Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering

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Abstract: In this review, flavonoids as classical examples of secondary metabolites are employed to highlight recent advances in understanding how valuable compounds can be regulated at various levels. With extensive diversity in their chemistry and pharmacology, understanding the metabolic engineering of flavonoids now allows us to fine-tune the eliciting of their production, accumulation, and extraction from living systems. More specifically, recent advances in the shikimic acid and acetate biosynthetic pathways of flavonoids production from metabolic engineering point of view, from genes expression to multiple principles of regulation, are addressed.

Flavonoid Biosynthetic Pathways in Plants: Versatile Targets for Metabolic Engineering

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Abstract

Plants, fungi, and microorganisms are equipped with biosynthesis machinery for producing thousands of secondary metabolites. These compounds have important functions in nature as a defense against predators or competitors as well as other ecological significances. The full utilization of these compounds for food, medicine, and other purposes requires a thorough understanding of their structures and the distinct biochemical pathways of their production in cellular systems. In this review, flavonoids as classical examples of secondary metabolites are employed to highlight recent advances in understanding how valuable compounds can be regulated at various levels. With extensive diversity in their chemistry and pharmacology, understanding the metabolic engineering of flavonoids now allows us to fine-tune the eliciting of their production, accumulation, and extraction from living systems. More specifically, recent advances in the shikimic acid and acetate biosynthetic pathways of flavonoids production from metabolic engineering point of view, from genes expression to multiple principles of regulation, are addressed. Specific examples of plants and microorganisms as the sources of flavonoids-based compounds with particular emphasis on therapeutic applications are also discussed.

Keywords: Flavonoids; Shikimic acid; Acetate; Metabolic engineering.

1. Introduction

Living organisms, particularly plants, fungi, and microorganisms, can synthesize various compounds known as primary metabolites, which are essential for vital processes, including photosynthesis and energy production/expenditure, as well as processes implicated in carbohydrate, fat, and protein metabolism. In addition to this group of fundamental compounds of life processes, there are a wide variety of molecules called secondary metabolites that include polyketides, terpenoids, phenylpropanoids, alkaloids, etc. They are generally known to enable living organisms to cope with environmental stresses against potential predators or competitors, or probable attacks by parasitic microorganisms, etc. (Demain, 1999; Keller et al., 2005; Makkar et al., 2007; Sanchez and Demain, 2008).

The biosynthesis of secondary metabolites in cellular systems proceeds through highly controlled enzyme-mediated reactions. While the pathways involved in biosynthesis and functions of carbohydrates, fats, proteins, and nucleic acids, or simply primary metabolites, are essentially the same in all living organisms, a great deal of diversity exists for secondary metabolites. Understanding these metabolic processes is critical for the regulation of desired plant phytochemicals biosynthesis and their potential exploitation in the food, medicine and nutraceutical industries (Dewick, 2002).

In plants, there are essentially two distinct biosynthetic pathways for the synthesis of flavonoid-based compounds. They include the shikimic acid pathway generating the phenyl propanoids (C6-C3) skeleton and the acetate pathway serving as a building block for polymeric 2-carbon units (Croteau et al. 2000). An overview of these biosynthesis pathways of flavonoids starting from primary metabolites is shown in Fig. 1.

The regulation of secondary metabolites production can be achieved by understanding the genes implicated in the metabolic pathways, environmental, and physiological processes that induce the activation of the relevant genes, and regulations that occur at various levels of

post-gene expression events. In this article, we present a systematic review of metabolic engineering of the flavonoids, addressing their chemistry, biochemistry, function, therapeutic application, and their regulation.

2. An overview of flavonoids chemistry

Flavonoids are the major group of phenolic compounds, and are by far the most diverse common secondary metabolites in plants. Structurally, they are constructed from a 15-carbon skeleton and composed of two benzene rings (ring A and ring B) connected by 3-carbon linking chain. Hence, they are represented as C6-C3-C6 compounds. In many flavonoids, the linking chain further forms a heterocyclic pyran or pyrone ring (ring C) (Corradini et al., 2011; Frs and Baxter, 1999) (Fig. 2). In the absence of this third ring system (ring C), the flavonoids are called chalcones, and mostly serve as precursors of the various flavonoid classes (see below).

The biosynthesis of flavonoids proceeds through two different pathways: the acetate pathway (ring A) and the shikimate pathway (ring B along with the linking chain (ring C) making the C6-C3 component). Ring A is synthesized from three malonyl-CoA molecules generated *via* the transformations of glucose while ring B is synthesized from 4-coumaroyl-CoA produced from phenylalanine *via* the shikimate pathway. Condensation of rings A and B generates chalcone, which subsequently undergoes isomerase-catalyzed cyclization to form flavanone. The later compound is utilized as the starting compound for the synthesis of other flavonoids. All known flavonoid compounds, approximately 7000, have this common biosynthetic pathway and therefore share the same basic structural skeleton. For the vast majority of flavonoids that included ring-C, the simple structural unit is 2-phenylchroman (flavan) while further structural diversity stems from to the degree of unsaturation and oxidation of ring C: (2-phenylchromenyliums (anthocyanidins/anthocyanins); 2-

phenylochromones (flavones, flavonols, flavanones, di-OH-flavonols); 2-phenylchromanes (flavans, flavan-3-ols, and flavan-3,4-diols (proanthocyanidins)); chalcones/dihydrochalcones; 2-benzylidene coumaranones (= aurones), etc. (Khoo et al., 2017). Due to their structural differences, flavonoids are mainly classified into different groups, such as flavanones, flavanols, flavones, and flavonols. Other classes of flavonoid compounds include isoflavones, biflavonoids, flavonolignans, prenylflavonoids, flavonoid glycosidoesters, aurones, and chalcones. Structurally all flavonoid compounds can be referred to the basic structures presented in Fig. 3.

Individual flavonoid differs in ring substituent(s) formed in the process of hydroxylation (large number of hydroxyl groups: -OH), methylation (methoxy groups being more common as substituents of ring B, compared with ring A), acylation, and glycosidation with mono- or oligosaccharides (e.g. glucose, galactose rhamnose, xylose, arabinose) at various ring positions. One -OH group in ring A may be situated in *ortho*-position to the side chain which may be methylated, glycosylated or bear other functional groups. In addition, classification of flavonoid compounds is also based on the presence or absence of a carbonyl group at carbon 4 (C-4) within ring C. The common hydroxylation pattern in flavonoids is at positions C-5 and C-7 in ring A, and C-4' in ring B which often forms a catechol function group in the presence of a further hydroxyl group at C-3' (ring B). Different positions of substituents in flavonoid compounds induce distinct chemical and physical properties leading to variable biological activities.

