyanins. While catechins
Fig 2.1.1.1. Formation of Naringenin catalysed by chalcone synthase (CHS)
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. The glutathionation of anthocyanins is depicted in Fig. 2.1.1.2. 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.

There is not much information about the intracellular site(s) of enzyme assembly and synthesis of various intermediates of flavonoid biosynthetic pathway. Ultrastructural details of the process of synthesis accumulation and mobilization into organelles are lacking. The enzymes of flavonoid and phenylpropanoid metabolism seem to be associated with the endoplasmic reticulum (Wagner et al, 1984). The flavonoid intermediates are proposed to be channeled through multienzyme complexes, and are organized in such a way that the compound produced by the preceding enzyme in the sequence becomes the substrate for the subsequent enzyme reaction (Hrazdina and Wagner 1985). The PAL activity was located on the lumen of the ER, where it has an easy access to the substrate pool of phenylalanine. The next enzyme in the complex, cinnamate-4-hydroxylase, remains embedded in the ER membrane but channels its product, p-coumarate to the cytoplasmic face of ER. The remaining
Fig. 2.1.1.2. Formation of glutathione intermediate catalyzed by Bz2 encoded Glutathione-S-transferase (GST)
enzymes of the pathway like p-coumarate Co-A ligase, CHS, CHI, DFR, are on the cytoplasmic face of the ER and are held by week forces. The phenyl propanoid and flavonoid pathway products are then sequestered in the ER lumen after glycosylation by specific glycosyl transferases present on the lumen face or in the lumen. From here the products are then transported to the plasma membrane or to the central vacuole mediated by GST where the pigments are ultimately localized (Hrazdina and Jenssen 1990).

Since CHS and glutathione-S-transferase are the subject of this dissertation work, a detailed account about these enzymes is reviewed below.

2.2 Glutathione S-transferases in higher plants

GSTs (E.C: 2.5.1.18) have been reported in several plant species GSTs in plants are associated with the detoxification of the potential alkylating agents, including pharmacologically active compounds (Habig et al., 1974). GSTs catalyse the transfer of -SH group of glutathione (γ-Glu-cys-gly; GSH) to the electrophilic sites of a variety of compounds, and thus neutralise and render the product more water soluble. These glutathione conjugates are hypothesized to be metabolized further by cleavage to glutamate and glycine residues, followed by acetylation of the resultant free amino group of cysteinyl residue and the final product mercapturic acid (Boyland and Chasseaud 1969; Wood. 1970).

The natural substrate of GST enzymes in plants, however, remains unclear. One of the well-worked out natural substrate is trans-cinnamic acid (Dean et al., 1991; Diesperger and Sanderman 1979; Edwards and Dixon 1991), an important intermediate of the phenylpropanoid pathway. Phytoalexins are also reported to be natural substrates for GST’s (Li et al., 1997).

In plants, GSTs are primarily involved in herbicide detoxification and most of the research is focussed on the ability of certain herbicides to serve as substrates for GST enzymes. However, many of the recently discovered GSTs are found to be involved in response processes to abiotic stress conditions.
2.2.1 Classification of Plant GST’s

To date, the recognized sixteen plant GSTs are classified into three major types based on an evolutionary tree and the gene organization, (Droog et al., 1995). Type I GSTs include all maize GSTs, characterized by the presence of two introns and three exons. These GST’s are responsive to environmental perturbations such as dehydration (Kiyosue, et al., 1993), wounding (Kim, et al., 1994), pathogen attack (Dudler et al., 1991), auxin responsive genes such as tobacco Par-B. Type II GST have nine introns, and to date there is only one representative example from carnation (Meyer et al., 1991, Itzhaki, and Woodson 1993). Type III GST are characterized with a single introa. Majority of the cDNAs/genes belonging to the type III category are originated from diverse sources, involved in various other functions with significant sequence similarities with known GSTs. Characteristically, the type III GSTs are responsive to numerous environmental stress conditions such as pathogen attack (Taylor et al., 1990), oxidative stress (Wingate et al., 1988; Levine et al., 1994; Tenhaken et al., 1995) and heavy metal stress (Czarnecka et al., 1988; Hagen et al., 1988; Wingate et al., 1988; Mauch and Dudler, 1993). Examples of this class are heat shock inducible genes such as Gmhs p26-A or GmGST26-A from soybean (Czarnecka, et al., 1988 Hagen et al., 1988), also responsive to a variety of chemicals including 2,4-D. ABA, cadmium and other heavy metals(Droog et al., 1995) and a number of other genes belonging to auxin-regulated tobacco genes (Takahashi et al., 1990; 1991; 1995).

