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Flavonoid Biosynthetic Pathway: Genetics and Biochemistry

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Plants are sessile organisms which are capable of producing a large array of metabolites, required for their adaption and survival. Flavonoids are low molecular weight metabolites with C6–C3–C6 carbon backbones and are categorised into different classes on the basis of structural organization and polymerization. The biosynthesis and distribution of flavonoids depends on the development stage of the plant as well as on diverse environmental conditions. They play a significant role as pigments, phytoalexins, attractants of pollinators and promotes auxin transport. In plants, antioxidant and antimicrobial activities are attributed to interaction of flavonoids with various enzymes, transcription factor and signalling pathways. This review aims to provide the current understanding of structure, their types, biosynthesis and regulation of flavonoid pathway that provide the insights to the key regulating factors and their interactions which makes them the most promising and interesting targets for plant breeding programs to enhance the value-added products in plants. In this review the deep knowledge of flavonoid regulation by micro-RNAs has been provided that attracts the biotechnologists to develop new molecular approaches so as to engineer various plant metabolic pathways to enhance the health-promoting metabolites in plants for human consumption.

Keywords: Flavonoids; Gene Expression; miRNAs; Transcription Factors.

Plants and their extracts have been used in traditional medicines for the treatment of various ailments as they have less or no side effects. Plants being sessile produce structurally diverse secondary metabolites so as to adapt themselves to various environmental conditions. Most of these metabolites are the natural end products of the primary metabolism[1]. They include coumarins, phenolics, tannins, lignins, isoflavonoids and flavonoids. Out of several metabolites, flavonoids are predominant and ubiquitously present in the plant kingdom[2]. These are the low molecular weight polyphenolic compounds that are not involved in the growth[3]. The unique physio-chemical properties of flavonoids enable their interaction with different targets, thus influencing various biological functions in plants[4,5]. Apart, they play a key role in imparting flower colour to various plant tissues.Even, Mendel used flower pigmentation as one of the major characters for the elucidation of inheritance of traits in genetics. Similarly, McClintock observed and reported the transposable elements that modulate the transcription of flavonoid genes, thus leading to discovery of gene silencing mechanism in maize kernels[6].

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Flavonoids are grouped into various families based on their structure, degree of polymerization and hydroxylation[7]. These include chalcones, flavonols, flavanones, flavones, isoflavones and anthocyanins[8] (Figure1). These compounds are synthesised by the flavonoid branch of the large phenylpropanoid pathway that leads to the formation of various phenylpropanoids including lignins, flavonoids, stilbenes, phenolics and isoflavonoids9. These compounds are formed by sequential elongation and cyclization of phenylalanine which is a product of shikimate pathway[10,11]. Also, several downstream enzymes such as chalcone isomerase (CHS), flavanone 3-hydroxylase (CHI), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose: flavonoid glucosyltransferase (UFGT) results in the formation of different classes of flavonoids (Figure 2). These flavonoids play specific functional roles in plants. Since the development of colour in plants is the result of co-pigmentation processwhich is due to interactions among colourless flavonoids (flavanone and flavonols) with coloured compounds (anthocyanins) but can beincreased by inducing substrate competition between flavonoid genes[12]. The biosynthesis of flavonoid compounds is controlled by various regulatory proteins that effect their activities and expression. Hence, the biosynthesis of flavonoids is studied in many plants in order tounderstand their role in plant physiology[13].

Various flavonoid biosynthetic enzymes function as multi- enzyme complex that are attached to the endoplasmic reticulum[14]. In contrast, the accumulation of different flavonoid pigments occurs in either cell wall or vacuole. It was observed that anthocyanins accumulate mostly in the vacuoles which requires a transporter (multidrug resistance associated protein; MRP type)[15]. The transcription of transporter is co-regulated with the expression of flavonoid structural genes, thus influencing the accumulation of anthocyanins[16]. In additions, accumulation and transport of flavonoids is also influenced by other factors such as light and circadian clock[17].

Flavonoids have key role in plant physiology where it plays major role as pollinator attractors and dispensers, protection against UV radiations, prevent pathogen attack[18] and promotes auxin transport[19]. Every plant has diverse flavonoids profile that makes them unique among species[20]. This involves biosynthesis of various types of anthocyanins which provide unique flavour and colour to each plant part. They also have nutraceuticals properties that contribute towards human health. Flavonoids compounds have antioxidants as well as pharmacological properties that are responsible for various biological activities in plants[21].