In plants, flavonoids are found in two different forms including free aglycones and glycoside-bound forms (Andersen and Markham, 2005). The addition of a sugar moiety to an aglycone molecule results in an increase in the polarity of the compound. Most commonly, sugar moieties are attached as *O*-glycosyl derivative at C-3, less commonly to positions C-4', C-3', C-5, and C-7. Glycoside-bound forms are the most common forms for flavonols and

flavones, which are usually ingested by humans. The most common forms of natural glycosides are the *O*-glycosidic derivatives, while *C*-glycosides are less common. They are not only monosaccharides (D-glucose, D-galactose, D-apiose, L-arabinose, L-rhamnose or D-xylose) as well as D-glucuronic and D-galacturonic acids but often disaccharides and even higher saccharides are also found in association with free forms of flavonoids. In addition, flavonoid glycosidoesters, flavonolignans, prenylflavonoids, and biflavonoid derivatives can be present in nature (Brodowska, 2017). Acylation of the glycosides, in which one or more of the sugar hydroxyls are derivatized by acids, such as phenolic acids, is also occasionally observed (Kumar and Pandey, 2012). Another class of flavonoids comprises sulphate derivatives containing one or more sulphate residues attached to a phenolic or sugar hydroxyl.

A broad range of biological activities of flavonoids is due to their physico-chemical properties (Andersen and Markham, 2005). Flavonoid glycosides are soluble in solvents like ethanol and water, but are not soluble in solvents with less polarity, such as benzene, ether, and chloroform. Aglycones are characterized by low-polarity and are therefore well soluble in organic solvents. As a general trait, flavonoids are well soluble in alkaline solutions, yielding yellow colour. A range of colour-forming (complexation) reaction is also utilized for their preliminary identification. Flavonoids may undergo hydrolytic degradation and are capable of ultraviolet fluorescence, features which are used in their structural identification. State-of-the-art analytical techniques facilitate the separation process and accelerate the speed of identifying flavonoids, often present in complex mixtures following their isolation from plant materials. Various chromatographic techniques such as TLC/HPTLC, HPLC or UPLC are widely used techniques for the separation of flavonoids. The detection and structural identification of this class of natural products are based on a number of analytical methods, including UV/VIS, IR, as well as 1D/2D-NMR (Harborne and Roberts, 1994).

3. Flavonoid functions in plants and humans

Flavonoids are synthesized by plants to serve many actions as visual attractors, feeding repellents, photoreceptors, oviposition stimulants, allelopathy, phytoalexins, antioxidants, and antimicrobials (Iwashina, 2003; Jeandet et al., 2013 and 2014; Ren et al., 2003; Shirley, 1996; Srivastava and Bezwada, 2015).

It has been demonstrated that flavonoid biosynthesis is stimulated by oxidative stress, which is triggered by reactive oxygen species (ROS) generated through the plants' metabolic functions. Flavonoids not only inhibit the production of ROS but also protects against damage induced by UV radiations (Agati and Tattini, 2010). The antioxidant effect and UV absorbability of flavonoids depend on the main backbone structure as well as substitution at the various sites. Although B ring dihydroxylated flavonoids display higher antioxidant activity, those monohydroxylated exert higher capacity to absorb UV wavelengths. Glycosylation of flavonoids further provides water solubility, prevents auto-oxidation of hydroxyl groups, and facilitates their transportation to cell membrane (Agati and Tattini, 2010).

Flavonoids are involved in the aluminum toxicity in maize, where their production is induced in aluminium-exposed roots (Kidd et al., 2001). Flavonoids have also been reported to regulate the movement and catabolism of auxins, creatin, an auxin gradient that causes different morpho-anatomical characters of plants (Taylor and Grotewold, 2005). Moreover, flavonoids regulate indole-3-acetic acid oxidase effect and modulate protein activity implicated in cell growth (Mathesius, 2001; Naoumkina and Dixon, 2008; Saslowsky et al., 2005).

Plant flavonoids do also serve as active ingredients in a variety of pharmaceutical, nutraceutical, and cosmetic preparations for human uses. This aspect is associated with their broad spectrum health promoting actions, including anti-allergenic, antimicrobial, anti-

inflammatory, vasodilator, anti-mutagenic, and anti-carcinogenic activities (Hossain et al., 2017; Kim et al., 2004; LeJeune et al., 2015; Snijman et al., 2007; Srivastava and Bezwada, 2015). Among these biological features, the most renowned property of flavonoids is antioxidant effects that are often much higher than those of vitamin E and vitamin C (Prior and Cao, 2000). Hence, they are able to prevent oxidative stress (Haraguchi et al., 1996; Hertog et al., 1997; Ishikawa et al., 1997; Katan and Hollman, 1998; Kitagawa et al., 1992; Lale et al., 1996; Zeghad et al., 2016), by suppressing and scavenging ROS along with upregulating antioxidant enzyme systems, according to the flavonoid type (Halliwell and Gutteridge, 2015; Mishra et al., 2013a). While they are capable of directly scavenging free radicals by donating the hydrogen atom (Procházková et al., 2011), they are also able to inhibit some enzymes, including NADH oxidase (NOX), glutathione-*S*-transferase, mitochondrial succinoxidase, and microsomal monooxygenase, which are important sources of ROS production (Brown et al., 1998). Flavonoids can also chelate free metal ions, which enhance the formation of highly reactive hydroxyl radicals (Mishra et al., 2013a; Mishra et al., 2013b). In addition, they are able to prevent the peroxidation of lipids and the oxidative damage of membrane lipids (Kumar, A. et al., 2013; Kumar and Pandey, 2012).

Through combined antioxidant and antiinflammatory properties, among other effects, the health benefit of flavonoids is widely known. For example, epidemiological studies revealed that flavonoid-rich diets are associated with low incidence of cardiovascular diseases (CVD) (Daubney et al., 2015; Del Rio et al., 2013); they display hepatoprotective (Tapas et al., 2008; Wu et al., 2006; Zhu et al., 2012); neuroprotective including therapeutic options for neurodegenerative diseases (Spencer, 2007, 2008; Vauzour et al., 2008) and improvement of cognitive function (Lee et al., 2004; Lund et al., 2001; Pan et al., 2000); broad spectrum antimicrobial (Mishra et al., 2011; Mishra et al., 2013a; Mishra et al.,

2013b; Pandey et al., 2010) including against the human immunodeficiency virus (HIV-1) (Critchfield et al., 1996; Cushnie and Lamb, 2005) and various other effects.