The maize Bz2 belongs to the type III GST, and is responsive to cold stress (Droog et al., 1995; Christie et al., 1994). In addition, Nicotiana plumbaginifolia LS 216, the tobacco C7, potato PrpI or GST1 and soybean GmGXI also belong to this category of GSTs. Though the above classification of GSTs into different classes is based broadly on the substrate specificity and gene organization, nevertheless, closely related GSTs have distinct substrate preferences and conversely, the highly divergent enzymes recognize the same substrates (Alfenito et al., 1998). For instance, An9 and GST III belonging to type I GST and soybean GmGST26A (type III) could complement Bz2 mutation (type III) (Alfenito et al., 1998). On the other hand, other type I GSTs, including the Arabidopsis Gst EST H36860 (cloned as most closely related to Petunia An9), maize GSTl and GSTIV failed to complement the Bz2 mutation and restore
pigmentation in transient expression studies, thus indicating the differences in substrate preferences. The proof for the involvement of GST in abiotic stress resistance in shown in transgenic tobacco seedlings over-expressing both glutathione S-transferase and glutathione peroxidase (GPX) cDNAs resulting in an increase in the GST- and GPX-specific enzyme activities showing an improved tolerance to chilling or salt stress (Roxas et al., 1997).

GSTs from pumpkin sarcocarp are identified and biochemically characterized (Fujita et al., 1994), however, these GST's could not be included in the above classification due to the lack of information about gene organization.

2.2.2 GST's and their role in anthocyanin sequestration

Anthocyanins synthesized in the cytoplasm are ultimately sequestered into the vacuole with the aid of Bz2 encoded GST which catalyses the formation of anthocyanin - GSH conjugates forming a transfer intermediate allowing the transport into the vacuole and thus impart a deep red or purple color. The glutathionation of anthocyanins is presumed to transport the bioreactive molecules from cytoplasm and actively transport the conjugates through a Mg-ATP requiring GS-X pump (Marrs et al., 1995).

An9 locus, is involved in the last step of Petunia anthocyanin pathway, (Gerats et al., 1982) similar to Bz2. An9 and Bz2 exhibit extensive sequence divergence. On the other hand An9 cDNA shows significant sequence similarities with type I GST, which encodes a protein of 26kDa protein showing GST activity. Interestingly, An9 cDNA was shown to functionally complement maize Bz2 and produce anthocyanins in aleurone. Other Gsts that could complement Bz2, though less efficiently are GmGST26A and maize GST III (Alfenito et al., 1998).

2.3 Genetics and Molecular Biology of the flavonoid pathway

2.3.1. Genetics of the flavonoid pathway

Much of the advances in understanding this pathway in plants is mainly because of brightly colored, non-lethal and visually scorable flavonoid /anthocyanin mutant phenotypes and several transposon induced mutants in maize, which served as "ready
made" genetic variability. In addition, simple and reproducible chemico-physical techniques, coupled with well-established genetic/molecular methodologies have contributed to the identification of genetic determinants governing the synthesis of specific flavonoid molecules.

Genetic-biochemical and molecular analysis of *Zea mays* flavonoid biosynthesis revealed functionally distinct classes of genes dispersed in the genome, acting sequentially in a temporal and spatial manner (Coe *et al.*, 1988; Neuffer *et al.*, 1997). Broadly, the maize genes involved in the anthocyanin pathway can be grouped into three classes. Class I genes are structural genes encoding enzymes that control the single steps in the biosynthesis and subsequent modification. Class II genes are the regulatory genes encoding transcription activator proteins that interact among themselves and with the promoter elements that switch on the whole pathway or parts of the pathway, and determine the tissue specific distribution of the pigments. Lastly, class III genes are largely known as modifying genes that influence the flavonoid concentration, intensity and distribution of pigments. Most of the known flavonoid genes are thus classified into one or the other class and exhibit clear phenotypes. Gene-enzyme relationships of the individual step reactions in the pathway have been well characterized in maize, *Petunia*, and *Antirrhinum*. Almost all the genes involved in the pathway in these plants are isolated and characterized enabling one to understand the molecular basis of expression and regulation.