Since the flavonoid biosynthesis in plants is the crucial pathway that draws much scientific attention due to contribution to visual appeal to various plant parts and their nutritive value. The complicated metabolic network needs to be deciphered for better understanding the nature of interaction and its role in biosynthesis of different flavonoids. This review covers the major integrated findings related to flavonoid biosynthetic pathway that was studied in last decades. The numerous scientific articles already published in NCBI in last two decades were thoroughly studied and analysed to elucidate the role of key flavonoid genes in plant metabolism. The published reports provide core understanding of the structure-activity relationship of flavonoids and their complex regulation at transcriptional and post-transcriptional level.

Classification and chemistry of flavonoids

Flavonoids are the polyphenolic metabolites that have a benzo-?-pyrone structure. These are 15-carbon compounds which are derived from a C6-C3-C6 backbone. They have two benzene rings (designated as A and B rings) which are linked by a heterocyclic pyrane ring (designated as C ring) except in chalcones where A and B rings are linearly linked by three carbon chain[22]. Flavonoids are planar in structure but hydroxylation, methoxylation and glycosylation of hydroxyl group leads to their modification. The degree of hydroxylation at special sites of C6-C3-C6 backbone is favourable for many biological activities of flavonoids[23]. Flavonoids are classified into different categories depending upon oxidation and degree of unsaturation of the heterocyclic C-ring. These include chalcones, flavones, flavonols, flavanones, dihydroflavonols and anthocyanins.

Chalcones

These are the open chain flavonoids which are common in edible plants. They have A and B aromatic rings that are joined by carbon chain of a, ß-unsaturated carbonyl systems which are linear

in shape. The formation of chalcones occurs by the condensation of phenylalanine and p-coumaryl CoA[24]. There are two types of chalcones which are classified on the basis of the presence or absence of OH group at 6th position. The first class is hydroxychalcones (naringenin chalcones) which acts as precursors for the synthesis of downstream classes of flavonoids. The second class is 6'-deoxychalcones which lacks hydroxyl group at 6th position and leads to the formation of 5-deoxy flavonoids and are less abundant in plants25. Production of hydroxychalcones is due to the expression of CHS gene while 6'-deoxychalcones requires expression of both chalcone synthase and chalcone reductase[26]. The natural occurring chalcones are present in monomeric form which differs from each other in the substitution pattern. The different substituents present are hydroxyl groups, methoxy group, methyl or prenyl groups.

Flavanones and dihydroflavonols

These are formed by the condensation of six membered rings with either a-pyrone or its dihydro-derivatives. Presence of hydroxyl group on 3rd position and double bond on the C-ring differentiates flavonolsfrom flavanones that results in non-planer skeleton of flavanone. Flavanones are formed by the stereospecific cyclization of chalcones that are catalysed by chalcone isomerase (CHI) enzyme which results in the production of 2S-flavanones,which is common substrate in the biosynthesis of various flavonoids. Aglycones and glycosides are the two naturally occurring flavanones present in plants. Dihydroflavonols are the flavanones with a hydroxyl group present at 3rd position. It is a crucial intermediate which bifurcate the flavonoid biosynthetic pathway into two routes. One route lead to the formation of flavonols which is catalysed by leucoanthocyanidin reductase (LAR) and another route results in anthocyanin production catalysed by UFGT gene[12]. Naringenin is a colourless flavanone which is reduced to dihydroflavonol by the action of DFR enzyme. It catalyses the formation of dihydroflavanol by saturating the C3-C4 double bond[27].

Flavonols

These are the flavonoid compounds that are formed by the attachment of hydroxyl group at 3rd position of flavones[28]. There are two classes of naturally occurring flavonols namely, aglycone and glycosides. There are about 450 types of aglycones and 900 kinds of glycosides. Most of the flavonols exist as O-glycosides which may differ due to the type of sugar moiety attached to them. Flavonols are formed by the oxidation of dihydroflavonols and action of flavonols synthase (FLS) enzyme. The FLS enzyme competes with DFR enzyme for the substrate (2R, 3R-dihydroflavols) for the formation of flavonols[29].

Anthocyanins

These are the abundant class of flavonoids responsible for imparting various colours to different plant parts. More than 600 anthocyanins have been identified and reported in various plants[30]. Apart from colour development, they act as regulators at different development stages, aids in pollination and UV protectants to the plants. The basis of core structure of anthocyanin is 7-hydroxyflavyllium ion. These are 15 carbon compounds (C6-C3-C6) with one fused aromatic ring A, second ring B at position 2 and benzopyran ring C[31]. The various sugar molecules attached to 3-OH group are present in ring C. Numerous coloured anthocyanins pigments, ranging from yellow to purple colour are due to different glycosylation pattern. The production of anthocyanins is pH dependent and requires metal co-factors for their accumulation in various tissues. Different anthocyanins responsible for colour development in plants are pelargonidin, cyanidin, delphidin and malvidin[32].