4. New advances in the biochemistry of the flavonoid pathways

As explained in the preceeding section, the synthesis of flavonoids involves the phenylpropanoid metabolic pathway. First, *p*-coumaryl-CoA is synthesised from the aromatic amino acid, phenylalanine (Phe), using the following enzymes: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA ligase (4CL). These enzymes catalyzed the deamination reactions of Phe to coumaric acid (by PAL), the following oxidation to 4-coumaric acid (by C4H), and finally, the activation to *p*-coumaroyl-CoA (4CL) by the addition of a co-enzyme A (CoA) unit. In certain plants, the amino acid tyrosine is directly converted to 4-coumaric acid by TAL (tyrosine ammonia lyase) (Rosler et al., 1997).

While the above-mentioned phenypropanoid pathway leads to a range of phenolic acids as secondary metabolites, the action of chalcone synthase (CHS) represents the beginning of the specific flavonoid pathway. This enzyme is responsible for the generation of a chalcone from one *p*-coumaroyl-CoA and three malonyl-CoA molecules to form the two phenyl rings (i.e., A and B rings) of the flavonoid skeleton (C6-C3-C6). The formation of the heterocyclic C-ring is catalysed by chalcone isomerase (CHI), generating naringenin (flavanone) as an intermediate compound. The hydroxylation of naringenin by F3H (flavanone 3 β-hydroxylase) generates the dihydroflavonols. Reduction of these compounds through a catalytic action of dihydroflavonol-4-reducatse (DFR) gives rise to leucoanthocyanidins (named also flavan 3,4-diols) while oxidation to flavonols is catalysed by flavonol synthase (FLS). Flavanones (e.g., naringenin) can be converted to flavones that bear a double bond between the C-2 and C-3 (of the C ring) through the enzymatic action of flavone synthase (FNS). The last steps of the flavonoid pathway produce flavan-3-ols:

leucoanthocyanidin reductase (LAR) directly reduces leucoanthocyanidins to the corresponding flavan-3-ol, while anthocyanidin synthase (ANS) converts leucoanthocyanidins to anthocyanidins. The resulting metabolites are further reduced by the enzyme called anthocyanidin reductase (ANR).

The genes encoding enzymes that are involved in the biosynthesis of flavonoids and the biochemistry, molecular biology, genetics, and gene expression, have been studied in several model plants. Good examples are, *Arabidopsis thaliana* (L.) Heynh (Saito et al., 2013), and economically important plant species such as *Vitis vinifera* L. (Petrussa et al., 2013) and *Zea maize* L. (Andersen et al., 2008). The study of flavonoid metabolism is constantly growing and non-model medicinal crop species or exotic plant species, which produce secondary metabolites such as flavonoids or even other pharmacologically-active compounds, are involved (Svobodová et al., 2013).

Recent reports on the CHS, a critical enzyme for flavonoid biosynthesis, have identified and characterized a novel *Morus atropurpurea* Roxb chalcone synthase, MaCHS (Wang et al., 2017). MaCHS genes have important functions in defence responses and MaCHS overexpression in transgenic tobacco modulated metabolite profile, and confer resistance to environmental stresses, such as salt, drought and heat. To date, two CHS isoforms have been identified from the CHS multigene family in *Grewia asiatica* L., GaCHS1 (NCBI acc. KX129910) and GaCHS2 (NCBI acc. KX129911) were also characterized(Wani et al., 2017). *Ga*CHS2 was abundantly expressed in reproductive tissues, compared with *Ga*CHS1 normally expressed in vegetative plant parts. Furthermore, *Ga*CHS2 was highly expressed in male parts (stamens) of the flower, suggesting its potential role in the process of pollen maturation, in which *Ga*CHS2 expression is tightly regulated spatiotemporally. Yahyaa et al. (2017) characterized three chalcone synthase-like genes from apple (*Malus x domestica* Borkh.) associated with the levels of phloretin in apple leaves.

Numerous genes implicated in flavonoid pathways have been identified in several plant species, including *Camellia sinensis* L. Kuntze (Lin et al., 2007; Wang et al., 2018), *Citrus unshiu* Marc. (Wellmann et al., 2002), *Fagopyrum tataricum* L. Gaertn. (Li et al., 2012), *Ginkgo biloba* L. (Xu et al., 2012; Wu et al., 2018), *Allium cepa* L. (Park et al., 2017), *Paeonia suffruticosa* Andr. (Zhao et al., 2015), *Sophora japonica* L. (Singh et al., 2018), *Carthamus tinctorius* L. (Chen et al. 2018), *Salvia miltiorrhiza* Bunge (Deng et al. 2018), *Artemisia annua* L. (Liu et al., 2017b), and *Mangifera indica* (Bajpai et al., 2018). Uridine 5'-diphospho (UDP)-glycosyltransferases (UGT) are the final decorating enzymes produced in anthocyanin and flavonol biosynthesis. New advances suggest that a recombinant UGT716A1 protein (from *Gingko biloba* L.) produce multiple glycosylated products towards flavanol gallates, including (−)-epicatechin gallate, (−)-catechin gallate, (−)-gallocatechin gallate, and (-)-epi-gallocatechin gallate (Su et al., 2017). UGT716A1 is different from UGT72L1 (which is identified in *Medicago truncatula* Gaertn.) that was previously shown to glycosylate only non-galloyated epicatechin (Pang et al., 2008). The Arabidopsis UDPglycosyltransferases, UGT79B2 and UGT79B3 functions as anthocyanin rhamnosyltransferases, which confer abiotic stress tolerance by the modulation of anthocyanin accumulation (Li et al. 2017). The identification of key genes in *Camellia sinensis, CsMYB6A* and *CsUGT72AM1,* allowed to regulate the accumulation of anthocyanins and flavonols in purple-leaf tea; hence providing opportunities in understanding flavonoid biosynthesis in non-alcoholic beverage crops (He et al., 2018).