In maize, the well characterized structural genes include, the *Pal* (phenylalanine ammonia lyase), the *C2* (chalcone synthase), the *CHI* (chalcone isomers), the *Al* (dihydroflavonol reductase), the *A2* (anthocyanidin synthase), the *Bzl* (flavonoid 3-O-glycosyltransferase) and the *Bz2* (glutathione -S-transferase). Activity of all the enzymes are obligatory for full expression of color phenotype and absence of any one of the enzyme leads to non-color phenotypes. For instance, maize tissue devoid of F3GT, and GST is brown, where as the wild type is purple. Dominance-recessive relationship between alleles at individual loci, complementation pattern between non-allelic gene loci and the regulatory interaction all leading to predictable phenotypes have been elegantly demonstrated in maize (Coe *et al.*, 1981; Neuffer 1997). For instance, in
maize, the regulatory loci, \( B \) (booster), \( C1 \) (colored-1), \( P \) (pericarp), \( Pl \) (plant color), \( R \) (red) and \( Vp1 \) (viviparous) encode trans-activating factors governing the expression of the pathway genes (Forkmann 1993; Neuffer et al, 1997) Well-characterized regulatory genes in \textit{Antirrhinum} include \textit{Delila, Eluta} and \textit{Rosea} (Martin et al, 1991) and \textit{An1, An2, An3, and An4} in \textit{Petunia} (Gerats et al., 1982b; Beld et al, 1989).

In addition, a range of mutants belonging to individual steps of the flavonoid pathway are characterized enzymatically in several ornamental plants which lack clear cut genetic information. For example in \textit{Gerbera, Dahlia, Helichrysum, Sinningia cardinalis, Saintpaulia} and \textit{Zinnia} a deficiency or reduction of CHS enzymatic activity has been demonstrated (Forkmann et al, 1989). Similarly, \textit{Petunia RL101} line is a mutant for \textit{Dfr} gene (Meyer et al, 1987).

The structural genes, enzymes, biosynthetic steps, and the regulatory features have been clearly elucidated in several other plant species such as barley (Jende strid et al, 1993; Kristianten 1984), \textit{Arabidopsis} (Shirley et al, 1993; 1995; Martin et al, 1991; Feinbaum and Ausubel 1988; Feinbaum et al, 1991; Kubasek et al, 1992). \textit{Antirrhinum} (Martin et al, 1991; Jackson et al, 1992) and \textit{Petunia} (Wiering 1974; de Vlaming et al, 1984; Cornu et al, 1990; Van Tunen and Mol 1991, Gerats et al, 1992). In maize, \textit{Petunia} and \textit{Antirrhinum}, the regulatory genes, their gene families have been isolated, and the molecular phenomenon of regulation has been extensively reviewed in maize (Coe et al, 1988; Dooner et al, 1991; Neuffer et al, 1997) \textit{Petunia} (de Vlaming et al, 1984) \textit{Antirrhinum} (Gerats et al., 1992; Martin and Gerats 1993) and an overview of all the above plants can be found in (Holton et al, 1995). Table 2.1 summarizes the available information on the structural genes in representative plants.

In rice, the information on purple/red pigmentation has been restricted largely to the phenotypic descriptions, the inheritance of specific loci governing the pigmentation pattern and their classical map positions (Ramaih and Rao 1953; Kinoshita and Maekawa 1986; Kinoshita and Takahashi 1991; Reddy et al, 1994; 1995; 1996a; 1998; Reddy 1996). The anthocyanin gene pigment system in rice is not clearly elucidated Based on the limited information, the rice anthocyanin pathway is explained
to consist of structural genes, the C (Chromogen), \( A \) (Activator) \( Rc \) and \( Rd \) (determining the brown pericarp), and the regulatory genes \( P \) (purple) and \( Pl \) (purple leaf), with a number of alleles, determining the distribution of purple pigments in various plant organs. Apart from the above-mentioned genes, rice genome has a class of dominant inhibitor loci that eliminate color expression in diverse tissues. In the presence of these inhibitor genes, a typical wild type dominant anthocyanin gene behaves as a recessive. The presence of such multiple inhibitor alleles is an interesting feature in rice, though the significance of this observation is obscure at present. Detailed phenotypic effects of the inhibitor alleles in rice have been described (Reddy et al., 1995) though the mechanism of action is not yet clarified. Such a dominant inhibitor allele, the \( CI-J \) which eliminates color in heterozygous state, in maize tissues, has been extensively characterized (Coe et al., 1962; Paz -Ares et al., 1987;1990). The \( CI-I \) protein is a truncated one arising out of an insertion of a 8bp sequence in the third exon coding the C-terminus of the \( CI-I \) protein. The truncated version is short by 21 amino acids (Paz-Ares et al., 1990).

Recent studies on biochemical and molecular aspects of the pathway in rice are beginning to add much needed information to this pathway. The rice pathway has been demonstrated to be UV-B responsive and this property has been exploited by the molecular analysis of the pathway (Reddy et al., 1994). Several structural cDNA sequences have been cloned and sequenced (Reddy et al., 1996; 1998). Table 2.1 includes the status of rice structural genes isolated and characterized so far. Such studies have naturally paved the way for genetic engineering of rice for altered pathway (Madhuri et al., 1998).