Flavonoids in plants are mainly in glycosidic form which is mediated by the action of glycosyl transferase. Glycosylation of flavonoids tends to increase their solubility and stability[33]. Some common glycosylation sites in different flavonoids are: 7-OH in flavones, 3-OH in flavonolsand 5-OH in anthocyanins. Depending upon the attachment of the sugar moiety, flavonoid glycosides are of two types: O-glycosides and C-glycosides. When sugar moiety is attached to the OH group of flavonoid skeleton, it leads to O-glycosides biosynthesis. In contrast, C-glycosides are formed by the attachment of sugar group to the flavonoid skeleton by the C-C linkage[34]. Similarly, attachment of rhamnose sugar on the 2-OH group of naringenin results in the bitterness of grapes[35]. Flavonoid glycosides act as phytoalexins and are involved in anthocyanin and proanthocyanidins formation[36].

Flavonoid biosynthetic pathway

The flavonoid biosynthetic pathway helps in understanding the chemo-diversity and

their potential role in plant development.The first report on isolation of flavonoid biosynthetic genes was from Arabidopsis thaliana[37]. Flavonoids are produced by phenylpropanoid pathway which in turn is tightly linked to shikimate acid pathway that forms an important aromatic amino acid, phenylalanine. Flavonoid biosynthesis is controlled and regulated by various structural genes and regulatory genes[38].

Flavonoid biosynthetic enzymes function as multi- enzyme complex that are attached to the endoplasmic reticulum, while the accumulation of different flavonoid pigments occurs in either cell wall or in vacuole. Anthocyanins are accumulated mostly in the vacuole as they require a transporter, multidrug resistance associated protein. The transcription of transporter is co-regulated with the structural genes expression thatcontrols theanthocyanin accumulation[39]. Some other factors like light and circadian clock also influences the accumulation and transport of flavonoids[40]. Several transcription factors modulate thestructural gene transcription, thus regulating the flavonoids accumulation in various tissues. These regulatory enzymes are MYB, bHLH and WD40 transcription factors that mediate their functions by forming a ternary MYB-bHLH-WD40 (MBW) complex[41]. In addition, some non-coded RNAs (miRNAs) also post-transcriptionally regulate flavonoid biosynthesis.

Chalcone synthase

Chalcone synthase (CHS) belongs totype III polyketide synthase family that catalyse the first crucialreaction in flavonoid biosynthetic pathway. This enzyme functions as a symmetric dimer (where each monomer is ? 42KDa polypeptide) and contains two independent active sites. The structure and catalytic machinery of this enzyme first was revealed from the X-ray crystal structure and the functional studies were performed on Medicagosativa CHS protein[42]. CHS enzyme has conserved cysteine residue that play the role of nucleophile bymediating the movement of intermediates through CoA molecules. Each CHS monomer have upper and lower structural domains. The upper domains contain pseudo-symmetric motif, which is also present in fatty acid ß-ketoacyl synthase[43]. The structural differences between upper and lower domains creates a larger active site that is buried in the interior cavity and provides the space for the intermediates that are involved in chalcone synthesis44,45. The CHS homodimer contains two active sites with each active site containing the residues from a single monomer except Met 137 which is derived from adjoining monomer. Each active site contains Cys 164, Phe 215, His 303 and Asn 336 residues which are responsible for enzymatic function of CHS46. Cysteine 164 play the role of nucleophile by shutting polyketide intermediate while His 303 functions as a general base catalyst resulting in formation of a nucleophilic thiolate anion. Phe 215 and Asn 336 function during decarboxylation reactions thus providing the Van-der Waals interactions favouring the release of CO2 molecules[47].

CHS enzyme catalyses the three-step reaction, which involves p-coumaroyl CoA and malonyl CoA condensation for the production of naringenin chalcones (4,2',4',6'–tetrahydroxy chalcone). The first step involves the loading of p-coumaroyl moiety from the CoA to the Cys16448. The second step includes a decarboxylation reactionwhere malonyl moiety (obtained from the malonyl CoA) binds with the carbonyl group of coumaroyl thioester. This creates a non-polar environment and facilitates the removal of CO2. The final step involves an intramolecular Claisen condensation wherethree acetate units condense with coumaroyl moiety. This results in the formation of tetraketide intermediate that further undergoes subsequent breakdown and aromatization to yield chalcones[49]

Chalcones are the important secondary metabolites that along with anthocyanin biosynthesis are also involved in biosynthesis of antimicrobial phytoalexins and flavonoid inducers. These provide plant defence during microbial attack. CHS promoter sequence was first studied in petunia that helped in understanding gene expression pattern in different tissues[50]. Later, CHS sequences were characterized in several plants like raspberry[51], Arabidopsis thaliana[45], crabapple[52] and mulberry[53]. The expression of CHS gene has revealed its pivotal role inflavonoid formation during plant development[54-57].