Zhang et al. (2017a) performed RNA-sequencing for two types of Pak Choi (*Brassica rapa chinensis* (L.) Hanelt.) with varying contents of anthocyanins. This study identified nine structural genes which are critically involved in the biosynthetic pathway of anthocyanins These genes were increased in the purple-leafed variety. Among these genes, the late

biosynthetic genes (*Bra013652, Bra019350, Bra027457, Bra003021, Bra038445, and Bra035004)* could possibly be more critical in the biosynthesisof anthocyanins.

Epigenetic changes in plants can be considered as novel regulators of fine biochemical events in both normal growth and upon exposure to environmental stress. The biosynthetic pathways of flavonoids played an important role in mediating plant defence following chronic exposure to environmental alterations such as salt stress, temperature, light- and drought-induced changes (Bharti et al., 2015; Hichri et al., 2011; Russo et al., 2015; Singh et al., 2017). Flavonoid biosynthesis and diversity are controlled by several different transcription factors through gene expression regulations involved in this metabolic pathway. The enforced manipulation of gene expression represents a useful tool to increase the contents of flavonoid constituents especially in some food products such as fruits and/or vegetables which are consumed in large volumes (Hichri et al., 2011).

Plant cells can regulate flavonoid biosynthesis by acting both transcriptionally and post-translationally. Mechanisms at post-transcriptional and post-translational levels have been recently studied and represent important targets for developing novel phenotypes or genotypes associated with stress tolerance. In fact, these could lead to a new generation of transgenes as well as support the molecular breeding of new genotypes (Guerra et al., 2015). The transcriptional regulation of flavonoid pathways has been intensively investigated in *Arabidopsis thaliana* L. Heynh. (Xie et al., 2017; Xu et al., 2014) and also in other plant species (Bemer et al., 2017; Bharti et al., 2015; Dastmalchi et al., 2017; Deng and Lu, 2017; Henry-Kirk et al., 2018; Pandey et al., 2016a; Pérez-Díaz et al., 2016; Sofo et al., 2010; Zhang, et al., 2017b). In some major species, the expression of genes involved in plant structure is mainly regulated by two transcriptional factors (including MYB, and basic helix-loop-helix (bHLH)), as well as by WD40 proteins (Hichri et al., 2011). MYB receives intense research interest as it is the main family of the transcription factors that are implicated in

inhibition or activation of gene transcription in the plant kingdom. The regulation of anthocyanin, proanthocyanin, flavonol, and other flavonoid biosynthesis is controlled by MYB, which is largely conserved in plants (He et al., 2018; Henry‐Kirk et al., 2018; James et al., 2017; Liu et al., 2017a; Sun et al., 2018).

The molecular basis involved in the potential post-translational regulatory processes has been narrowly described. Post-translational modifications include processes such as phosphorylation (Yin and Komatsu, 2017), *S*-nitrosylation (Romero-Puertas et al., 2013), ubiquitination (Manzano et al., 2008), and Small Ubiquitin-like Modifier (SUMO)-ylation (Miura et al., 2007), to regulate numerous cellular functions.

Recently, ubiquitin and SUMO conjugations have been described as the main post-translational regulatory processes that play a key role in all eukaryotes including plants (Guerra et al., 2015). Ubiquitination is one of the most prevalent post-translational modifications, which leads to the degradation of proteins by the proteasome (Wilkinson, 2000). It was found to be involved in several aspects of plant biochemistry and biology, from growth regulation and tissue development to responses to biotic and abiotic stimuli (light, drought and temperature changes, salt stress) (Verma et al., 2013). In addition, it can lead to the attenuation of protein accumulation through proteasome-dependent degradation or it can alter transcription factors activity. The ubiquitination of target proteins is catalysed by three major ubiquitination enzymes (Callis, 2014): E1 ubiquitin-activating enzyme (which catalyzes the formation of an E1–Ub intermediate), E2 ubiquitin-conjugating enzyme (conjugation of Ub to a conserved cysteine in E2), and E3 ubiquitin ligases (which transfers Ub units from E2 to target proteins). The E3 ubiquitin ligases are responsible for recruiting the proteins to the 26S proteasome complex to be degraded or modified. This family of ligases includes HECT-type (Homologous to the E6-AP Carboxyl Terminus), U-box type, Ring-type, and the SCF-complex (Skp1-Cullin-F-box), all of which play pivotal roles in

regulating plant growth and environmental stress response. In the SCF complex, Cullin physically interacts with two complex components, SKP1 and RBX1, providing a scaffold where the F-box protein can assemble; the specificity of target proteins is achieved by this particular F-box. One of F-box proteins is the KFB (Kelch domain (repeat)-containing F-Box) (Sun et al., 2007). Recent studies (Zhang et al., 2013; Zhang and Liu, 2015) demonstrated that *Arabidopsis* KFB 01, 20, 39, and 50 (KFB^{PALs}) interact with the rate-limiting step of phenylpropanoid PAL biosynthesis pathway. This interaction is able to regulate the PAL protein expression via ubiquitination-dependent degradation.

The KFB^{PALs} have been demonstrated to be negative regulators in the control of the biosynthesis of phenylpropanoids. Subsequently, by performing a yeast-two-hybrid screening assay, a KFB was identified to interact with CHS, the key enzyme for the flavonoid biosynthesis, which leads to its ubiquitination and degradation (Zhang, X. et al., 2017). KFB can be considered as a proteolytic regulator that is essential for negative control of phenolics, it is also able to coordinate with transcriptional regulation mechanisms of the secondary metabolite biosynthesis during plant growth and in response to environmental factors, such as UV-B radiation and some light signals (Zhang, et al., 2017b).

In vitro and *in vivo* studies in apples (An et al., 2017) indicated that MdMIEL1 is an E3 ligase which ubiquitinates MdMYB1, followed by 26S proteasome-dependent protein degradation. Moreover, apple calli and *Arabidopsis* have been utilized for transgenic research and results exhibited that MdMIEL1 could negatively regulate the accumulation of anthocyanin by altering MdMYB1 protein degradation.

E3 ligase Constitutive Photomorphogenic1 (COP1) negatively regulated the accumulation of anthocyanin in both apples and *Arabidopsis* by impacting the degradation of transcription factor anthocyanin-associated MYB (Li et al., 2012; Maier et al., 2013). A recent study (Kim et al., 2017) has confirmed the attenuation of anthocyanin biosynthesis

through COP1 which is balanced by elongated hypocotyl5 (HY5)-the positive regulatory factor of the biosynthesis of anthocyanins. Interestingly, it has also been demonstrated that high temperatures induce the degradation of HY5, without significant influence on *HY5* gene expression, in a COP1-dependent manner. One plausible explanation is that COP1 was implicated in destabilizing the HY5 protein, thereby, mediating high temperature induced repression of anthocyanin production. Keeping with this evidence, the biosynthesis and accumulation of anthocyanin fail to respond to temperature alternations in plants carrying *cop1* and *hy5* mutants (Kim et al., 2017; McNellis et al., 1994).