2.3.2 Molecular biology of the flavonoid biosynthesis

Maize is a model system to understand the molecular basis of anthocyanin synthesis, accumulation and metabolism in plants. Anthocyanin pigments owing to their brightly colored visible phenotype enabled one to decipher the physiological/biological meaning of this pathway in plants, to dissect various molecular genetic phenomenon such as transposon activity, \textbf{breakage-fusion} bridge cycle, genetic and cytologic crossing over, and also served as markers in molecular mapping and
Table 2.1. Structural genes encoding anthocyanin biosynthetic enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Maize</th>
<th>Rice</th>
<th>Petunia</th>
<th>Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Locus</td>
<td>Locus</td>
<td>Clone</td>
<td>Clone</td>
</tr>
<tr>
<td>Chalcone synthase (CHS)</td>
<td>$c^2$</td>
<td>$ch$</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Chalcone isomerase</td>
<td>$Wh$</td>
<td>$Po$</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavonone 3'-hydroxylase</td>
<td>NA</td>
<td>NA</td>
<td>$ani$</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid 3'-hydroxylase</td>
<td>$pr$</td>
<td>NA</td>
<td>$Ht1/Ht2$</td>
<td>+$^e$</td>
</tr>
<tr>
<td>Dihydroflavonol reductase (DFR)</td>
<td>$al$</td>
<td>$Dfr$</td>
<td>$An6$</td>
<td>+</td>
</tr>
<tr>
<td>Anthocyanidin synthase</td>
<td>$a^2$</td>
<td>$ans$</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid 3'-glucosyl transferase</td>
<td>$Bz1$</td>
<td>NA</td>
<td>$Rt$</td>
<td>+</td>
</tr>
<tr>
<td>UDP rhamnose: anthocyanidin 3'-glucoside</td>
<td>NA</td>
<td>NA</td>
<td>$Gf$</td>
<td>NA</td>
</tr>
<tr>
<td>Anthocyanin acyl transferase (AAT)</td>
<td>NA</td>
<td>NA</td>
<td>$Mf1/Mf2$</td>
<td>+$^e$</td>
</tr>
<tr>
<td>Anthocyanin methyl transferase (AMT)</td>
<td>NA</td>
<td>NA</td>
<td>$An1$</td>
<td>+</td>
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<td>Glutathione S-transferase(GST)</td>
<td>$Bz2$</td>
<td>NA</td>
<td>$An1$</td>
<td>+</td>
</tr>
</tbody>
</table>
transformation. Investigations into the molecular organization of the genes governing the anthocyanin pathway revealed that the structural genes of anthocyanin pathway are under the control of regulatory genes that determine the intensity, and tissue specific expression of the purple color. The protein products of the regulatory genes of Zea mays, such as those belonging to the C/Pl and RIB family encode transcription activators with functional domains interacting among themselves and also the promoters of the structural genes leading to the regulation of gene expression. Such regulatory protein products are related to the myb and myc class of transcription activators having an acidic domain and a basic bHLH domain. The bHLH domain is conserved among various species.

The C1 gene product having an amino terminal basic myb-homologous domain binds to the DNA of promoters of structural genes while the C-terminus containing the amphipathic acidic alpha helix is thought to be responsible for transcription activating function (Paz-Ares et al., 1987). Thus, anthocyanin biosynthesis requires at least one bHLH protein (RIB) and one Myb homologous protein (Goff et al., 1992). Another regulatory locus, P gene encodes a myb-homologous protein that binds and activates a set of structural genes in floral tissues of maize. A different class of regulatory genes such as Vpl has pleiotropic effects which facilitates seed germination but blocks anthocyanin biosynthesis (Robertson, 1955). Vpl regulates anthocyanin biosynthesis in seed by regulating the C1 promoter (Hattori et al., 1992). Other regulatory/modifying genes include the In, (Intensifier) which determine the intensity of purple color in aleurone. In maize, the timing, the distribution and amount of anthocyanins in a given tissue are determined by a complex regulatory hierarchy comprising mainly two gene families are the C1, and R /B and In. R genes constitute a small gene family which includes R(S) (Perrot and Cone 1989), R (Sn) (Tonelli et al, 1991), R(Lc) (Ludwig et al, 1989) determining the temporal and spatial pattern of anthocyanin accumulation (Coe 1962). Members of the B family include Bl-Peru (Chandler et al., 1989), Bl-I (Radicella et al., 1992). Both R and B act as duplicate genes (Goff et al, 1990). Similarly, C1 regulates the expression in kernel, that is scutellum and aleurone of maize while its homologue Pl regulates the expression in plant body (Paz-Ares et al., 1986,1987; Cone et al, 1989; Scheffler 1994). Also, different regulatory genes in
maize have specific target genes. The transcription of the structural genes C2, Chi, F3H, Al, A2, Bzl and Bz2 is regulated by RIB and C1 genes in various plant tissues (Dooner and Robbins 1991; Bodeau and Walbot 1992; Deboo et al, 1995). The P locus governs the expression of C2, Chi and Al in pericarp tissue (Grotewald et al, 1994) and in cultured maize cells (Grotewald et al, 1998).