Chalcone isomerase

CHI catalyse the intramolecular cyclization of the chalcones into flavanones58. Presence of 2'OH in chalcone is the perquisite requirement for intramolecular cyclization which is

mediated by CHI enzyme. The reaction mechanism begins with the formation of 2'-oxyanion that resulted due to the loss of proton from the 2'OH group of chalcones. The newly formed 2'-oxyanion attacks a, ß-unsaturated double bond of the substrate resulting in the formation of 2'acidic OH group[59]. Mechanistic studies revealed that the deprotonation of the chalcone 2'- hydroxyl group is pH dependent and the formation of flavanone is a diffusion limited reaction. Presence of van-der wall forces and extensive H-bonding in the active sites mediates the formation of flavanones.

CHI was first isolated from pea60and is grouped in two types namely, Type I CHI and Type II CHI61. Type I CHI catalyses the conversion of hydroxychalcone into hydroxyflavanone and is ubiquitously present in plant kingdom. Type II CHI catalyses the conversion of both 6'-hydroxychalcone and 6'-deoxychalcone into 5-hydroxyflavanone and 5-deoxyflavanone respectively. CHI is mostly present in leguminous plants. The size of CHI enzyme ranges from 35-465 amino acid residues having molecular weight 23-26 KD.

CHI has been cloned using conserved primers in order to understand its expression profile in different tissues during fruit development. In Arabidopsis, CHI enzyme plays a role of enhancer in flavonoid biosynthetic pathways thus improving the accumulation of flavonoids[62], while in Chinese water-chestnut it functions as a promoter that controls its ripening, thus playing an important role in development of this fruit[63].

Flavanone 3-hydroxylase

F3H belonging to dioxygenase family catalyse bifurcation of flavonoid pathway into two branches, anthocyanin and flavonols branch[64]. It catalyses the stereospecific hydroxylation of flavanones to form different types of dihydroflavonols[65]. Amino acid analysis revealed that the conserved amino acid motifs (His 233, Asp 235, His 289, Arg 299 and Ser 301) binds to the Fe11 ions and 2-oxogluarate thatmediate the redox reaction. F3H belongs to 2-oxoglutarate dependent dioxygenase family, as revealed by three dimensional studies and is based on its absolute requirement of 2-oxoglutarate, molecular O2, ferrous ion and ascorbate for the complete activity of the enzyme[66].

Gene expression of F3H has been studied in several plants. In-vitro enzyme assay studies indicated that F3H is effective in catalysing the conversion of naringenin into dihydro kaempferol, thus proved the participation of F3H in the flavonol biosynthesis pathway. Also, the subcellular localization studies confirmed the presence of F3H in the nucleus and cytosol as these sites leads to stabilization of distorted core of 2-ODD and protein-protein interactions[67]. F3H being a non-heme iron protein, has a significant role in the post translational processing of collagen, plant hormone biosynthesis (gibberlins) and production of ß- lactam antibiotics[68,69].

Dihydroflavonol-4-reductase

DFR is an oxidoreductase enzyme that stereo-specific reduce thedihydroflavonols into leucoanthocyanidins (Flavan-3,4-diol) which serve as the substrates for the formation of anthocyanidin and proanthocyanidins11. DFR results in the production of colourless, unstable leucoanthocyanidin (leucopelargonidin, leucocyanidin and leucomyricetin). DFR also affects the biosynthesis of other flavonoid compounds such as flavonols and proanthocyanidins. This enzyme controls the carbon flux during flavonoid biosynthesis.

The activity of DFR was first studied and reported in maize plant[70]. DFR displays a substrate specificity that leads to the production of three different derivatives of anthocyanin pigments such as delphinidin, cyanidin and pelargonidin. The structural information of DFR gene was confirmed by predicting the crystal structure on the basis of its sequence which confirmed that DFR belongs to the short chain dehydrogenase and reductase superfamily that includes YXXXK motif, a C- terminal domain having 4a helices and N-terminal glycine rich motifs that adopts rosamann fold[71]. This rosamann fold spans the cleft that acts as coenzyme binding site. DFR display different degree of hydroxylationwhich is due to the substrate specificities provided by specific amino acids[72]. Amino acid Asp134 make DFR enzyme to accept dihydrokaempferol as a substrate whereas presence of aspartic acid showed increased preference for dihydroquercetin[73].