Small ubiquitin-like modifiers (SUMOs)-mediated sumoylation is a post-translational modification of proteins that regulates protein expression, stability, localization, as well as protein/protein interaction (PPI). SUMOylation consists of three major steps: E1 SUMO-activating enzyme (including SAE1 and SAE2), E2-conjugating enzyme (such as SCE1), and E3-ligases (such as MMS21 and SIZ1). SIZ1 mediates the sumoylation of protein substrates and regulates their physiological functions. SIZ1 has also been found in *Arabidopsis* (termed as AtSIZ1) (Catala et al., 2007), rice (OsSIZ1 and OsSIZ2) (Park et al., 2010; Wang et al., 2015), soybean (GmSIZ1) (Li et al., 2014), apple (MdSIZ1) (Zhang et al., 2016), *Dendrobium* (DnSIZ1) (Liu et al., 2015) and tomato (SlSIZ1) (Zhang et al., 2017b).

Interestingly, it was demonstrated that overexpression of MdSIZ1 has a positive effect on the accumulation of anthocyanins and the coloration of red fruits (Zhou et al., 2017). Particularly, abiotic stress as low temperature (17 °C) leads to the SUMOylation of transcription factors R2R3 MYB and MdMYB1, via increasing MdSIZ1 gene expression. The SUMOylated-MdMYB1 hinders the binding of ubiquitination of MdMYB1, thereby increasing the protein stability of MdMYB1. SUMOylated MdMYB1 promotes the binding of other transcription factors (for example, MdbHLH3) to anthocyanin-specific gene promoters as well as MdDFR (Xie et al., 2012) and MdANS (Ban et al., 2007) to stimulate

the biosynthesis of anthocyanins in apples (Fig. 4). Post-translational mechanisms are important in regulating flavonoid metabolic pathways. So far, ubiquitination and SUMOylation processes seem to attract researchers' attention, but much has not been identified yet. Future studies could be addressed to unravel other target substrates and new post-translational mechanisms involved in the metabolic flavonoid pathways for their accumulation.

5. Metabolic engineering of flavonoid compounds

Several plants and microorganisms (bacteria and yeasts) were successfully used as model organisms for flavonoid engineering.

5.1. Plants

For more than 30 years, engineering of flavonoids pathways (EFP) in plants has been the focus of scientific attention. The process has four main aims: engineering of (a) model plants in order to explain the general biosynthetic pathways, (b) florticultural plants in order to produce different ornamental plants, (c) plants for increased tolerance to pathogens (phytoalexin engineering) and (d) crop plants for flavonoid enrichment. As a recent paper by Tohge et al. (2017) elegantlly summarized current knowledges about flavonoid engineering in model plants, the subject is not detailed herein.

Anthocyanins are pigments from the natural environment which are responsible for the different colors of various flowers and fruits. Thus, they serve as excellent targets for engineering with the purpose of creating plants with different or desired colors. The first example of successful engineering was reported in late 1980´s when scientists introduced the gene of dihydroquercetin 4-reductase from maize into petunia. This led to a change in flowers’ color due to increased production of anthocyanin (pelargonidin) in the transgenic

plants (Meyer et al., 1987). Since then, genetic engineering produced a wide range of petunia plants with different flower color, pattern, and shape (Nakatsuka et al., 2007; Shimada et al., 2001; Tsuda et al., 2004). Lately, numerous successful modifications of anthocyanin's production and composition in different flowers, by expressing or silencing several critical flavonoid genes, such as F3H, CHS, DFR, and F3´5´H, led to the production of plants with modified floral colors (Table 2) (Mouradov and Spangenberg, 2014; Zhang et al., 2014). Furthermore, the creation of such floral phenotypes would not be possible by normal hybridization or breeding experiments.

Phytoalexins are antimicrobial secondary metabolites of low molecular weight produced by plants as a response to various stress factors. Plants from the Leguminosae family in particular can accumulate a considerable number of various flavonoid phytoalexins belonging to the isoflavone (daidzein, luteone), isoflavanone (kievitone), pterocarpan (maackiain, pisatin, phaseollin and glyceollin) and isoflavan (sativin) classes (Ingham, 1980). Due to the complexity of the flavonoid phytoalexin biosynthetic pathways and facing the crucial problem to introduce the right gene encoding the right enzyme of the limiting step of the pathway, there are relatively few reports of flavonoid phytoalexin gene transfer in plants, as compared to stilbene phytoalexins (Jeandet et al., 2002, 2010, 2012, 2013, 2014, 2017). Transformations regarding flavonoid phytoalexins have mainly concerned the genetic manipulation of flavonoid phytoalexin glycosylation (Chong et al., 2002; Gachon et al., 2004) and methylation (He and Dixon, 2000). For example, disease resistance of alfalfa plants to *Phoma medicaginis* was observed upon overexpression of the isoflavone 7-*O*-methyltransferase through the enhancement of the production of the 4'-*O*-methylisoflavonoid phytoalexin (He and Dixon, 2000). Moreover, loss-of-function genetic approaches have been developed to highlight the role played by flavonoid phytoalexins in the resistance of plants to various pathogens (Chong et al., 2002; Graham et al., 2007; Ibraheem et al., 2010). Down-

regulation of the 6-α-hydroxymaackiain-3-*O*-methyltransferase in pea was accompanied with a decreased resistance to *Nectria haematococca* (Wu and VanEtten, 2004). In the same way, the RNA interference (RNAi)-mediated silencing of the isoflavone reductase or chalcone reductase decreased the resistance of soybean to *Phytophthora sojae* (Graham et al., 2007). Deletion of the yellow seed1 gene implicated in the biosynthesis of the 3-deoxyanthocyanidin phytoalexin from sorghum also reduced the plant resistance to anthracnose disease (Ibraheem et al., 2010).