In snapdragon, the regulatory mutants of delila and eluta show distinct patterns of Chs and Chi mRNA accumulation from those of F3H and Dfr (Martin et al, 1991; Jackson et al, 1992) while the regulatory mutants anl, an2 and an11 affect the anthocyanin levels in Petunia floral color (Quattrocchio et al, 1993). Moreover, the regulatory loci also govern the type of pigments accumulated. For instance, C1/R family of genes control the production of anthocyanins. 3-hydroxyflavonoids, proanthocyanidins and flavonol glycosides, and whereas the P locus controls 3-deoxyflavonoids and 3-deoxyanthocyanidins, C-glycosylflavones, and phlobaphanes. Thus, there appears to be specific regulatory elements governing the sets of genes in different parts of maize plant leading to the tissue specific phenotypes. Most of such genes involved in the synthesis and regulation of anthocyanins/flavonoids have been identified, cloned and characterized from a number of plant species [reviewed in Holton and Cornish 1995).

With the elucidation of regulatory mechanisms, the genes and the corresponding enzymes, the genetic engineering of plants for modified pathway has become possible. Again rice lags behind in this area of gene regulation. So far not a single regulatory gene in rice has been shown to specifically influence the anthocyanin expression. The efforts of this lab in that direction yielded the isolation of several rice homologues of maize anthocyanin genes (Reddy et al, 1998). Particularly, the prospects of using well defined regulatory genes of model plants such as maize across many other plant species has become indeed a reality. The present work further tested this possibility in rice with success.
2.4 Biological functions of flavonoids

2.4.1 Flavonoids as UV protectants

One of the strategies developed by plants to overcome the increased UV-B radiation is the production of UV-B absorbing compounds such as flavonoids and anthocyanins. The production of such flavonoid compounds as a protective measure against damaging UV-B radiation is also seen lower organisms such as ferns and bryophytes (Markham et al., 1998; Veit et al., 1996). Flavonoids with their light absorbing, light scattering, UV filtration and photo-repair properties protect plants from the damaging UV-B radiation (Rozenna et al., 1997; Lois, 1994; Tevini et al., 1991; Reuber et al., 1993; Li et al., 1993). Flavonoids localized in the upper epidermal cells absorb most of the active UV-B radiation and effectively protect the sensitive inner cells from the damaging effects of UV-B radiation (Caldwell et al., 1983; Beggs et al., 1994). An important physiological consequence in response to UV-B light in plants is thought to be the differential induction of the flavonoid genes, resulting in the production of flavonoids, flavones, iso-flavonoids and anthocyanins (Taylor and Briggs, 1990; Jordan, 1996; Fiscus and Booker, 1995; Reuber et al., 1996; Klapper et al., 1996). 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 simple UV-B screens (Ken Markham et al., 1998). There are exceptions to the rule of the involvement of flavonoids in affording plant protection as presence of \( Lc \) in transgenic Petunia did not afford any protection against UV-B, even though increased ratio of quercetin to kaempferol was observed in all the transgenic lines (Ryan et al., 1998).

2.4.2 Flavonoids and their role in male sterility and pollen development

One of the important function of flavonoids is the association of flavonoids with pollen fertility. The involvement of flavonoids in pollination was first uncovered in a maize mutant namely Whp (White pollen) at the duplicate locus, of the \( C2 \) also encoding the chalcone synthase. While \( C2 \) is expressed in seed and other plant parts, whp is expressed in reproductive organs such as anthers, pollen, and stigma. Maize plants deficient in CHS activity produce white sterile pollen in contrast to wild type yellow pollen (Coe et al., 1981). It is a general observation that in maize and Petunia
the fertile pollen contain abundant amounts of flavonols (Styles and Ceska 1977; Coe et al., 1981; Koes et al., 1989; Davies et al., 1993; Pollak et al., 1993; Ylstra et al., 1995)