UDP glucose: flavonoid 3-O- glucosyltransferase

In plants, glycosylation plays an important role during the biosynthesis of secondary metabolites. UDP glycosyltransferase (UGT)

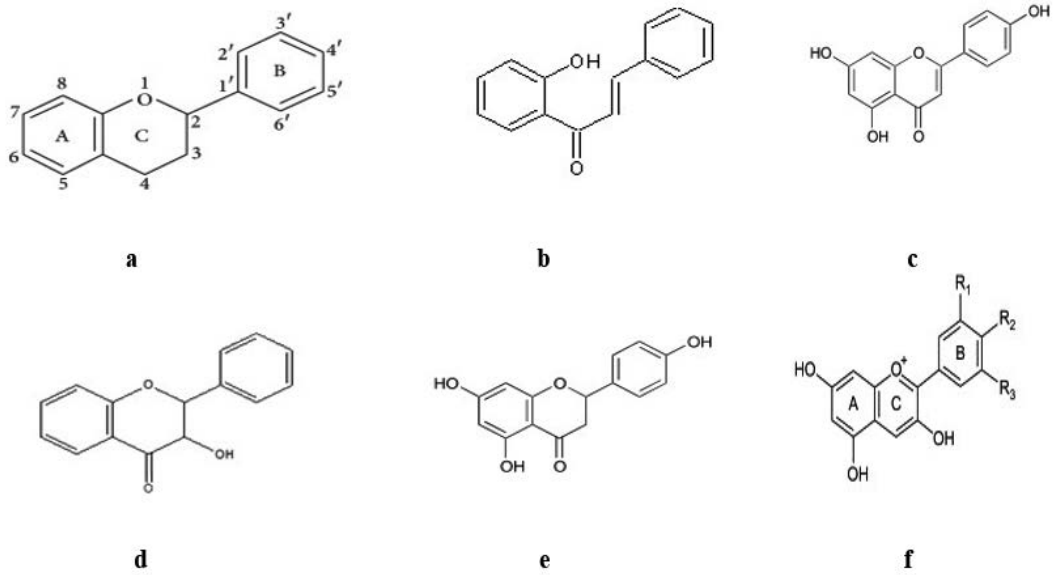

Fig. 1. Structure of flavonoids. (a) Basic structure of flavonoid having C6-C3-C6 backbone (b) chalcones (c) flavanone (d) dihydroflavonols (e) flavonols (f) anthocyanins

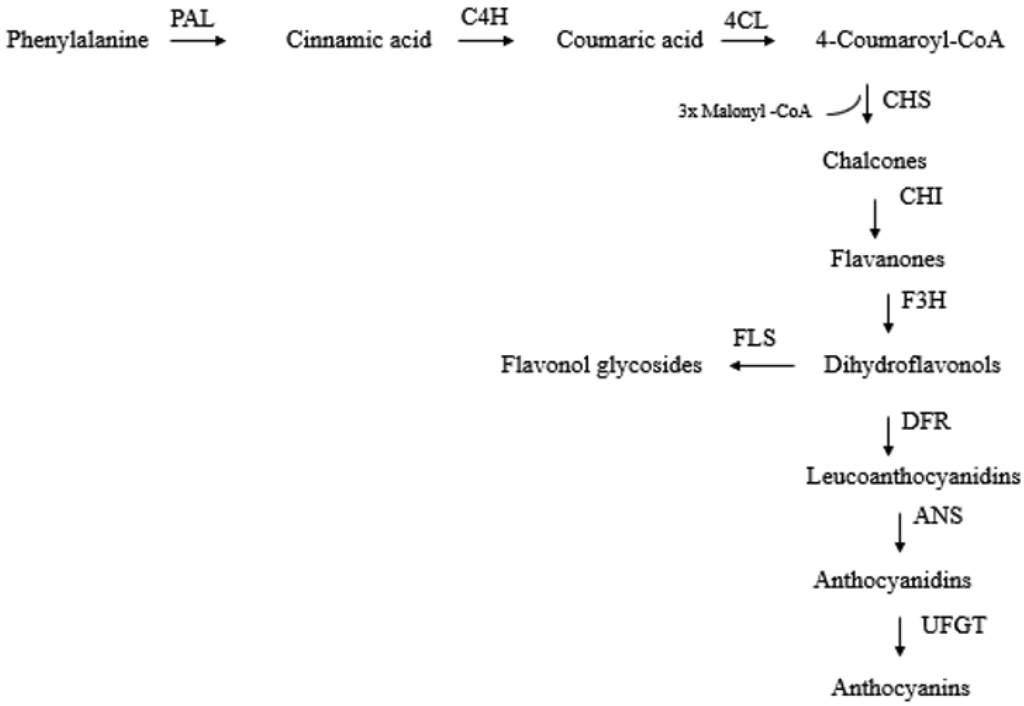

Fig. 2. A simplified representation of flavonoid biosynthetic pathway. Enzyme abbreviations: PAL- phenylalanine ammonia-lyase; C4H- cinnamic acid 4-hydroxylase; 4CL- p-coumarate:CoA ligase; CHS- chalcone synthase; CHI- chalcone isomerase; F3H-flavanone 3-hydroxylase; FLS- flavonol synthase;DFR- dihydroflavonol 4-reductase; ANS- anthocyanidin synthase; UFGT- UDP-glucose-flavonoid 3-O-glucosyltransferase