Anthocyanin's presence in food is associated with health benefits due to their protective effects against several chronic human disorders, including certain types of tumors, and cardiovascular diseases. Therefore, anthocyanin enrichment has been another aim of flavonoids engineering in crop plants. A good example is the creation of purple tomatoes with high anthocyanins content by simultaneously expressing R2R3MYB and bHLH proteins from snap dragon (Butelli et al., 2008). Similarly, a transposon insertion into the regulatory region upstream of R2R3 MYB TF led to increased levels of purple anthocyanins in cauliflower (Chiu et al., 2010). Furthermore, red apples were created by rearranging MYB10 promoter regions which generates a mini-satellite-like structure consisting of five tandem repeats of a 23 nucleotide sequence. This leads to the expression of anthocyanins in the fruit flesh (Espley et al., 2009). Recently, Zhu et al. (2017) created Purple Endosperm Rice via wild rice transformation using constructs which contain eight anthocyanin-related genes. These genes are driven by the endosperm-specific promoters, together with a selection marker and a marker excision gene.

Beyond anthocyanins, successful engineering of condensed tannins contributes to the bitterness and astringency of many fruits-derived products (Dixon et al., 2013). The transcription factors of the MYB family are key players in coordinating the activation of genes in the tannin pathways. Altered expression of these factors can change condensed

tannins' level in grapes, persimmon, kiwifruit, poplar, etc. (Dixon et al., 2013). Isoflavonoids, the legume subclass of flavonoids in particular (or so-called phytoestrogens), were engineered in alfalfa (*Medicago sativa*) by expressing MtIFS1 (isoflavone synthase from *Medicago truncatula*), which increases the accumulation of genistein and their derivatives such as biochanin A and pratensein (Deavours and Dixon, 2005). In addition to anthocyanins, other compounds from the flavonoid sub-group were successfully engineered in different crop plants. When genes LC and C1 were ectopically expressed in tomato or potato, increased amount of flavonoids from the flavonol group were observed in several studies (Schijlen et al., 2004). Tomato is a well-known model for flavonoid engineering. According to Bovy et al. (2007), three main approaches can be utilized: (a) increasing the production of endogenous tomato flavonoids using regulatory or structural genes; (b) using RNA interference (RNAi) strategies to block key steps in flavonoid pathway; (c) producing novel tomato flavonoids by introducing novel branches/genes in the flavonoid pathway. These approaches led to the creation of tomatoes that accumulated flavonoids, such as kaempferol glycosides and naringenin or fruits with increased or decreased levels of flavonoids (Bovy et al., 2002). Engineering of critical enzymes involved in the flavonoid pathway, such as CHI dihydroflavonol reductase (DFR), and CHS from *Petunia hybrida* into flax (*Linum usitatissimum*) resulted in flaxseeds rich in antioxidant products (flavonoids, phenolic acids, and lignin) derived from the phenylopropanoid pathway (Wu et al., 2014).

5.2. Microorganisms

The increasing market demand for plant flavonoids has become a challenge that cannot be met by the traditional laborious and rather expensive extraction methodologies. Therefore, biotechnological production of different plant secondary metabolites using microorganisms came into fashion in recent years (Park et al., 2018). Biotechnological

advances in microorganisms have further accelerated the synthesis of high quality products. However, metabolic engineering of microbes for production of flavonoid compounds needs a lot of techniques such as selection or optimization of host strains, determination of targets for gene manipulations, and also knowledge of all the enzymes involved in the biosynthesis (Chouhan et al., 2017). Until now, the most common microorganisms regarded as cellular factories of flavonoids are *Saccharomyces cerevisiae*, *Escherichia coli* and in some cases other species such as *Streptomyces venezuelae*. Very comprehensive reviews in this area have been published (Pandey et al., 2016b; Trantas et al., 2015). Flavonoid engineering using bacteria started in 2003 when *PAL*, *CHS*, and a 4CL from *Streptomyces* sp. were engineered in *E coli* to increase the production of flavanones such as naringenin or pinocembrin (Hwang et al., 2003). Lately, different types of flavonoids such as flavones, flavonols, flavanones, and isoflavones were successfully produced by engineering of either *E. coli* or *S. cerevisiae* (Wu et al., 2006; Fowler and Koffas, 2009; Wang et al., 2011; Trantas et al., 2015).

Building of the platform of flavonoids includes engineering the pathway targeting the B ring of flavonoids on one hand, and engineering the malonyl-CoA pathway (upstream and sink pathways), leading to the A ring on the other (Pandey et al., 2016b; Trantas et al., 2015). B ring formation of flavonoids follows the universal phenylpropanoid route starting from the aromatic amino acid precursor, phenylalanine. In this case, introduction of the complete pathway in *S. cerevisiae* including the *PAL*, *C4H*, *4CL*, *CHS* and *CHI* genes was needed to obtain naringenin (Trantas et al., 2009). As C4H requires the co-expression of a cytochrome P450 reductase (CPR) to achieve the hydroxylation step from cinnamate to *p*-coumarate, an electron-donor CPR must also be included (Koopman et al., 2012; Trantas et al., 2009). To by-pass this critical hydroxylation step, introduction of the *TAL* gene using the aromatic amino acid tyrosine as a precursor has been envisaged as an interesting option for the biosynthesis of naringenin in *E. coli* including the *4CL*, *CHS* and *CHI* genes (Miyahisa et al.,

2005). *P*-coumarate has been used in numerous studies as a precursor for the production of various flavonoid compounds (naringenin and apigenin) (Leonard et al. 2007 and 2008). For apigenin, engineering the pathway only requires the introduction of four genes: *4CL*, *CHS*, *CHI* and *FS1* (flavone synthase 1) (Leonard et al. 2008). Other flavonoids such as kaempferol and quercetin were obtained from *p*-coumarate with *4CL*, *CHS* and *CHI* and further genes operating on the pathway: *FLS*, *F3H* (flavanone 3β-hydroxylase) for kaempferol, and *FLS*, *F3H* and *F3',5'H* (flavonoid-3',5'-hydroxylase) for quercetin (Leonard et al., 2006; Trantas et al., 2009). To achieve further hydroxylation steps on the B ring of flavonoids, F3',5'H was fused to a CPR (Leonard et al., 2006).