In maize and Petunia, pollen deficient in chalcone synthase and therefore chalcones and flavones are not functional on similar CHS deficient stigmas, but are normally functional on wild type stigmas and thus exhibit a phenomenon of conditional male fertility, (Coe et al., 1981; Pollak et al., 1993; Ylstra et al., 1995; Mo et al., 1992.). Similarly, Petunia transgenics for Chs exhibiting co-suppression or anti-sense suppression are also male sterile (Taylor and Jorgenssen 1992; van der meer et al., 1992). The fertility function in such plants is chemically restored by spraying the infertile pollen with low concentrations of a flavonol, the kaempferol. However, the requirement of flavonoids for pollen fertility/sterility is not universal. Several flavonoid mutants of Arabidopsis, including a null mutation with impaired CHS enzyme and protein did not affect male fertility (Burbulis et al., 1996; Ylstra et al., 1995; Shirley et al., 1995). Also, a transposon induced mutation in Antihirinnum did not effect fertility (Sommer and Saedler 1986).

2.4.3 Flavonoids and their role in plant defense

Flavonoids have a key role in stress response mechanisms in plants. There is an impressive body of information on the anti-bacterial, anti-fungal and anti-viral properties of flavonoids indicating the role of these compounds in defense response (Bloor, 1995; Kramer et al., 1984; Martin 1995; Beretz et al., 1978; Musci and Pragai 1985; Weidborner and Jha 1993; French and Towers 1992; Kodama et al 1991). While anthocyanins by themselves are toxic in some plants, the aglycone moieties or other intermediates of the pathway are toxic in others. The accumulation of stress induced prenylated isoflavonoids, isoflavans, furanocoumarins, 3-deoxyanthocyanidins and flavonols was described in detail (Treutter and Feucht, 1990). Deoxyanthocyanidins from Sorghum have been shown to be toxic to the pathogen Colletotrichum graminicola (Snyder and Nicholson 1990; Tenkuano et al 1993). Sakuranetin, a methylether derivative of naringenin was also implicated in the resistance of rice plants against blast infection (Kodama et al., 1992; Dillon et al., 1997). Maysin, a C-glycosylflavone was demonstrated to confer resistance of maize plants to corn earworm Helicoverpa zea.
Polymerised anthocyanins - proanthocyanidins are also reported to exhibit anti-microbial activity (reviewed in Scalbert 1991). Further, anti-fungal properties of flavonoids have been demonstrated in vitro against many fungal pathogens. Antibacterial nature of naringenin was demonstrated in vitro against a major rice pathogen, Xanthomonas oryzae (Padmavathi 1997). The role of iso-flavonoids, as a class of phytoalexins (low molecular weight anti-microbial compounds) produced due to attempted infections, is shown in Leguminaceae members including Medicago sativa (Dalkin et al, 1990), soybean (Zacharius et al, 1989) and bean (Gnanamanickyam 1977). The rice phytoalexins oryzaalexins and momilactones are shown to inhibit in vitro the growth of the fungal pathogen Magnaporthe griseae (Cartwright et al., 1981).

2.5 Transgenic approaches to modify secondary metabolic pathways in plants

Metabolic pathway-engineering is aimed at enhancing the production of target compounds in a multi-step biosynthetic pathway. In order to use such a powerful tool, knowledge about the genetic and molecular basis of the entire pathway is absolutely essential. Secondary metabolic pathways in plants are receiving increasing attention as their study offers innovations in agriculture and horticulture besides being important in chemical and pharmaceutical industries. In this context, one of the most thoroughly analysed secondary metabolic pathway in plants is the anthocyanin biosynthetic pathway. Recent evidences indicate that pathway engineering is successfully applied in several plant species to alter the genetic makeup and produce novel flavonoids by introducing the missing gene/function or suppressing the activity of the endogenous gene. Such examples of flavonoid pathway engineering are discussed in the following paragraphs.

2.5.1. Flavonoid pathway and its role in biotic stress

The best example of metabolic pathway engineering is the introduction of grapevine stilbene synthase (Sts) gene into tobacco which produced resveratrol in transgenics showing an increased resistance to the bacterial pathogen Botrytis cinerea (Hain et al, 1993). Further, tomato and potato sts transgenics are also reported to
exhibit an improved resistance against the fungal pathogen, *Phytophthora infestans* (Thomzik 1995; Stahl et al., 1994). In addition, preliminary results from the analysis of the *sts-nce* transgenics also showed an improved resistance against *P. oryzae* (Lorenzen et al., 1997).