catalyse the glycosylation of various compounds which determine their availability and activity in plants[27]. Crystal structure analysis predicted that this enzyme belongs to the family 1- glycosyltransferase superfamily, on the basis of presence of GT-B fold which is a major characteristic of this superfamily[74]. It has two N and C terminal domains with a central ß sheet and a helices on its sides. The two domains form a cleft that acts as a binding site for substrate. The active site has highly conserved histidine and aspartate residues which interact with the acceptor thus resulting in the formation of an acceptor-His-Asp complex,that forms ß- glucosidic linkage product[75]. Flavonoids are mostly in glycosylated forms withdifferent sugar donor specificities. Glycosylation of flavonoid quercetin results in different sugar moiety at different positions thus forming 300 different quercetin glycosides, each with different bioactivity[76].

The main form of UGT found in plants is UDP-glucose: flavonoid 3-O-glycosyltransferase (UFGT) which has a major role in anthocyanin formation. UFGT is a decisive enzyme which attach sugar molecules to the C3- OH group of anthocyanidin in a direct displacement SN2- like mechanism that leads to formation of coloured pigments12. Colour diversity of anthocyanin depends on degree of hydroxylation on B ring. The important anthocyanins are cyanidin-3-glucoside, cyanidin-3-O rutinoside and cyanidin-3-O galactoside. UFGT geneis important structural gene whose up-regulation is closely associated with the accumulation of anthocyanin in various plant parts.

UFGTdisplay human health promoting benefits such as antioxidant and anticancerous activities[77]. MYB gene being a regulatory gene binds to the promoter region of UFGT gene and regulates its activity[78]. The expression of UFGT geneis also up-regulated by various plant hormones and sugars[79]. Plant hormones such as abscisic acid and ethylene increases the transcript levels of UFGT gene, resulting in colour variegation[80].

Flavonoid biosynthesis regulation by MBW complexes

The flavonoid biosynthesis involves early biosynthetic genes and late biosynthetic genes on the basis of structural gene expression and their regulation by various transcription factors. These structural genes are co-ordinately regulated by a MBW complex which consist of three transcription factors namely, basic helix loop helix, MYB transcription factors and WD40 proteins81. These three transcription factors are highly conserved among plants species and well-studied in various model and non-model plants[82]. MBW complex predominately regulates the late biosynthetic genes, but the degree of regulation differs from species to species and even within tissues[83].

The largest family of transcription factors are Myeloblastosis transcription factors (MYB TFs) which are present in all eukaryotes. MYB TFs

Table 1. miRNAs and their target proteins involved inflavonoid biosynthesis

miRNA family	Target protein(s)	Functions	References
miR828	MYB113, C1	Regulates anthocyanin biosynthesis	107
miR5538, miR477b	DFR	Regulates activity of flavanone 4-reductase, flower development	113
miR858	MYB	Regulates MYB-bHLH-WD40 complex	114
miR156, miR157, miR159	SPL	Fruit development and ripening, anthocyanin accumulation	111, 115
miR171	ANS	Anthocyanidin biosynthesis	116
miR535, miR396b,miR166i	FNS	FNS	116
miR396, miR845, miR829	CHS	Regulates the formation of naringenin	116
miR21miR167	CHIF3H	Flavonoid biosynthesisAnthocyanin accumulation	117
miR8181	SPL	Flavonoids biosynthesis	118
miR14, miR22, miR31	MYB33,WD 40	Fruit development, Anthocyanin formation, hormone signalling	119

regulates flavonoid biosynthesis at transcriptional level. These MYB TFs were first characterized from Avainmyeloblastosis virus that have oncogene v-myb and its cellular homologs c-myb[84,85]. In plants, Colorless1 (C1) was first MYB TF that was identified in maizewhich regulate anthocyanin pigments formation[86,87]. MYB TFs has a DNA binding MYB domain that acts as transcriptional regulatorin flavonoid biosynthetic pathway. The specificity of MYB domain is due to presence of four imperfect tandem repeats (R) which classify MYB TF into four classes namely 1R-MYB, R2R3-MYB, 3R-MYB and 4R-MYB88. In plants, only R2R3- MYB family is predominant among all the four classes[89]. R2R3- MYB TFs have been extensively characterized in Arabidopsisfrom where 126 R2R3-MYB TFs have been reported but only few of them act as regulators for flavonoid biosynthesis[90]. In addition to Arabidopsis, petunia and snapdragon are used for studying flavonoid biosynthesis in plants.