Production of flavonoids (naringenin and pinocembrin) could also be achieved in engineered yeast and bacteria using simple carbon sources such as D-glucose (Wu et al., 2013 and 2014; Santos et al., 2011). When microbial cells are supplemented with glucose or other simple carbon sources, the formation of the aromatic amino acid precursors, phenylalanine and tyrosine, originates from the shikimate pathway. This imposes engineering of the shikimate route in microorganisms, namely the introduction of enzyme versions resistant to the feed-back inhibition exerted by phenylalanine or tyrosine at the level of the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the chorismate mutase/prephenate dehydratase (CM/PDT) for phenylalanine and the chorismate mutase/prephenate dehydrogenase (CM/PDH) for tyrosine. (2*S*)-pinocembrin production from D-glucose in *E. coli* was optimized by a modular metabolic strategy using four modules comprising 1) module 1: the *aroF* gene encoding a DAHP synthase and the *pheA* gene encoding a resistant version of CM/PDT; 2) module 2: *PAL* and *4CL*; 3) module 3: *CHS* and *CHI*; 4) module 4: *matC* encoding a malonate carrier protein and *matB* encoding a malonyl-CoA synthase to increase the intracellular pool of malonyl-CoA (see below) (Wu et al., 2013). Using a similar approach, Wu et al. (2014) optimized the *de novo* synthesis of (2*S*)-naringenin in *E. coli* from

D-glucose using a construct composed of three modules; 1): module 1: *TAL* and *4CL*; module 2: *CHS* and *CHI*; 3) module 3: *matC* and *matB*. Two genes, *aroG* encoding DAHP synthase and *tyrA* encoding a resistant version of CM/PDH were also added.

One important feature regarding the building of the A ring of flavonoids also concerns the regulation of the intracellular pool of malonyl-CoA since this compound is a metabolic node at the intersection of the flavonoid pathway and the fatty acid biosynthesis. The pool of malonyl-CoA is the result of a balance between its anabolic pathway (from acetyl-CoA) and its downstream utilization towards fatty acid production. Regulating the intracellular pool of malonyl-CoA imposes the control of both its upstream and downstream pathways. The concentration of malonyl-CoA can be increased through overexpression of the acetyl-CoA carboxylase complex (ACC), a key enzyme in the acetate assimilation pathway. For example, overexpression of the *ACC* gene and the biotin ligase (*BirA*) gene was achieved to target flavonoid production in *E. coli* (Leonard et al., 2007; Fowler et al., 2009). The malonyl-CoA pool can also be enhanced by improving the assimilation of malonate into the cell. For example, *Streptomyces venezuelae* was successfully engineered for producing flavonoids by co-expressing two sets of genes involved in the assimilation of malonate. With the introduction of malonyl-CoA synthetase *matB* and the putative dicarboxylate carrier protein *matC* from *Streptomyces coelicolor*, the recombinant production of flavonoid compounds in *S. venezuelae* was substantially increased, reaching up to 40 mg/L of flavanones (pinocembin, naringenin) and 30 mg/L of flavones such as apigenin and chrysin (Park et al., 2011).

Otherwise, a Clustered Regularly Interspaced Short Palindromic Repeats Interference (CRISPRI) system was used to fine-tune the central metabolic pathways in *E. coli* (glycolysis, fermentation, tricarboxylic acid cycle and fatty acid synthesis) and to redirect the carbon flux into the production of acetyl-CoA and malonyl-CoA for the biosynthesis of

naringenin (Wu et al., 2015). The most efficient combination consisted of anti-*fabF* (β-ketoacyl-ACP synthase II)/*fumC* (fumarase C)/*fabB* (β-ketoacyl-ACP synthase I)/*sucC* (succinyl-CoA synthase)/*adhE* (acetaldehyde dehydrogenase) leading to the highest naringenin titer (421.6 mg/L) reported to date (Wu et al., 2015).

E. coli was not only transformed to synthesize different flavonoid aglycones, but also the plant-specific flavonoid glycosides, such as luteolin-7-*O*-glucuronide, quercetin 3-*O*-arabinoside, quercetin 3-*O*-xyloside, and quercetin 3-*O*-glucuronide, etc. were produced by engineering glucosyltransferases and nucleotide sugar pathways (Han et al., 2014; Kim et al., 2015).

Anthocyanins are a group of flavonoid compounds which are responsible for the red, orange, purple and blue color of flowers and fruits. As anthocyanins are colored pigments useful for the food technology, their production at an industrial scale is desirable. Engineered microorganisms for anthocyanin biosynthesis constitute valuable platforms for their production (Zhaa and Koffas, 2017). Building of the flavylium cation requires the combined action of DFR and anthocyanin synthase (ANS) or the sole action of ANS depending on the starting precursor (Yan et al., 2005a and 2008). Further modifications of the B ring of anthocyanins are under the control of decorating enzymes, mainly anthocyanin-*O*-glucosyltransferases (GT) and anthocyanin-*O*-methyltransferases (AOMT) using UDP-glucose (UDPG) and *S*-adenosyl-L-methionine (SAM) as glucose or methyl donors, respectively. For example, production of cyanidin 3-*O*-glucoside (Cn 3-G) was achieved from (+)-catechin using a protein fusion between ANS and a 3 *O*-glucosyltransferase (3-GT) in *E. coli* (Yan et al., 2005a and 2008). Cn 3-G was also obtained from naringenin with an *E. coli* strain bearing the *ANS*, *DFR* and *3-GT* genes (Yan et al., 2005b). More recently, peonidin 3-*O*-glucoside (Pn 3-G) was recovered in *E. coli* cultivated in shake flasks with a titer of 56 mg/L (Cress et al., 2017). The construct included the following genes: *ANS*, *AOMT*

and *3-GT*. A CRISPRi system was used as well, to silence the transcriptional repressor MetJ to improve the intracellular pool of SAM, thus insuring the efficient conversion of Cn 3-G to Pn 3-G (Cress et al., 2017).

Yan et al. also described the production of milligram quantities of flavanones from a *S. cerevisiae* recombinant strain that carries a plant-derived gene cluster (Yan et al., 2005b). In the same way, recent progress in yeast *S. cerevisiae* engineering has allowed *de novo* production of several flavonoids, including liquiritigenin, naringenin, resokaempferol, quercetin, fisetin, and kaempferol (Rodriguez et al., 2017). One prominent feature of this approach is that all products are generated directly from glucose, without the supplementation of expensive intermediate products (Rodriguez et al., 2017).

In addition to producing natural flavonoids, metabolic engineering can also be used to produce non-natural molecules by feeding with different precursors (Fowler et al., 2011; Mora-Pale et al., 2013; Pandey et al., 2016b). Although flavonoid production using microbial engineering is a vigilant and fast growing area, large industrial-scale production using bacteria continued to be a challenge (Wu et al., 2014).