In addition, the strategy of altering the metabolic flux by sense suppression is demonstrated in transgenic tobacco plants exhibiting *Pa*! sense suppression, showing a decreased levels of phenylpropanoids with a concomitant increase in the 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. Such transgenics with engineered pathways would prove to be extremely useful in generating crop plants with improved defense.

Our approach in rice is to alter the flow of metabolic flux of the anthocyanin biosynthetic pathway by directing the metabolism through over-expression or anti-sense suppression to regulate the products of interest. In effect, we aim at driving this important secondary metabolic pathway for the desired result- the improved defense response.

2.5.2 Flavonoids and flower color

Structurally diverse flavonoids and anthocyanins contribute to vivid floral colors observed in nature. Often, compounds with defined structures impart the expected color to the organ in which they are localized. For instance, 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. In addition, such differences in the floral color depends on 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 most importantly, the extent of co-occurrence of other flavonoids.

Flavonoid pathway genes are extensively used to modify floral color of several plant species. For instance, *Petunia* line RL101 transgenic line carrying the maize *Al*
gene results into a new bright red floral phenotype. This is due to the fact that RL101 line is deficient in the DFR but has the substrate accumulated in reasonable amounts in the petals upon which the maize enzyme can act (Meyer et al., 1987). Other factors like co-pigments influence the final color of these flowers. Further, different phenotypes, ranging from uniformly red to sectored and variegated phenotypes are produced by the Al transgene in the subsequent generations due to the hypermethylation of the introduced gene (Meyer and Heidmann 1994). Interestingly, Petunia flowers with the maize A\l transgene are paler red while flowers carrying the Gerbera Dfr are bright, although, both sequences encode dihydroflavonol-4-reductase (Elomaa et al., 1996). Thus, the expression of a single anthocyanin gene, namely Dfr, produces a range of flower color phenotypes in heterologous plants.

The Chs gene encoding the chalcone synthase, has been used extensively in the modification flower color in ornamentals. The floral color in such transgenics carrying the active chalcone synthase gene was altered through a number of genetic engineering excersises including antisense inhibition, sense suppression, and over-expression to produce a range of variegated Petunias (Napoli et al., 1990; Jorgensen 1994; 1995) or sectored phenotype (Van der Kro\l et al., 1988). In addition, modification of flower color in Chs transgenics has been demonstrated in several ornamental plants such as Chrysanthemum, Cyclamen, Pelargonium, Lisanthus, and Gerbera (Elomma et al., 1993; Courtney-Gutterson et al., 1994; Elomaa et al., 1996; Davies et al., 1997; 1998).

Other flavonoid genes such as chalcone reductase (CHR) are also used in the genetic engineering of floral 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). Another example of such a diversion of the flavonoid pathway is transgenic lisianthus, carrying the Anntirhinnum FLS transgene (encoding flavone synthase) that exhibits a novel flower phenotype (Elomma et al., 1993).

Many ornamental flowers, including roses, exhibit varying degrees of red
purple shades largely due to the accumulation of the cyanidin and pelargonidin derivatives. On the other hand, blue roses including other blue ornamental flowers are not normally found in nature. Production of blue roses might be possible by the 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). Blue colored pigments in Petunia have been generated with trade names Surfinia™ and Blue Moon™ which are in the process of commercialization. In fact, with the availability of rapidly developing transformation methodologies for rose, the commercial production of blue roses and other such novel flowers would soon be in the market place.

2.5.3 Anthocyanin genes as reporters in transformation

Genes encoding the structural or regulatory protein/enzymes of anthocyanin pathway have been used as reporters in transformation experiments in many cell types of maize and a few other plants owing to the visible, benign, dispensable and cell autonomous pigmentation property. Use of the anthocyanin genes as novel visible markers in transformation in maize aleurone was first shown with Lc (Leaf color) a member of R gene family (Ludwig et al, 1990). Lc, along with Cl from maize under the control of 35S promoter activates the anthocyanin pathway in heterologous plants such as Arabidopsis and Nicotiana (Lloyd et al, 1992). The R and Cl genes have been extensively used as reporters in maize transformation experiments as their activity results in the production of purple color (Bowen 1992). The maize Lc activates the anthocyanin pathway in tomato (Goldsbrough et al, 1996) and Petunia (Quattrocchio et al, 1993; Bradley et al, 1998) can complement itg mutation in Arabidopsis (Quattrocchio et al, 1993). Further, the R and B genes were used to generate stable sugarcane transgenic lines where the production of purple color was used as a non-destructible marker (Bower et al, 1996). Ectopic expression of Del (from Antirrhinum) also lead to the increased anthocyanin expression in tomato and tobacco using (Mooney et al, 1995). In addition, the ectopic expression of C1/R or P producing anthocyanins or 3-deoxy flavonoids in Black Mexican Sweet corn cell lines producing anthocyanins (Grotewald et al., 1998).
2.6. Rice as a choice plant for genetic engineering