In petunia, bHLH and MYB genes are encoded by Anthocyanin 1 (AN1) and Anthocyanin 2 (AN2) / AN4 respectively that are involved in the biosynthesis of anthocyanins in the floral tissues[91]. Another R2R2-MYB factor, DEEP PURPLE and PURPLE HAZE also regulates the pigmentation of floral and vegetative tissues. In Antirrhinum, three MYB genes known as Rosea1, Rosea2 and Venosa regulate the flavonoid biosynthetic pathway[92]. While in apple, three MYB genes (MdMYB1, MdMYB10 and MDMYBA) are co-expressed with bHLH factors to regulate the flavonoid biosynthesis in peel and flesh[93]. MYB proteins play diverse role in regulation of plant metabolism, stress responses and regulation of cell cycle[94].

In addition to MYB TFs, two other transcriptional factors, bHLH and WDR TFs lead to combinatorial regulation of flavonoid biosynthesis in plants. The first bHLH gene was identified in maize which was encoded by the Red 1 locus and is responsible for anthocyanin formation[95]. Several bHLH genes alongwith R2R3-MYB TFs coordinatethe transcription of flavonoid biosynthetic genes. The major characteristics of bHLH proteins are DNA binding domain, called bHLH domain that binds to specific DNA sequence. It has N terminal end consisting of 18 hydrophilic and 6 basic amino acid residues. In Arabidopsis, 162 bHLH TFs have been reported which are classified into 12 major groups and various subgroups[96]. In addition, bHLH proteins also regulate plant growth and development, cell patterning and regulation of various hormones signalling pathways[97].

WD repeat motif is the third transcription factor of MBW complex which is also known as WD40 as it contains 40 amino acid structural repeats of tryptophan-aspartic acid at its end. WD40 motif was first identified in G proteins that provide a platform for protein-protein interactions[98]. WD40 TFs have been reported in plants likeArabidopsis (TTG1), maize (PAC1), apple (MdTTG1) and grapes (VvWDR1/2).

Post-transcriptional regulation based on Micro-RNAs

In the recent years, research has been diverted towards understanding the post transcriptional regulation of the flavonoid pathways. There are two classes of endogenous non coding, small regulatory RNAs namely, small interfering RNAs and micro RNAs[99,100]. Among these non-coding RNAs, micro RNAs (miRNAs) are the non codingribo-regulators,[21-24] nucleotides in length that regulates the gene expression in eukaryotes. Several studies have conducted to understand the potential role of small RNAs in various processes of plant growth and development[101,102]. miRNA biogenesis is a step wise process where miRNA gene undergoes transcription and splicing to form pri-miRNA. After loading into RISC complex, it regulates the expression of its target genes post- transcriptionally. Micro-RNA acts as negative regulator by affecting the translation of coding mRNAs that showed perfect or near complementarity[103].

miRNAs regulatevarious metabolic processes, biosynthesis of secondary metabolites, hormone signalling and various stress responses[104]. More than 21 miRNA families reported are highly conserved in angiosperms such as miR156, miR159 and miR160. miR828 was first time reported in Arabidopsis and later, it has been also studied in many plants such as apple and popular. In Arabidopsis, miR828 regulates the anthocyanin accumulation with respect to phosphate deficiency[105]. Since, miR828 sequence is complementary to MYB transcription factor, hence it provides evidence for its key roles in regulating anthocyanin formation[106]. Over-expression of

miR828 also down-regulates the expression of various MYB genes resulting in reduced level of flavonoid accumulation in Arabidopsis. In tomato, the small tandem target mimic constructs were generated that blocked the miR858, leading to increase in MYB7 transcript levels[107]. Similarly, in apple and Arabidopsis, cleavage sites of miR828 and miR858 were observed in R3 domain of MYB transcription factor that target various MYB encoding genes thus suggesting the potential role of miR858 in anthocyanin biosynthesis regulation.

Squamosa promoter binding protein like (SPL) proteins play significant role in various processes of plant development including embryonic development, plant fertility and flowering induction[108]. These SPL9 proteins regulate the flavonoid biosynthesis by binding to the promoter region of regulatory genes[109]. An increase in the transcript levels of SPL gene along with the growing stem of Arabidopsis was due to decline in the transcription of miR156 during plant development[110]. In Arabidopsis, anthocyanin rich tissues contain higher levels of miRNA156 which in turn reduces the SPL9 activity. The reduction of miRNA156 targeting SPL9 activity is inversely correlated with the transcript levels of various flavonoid genes, thus affecting the accumulation of anthocyanins111. Therefore, increased expression of SPL genes results in reduced accumulation of anthocyanins and increased flavonol biosynthesis. Competition of SPL9 with bHLH transcription factor for their binding to PAP1 results in destabilization of the MBW transcriptional complex[112]. This in turn leads to the down-regulation of DFR and UFGT structural genes, thus leading to reduced anthocyanin production. Thus, an antagonistic relationship was observed between anthocyanin and flavonol formation. The expression of different miRNAshas beenquantified using real time polymerase chain reaction (qRT-PCR) in plant (Table 1). Thus, miRNAs are the potential target genesthat help in investigating their roles in plant growth development and other metabolic processes.

Biological functions of flavonoids in plant defence

Responses against UV-B radiation

The expression of flavonoid genes is regulated by UV light120 which is evident from studies on various grass species growing in regions with high levels of solar UV-B rays contains high amount of flavonoids especially flavones (orientin and luteolin) as it provides protection against UV-B radiations[121]. Flavonoids are produced in response to exposure to UV-light and provide defence against UV stress response. This is due to the open planer structure of flavonoids that provides the UV-absorbing characteristics to these compounds[122]. The 3-OH group present in flavonoid skeleton chelates various metal ions, thus inhibiting the formation of free radicals and reactive oxygen species (ROS). UV absorbing properties of flavonoids have been intensively investigated in Arabidopsis, grapes and petunia.

Resistance against biotic and abiotic stresses

Secondary metabolites biosynthesis is also affected by environment which in turn influences the plant defence and pathogen attack[123]. Abiotic factors such as UV light regulates the flavonoid biosynthesis and also influences the response of plant to biotic stress like pathogen attack. In Arabidopsis, the production of flavonoids in response to both biotic (bacterial elicitor flg22) and abiotic stress (UB-B radiation) provides a structural barriers for pathogen spread. This is due to high transcript levels of flavonol synthase that provide resistance to biotic stresses[124]. Similarly in maize plant, the resistance to corn earworm (Helicoverpazea) is associated with high concentration of flavones thus providing protection125. Flavonoid genes are also involved in adaptation to abiotic stresses due to activating signal transduction pathway of various plant hormones. In Arabidopsis, MYB is the first gene reported to be produced during water stress that induces the abscisic acid biosynthesis pathway and results in closure of stomata, thus helping plant to combat water stress[126,127]. In barley, MYB gene was identified to be the first gene that mediates gibberellins signalling pathway, thus controlling plant growth and development[128].

Roles of flavonoids in plant reproduction

Flavonoids imparts colour to the plant parts especially to pollens that aid in pollination and enhances plant reproduction[129]. The role of flavonoids in plant fertility was first studied in maize130 where it was observed that the mutants lacking chalcone synthase have non-functional pollen tube that leads to male sterility as well as flavonoid deficiency[131]. In tomato, parthenocarpy

was induced due to silencing of chalcone synthase gene that resulted in production of seedless tomatoes[132]. Similarly, silencing of FLS gene resulted in production of non-functional pollen tube in tobacco plant and further implication of flavonol reversed the condition, suggesting its role in pollen fertility and its germination[133].

CONCLUSION

Flavonoid metabolites play significant role in controlling the plant development and their interaction with various biotic and abiotic components. Due to the prevalence and beneficial health roles, flavonoid biosynthetic pathway has been intensively studied in many plants. Flavonoid biosynthesis and its accumulation is regulated in spatial and temporal manner and is also controlled by various genetic and development factors. About three decades of research is based on the conserved mechanism of flavonoid regulation that culminates in MBW complex, operating at transcriptional level controlling not only flavonoid pathway but also plant development pathway.

However, the underlying mechanisms that control the flavonoid regulation is still elusive and requires much investigation to dissect out the new perspective to understand the complexities involved in its regulation. The more in-depth study needs to be carried out to decipher the underlying mode of action, mode of intracellular transport, regulation and interaction of these flavonoid compounds in plants. This information would enable metabolic engineering of flavonoid pathway genes that can enhance their content, stability and bioactivity in plants in near future. Further, studies on various approaches such as constitutive over-expression or tissue specific expression aiming the miRNAs of interest and their corresponding target gene could be explored which could improve the response of plants to various biotic and abiotic stresses as well as their overall growth and development.

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Conflict of interest

The authors declare that they have no conflict of interest.

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