Rice constitutes the staple food for more than 50% of the world's population. Owing to its importance as the primary food source, rice breeding is an important farming enterprise of the world particularly in Asia. Conventional rice breeding strategies have led to the development of many rice cultivars with high yield and other important agronomic traits. However, traditional breeding methods are fraught with several limitations, such as, sexual incompatibility between species and long drawn genetic processes. Advent of genetic engineering and biotechnology provided immense opportunities and scope to overcome these limitations.

Rice plant is emerging as the favorite target for molecular biologists and genetic engineers owing to its smallest cereal genome (0.4x10 bases) with 1/10th of size compared to the human genome, and is thrice as complex as Arabidopsis genome (Shimamoto, 1995, Terada and Shimamoto 1993). In addition, recent advances in rice molecular biology, such as development of highly saturated molecular maps, extensive analysis of expressed sequence tags, cDNA sequences and numerous regulatory genes, coupled with the availability of well established transformation protocols makes it as an ideal cereal for experimental research. Also, rice is endowed with rich germplasm containing considerable genetic variation that can be used in genetic engineering exercises. Further, identification and isolation of several genes coding for resistance to diseases, pests, and abiotic stresses across many crop plants provided ample scope to transfer these traits into rice by genetic transformation. Among the cereals, rice occupies a pivotal position with respect to the application of modern biology tools for improving plant productivity and production.

2.6.1 Genetic transformation of Rice

There is an extensive work in the development of efficient, reliable and reproducible transformation system since the first report of plant regeneration from rice protoplast for Japonica by Fujimura (1985) and in Indica rices by Lee et al., (1989). Significant progress has been made on gene transfer of monocots using direct DNA delivery-methods such as electroporation and PEG mediated transformation of protoplasts (Potrykus, 1991). Several transgenic plants were obtained by the direct DNA uptake of
protoplasts in rice resulting in the successful transformation of *Japonica* rice lines (Toriyama et al., 1988, Shimamoto et al., 1989). In the meantime, several groups regenerated *Indica* and *Indica-type* varieties and thus developed transformation protocols for *Indica* rices (Christou et al., 1991, Datta et al., 1992; Ghosh Biswas et al., 1994a, Peng et al., 1992, Xu and Li 1994, Peng et al., 1992). There is a varying degrees of success among these labs with the transformation system being labour intensive and also having poor regeneration and/or low fertility mainly arising due to long tissue culture period. Ghosh Biswas *et al.,* (1994b) attempted to reduce the long tissue culture period by using the scutellar callus as the source of protoplasts. Paul Christou's group, have developed reasonably reproducible and efficient transformation system using immature embryos.

The advent of the microprojectile bombardment offered a much easier way of transformation of regenerable tissues (Sanford *et al.,* 1987). Thus, using microprojectile bombardment a number of *Japonica* and *Indica* rice cultivars have been successfully transformed (Christou *et al.,* 1991, Cao *et al.,* 1990, Li *et al.,* 1993, Sivamani *et al.,* 1996). Although, the *Indica* rices turned out to be relatively recalcitrant, alternative transformation methods such as *Agrobacterium*-mediated gene transfer (Chan *et al.,* 1993, Hiei *et al.,* 1994, Dong *et al.,* 1996) using embryogenic callus have also been developed.

Rice *in vitro* culture has strong genotype and culture dependence (Christou 1993). Sivamani *et al.,* (1996) have reported the genotype independent production of transgenic rice plants using the biolistic method, while Zhang *et al.,* (1996) have reported a genotype and environment independent transformation system for *Indica* rice using embryogenic suspensions. With the development of genotype independent, straight forward regeneration and transformation techniques, it is now possible to introduce genes of interest into rice- a first step for the development of rice via genetic engineering. In the recent past *Tp309* has extensively used as a transformation workhorse to genetically engineer the p-carotene biosynthesis in rice endosperm. Burkhardt (1996) demonstrated the genetic engineering of rice towards *β-carotene* by transforming *Tp309* with phytoene-synthase and phytoene-desaturase gene constructs.
Further, the biochemical investigations upto R1 generation of these transgenic plants revealed the expected enzyme products. Kumpatla et al. (1997), demonstrated the phenomenon of chimerism, gene silencing monocots using Tp309 as a model plant.