6. Conclusion and future directions

Flavonoids have diverse functions in plants, animals, and other living organisms that produce them. The presence of extended double bond and polyphenolic nature in their structure favours multiple mechanisms of action including antioxidant activities that serve as protective mechanisms in living system. Their common occurrence in various food ingredients (e.g., tea catechins) and potential applications from antimicrobial to chronic disease (e.g., neurodegenerative diseases) that we highlighted, necessitates detailed understanding of their potential exploitation through up-to-date research. We have reviewed recent advances in the understanding of the flavonoids biosynthesis, which allow us to

regulate their production. Transcriptional regulation by modifying transcription factors, such as MYB demonstrated how the biosynthesis of anthocyanins, proanthocyanins, flavonols, and other flavonoids is regulated. In addition to the environmental and chemical stressors that elicit the production of flavonoids, post-translational levels of regulation in flavonoids biosynthesis has become essential manipulation tools in recent years. Regulation of flavonoids' biosynthesis at the level of phenylpropanoids *via* ubiquitination mechanisms as well as SUMOylation processes, illustrates how the production of flavonoid compounds (for example anthocyanins) is modified. By employing genetic engineering both at transcriptional and pot-transcriptional regulations, maximising the yield of specific flavonoids compounds both in microorganisms and plants are now possible.

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Figures

Fig 1. An overview of flavonoids biosynthesis pathway

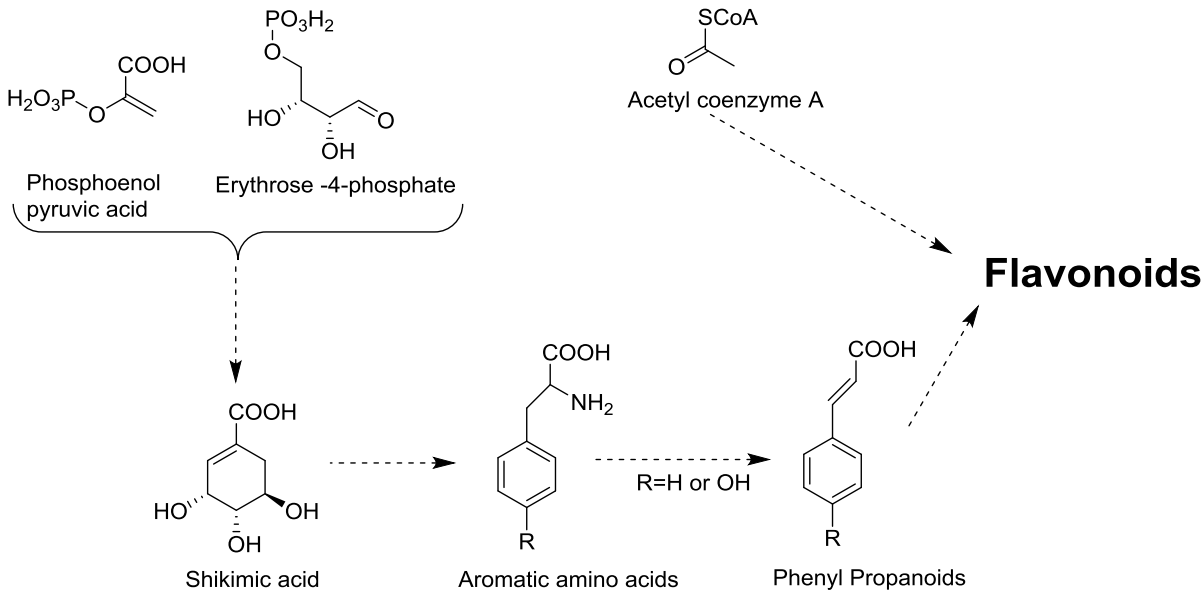


Fig 2. General structure of flavonoids

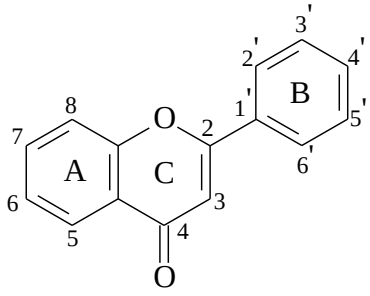
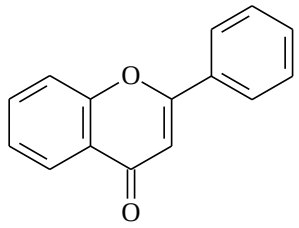
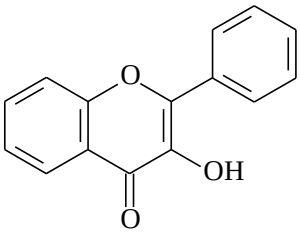


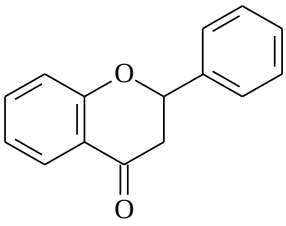
Fig 3. Most common types of flavonoid compounds



Flavone



Flavonols



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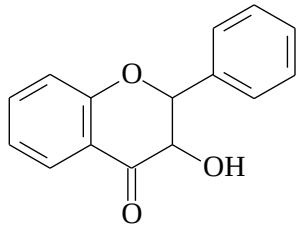
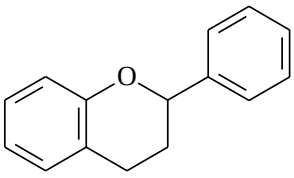


Flavanonol



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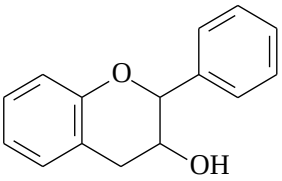
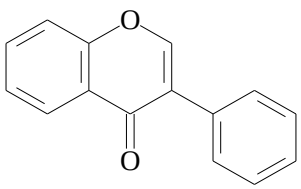
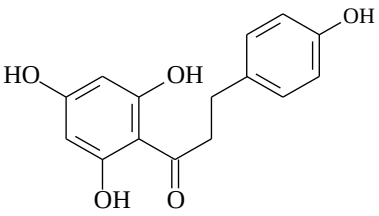


Flavanol





Isoflavone Chalcone

Fig 4. Schematic flavonoid pathway describing the whole regulation of biosynthetic process.

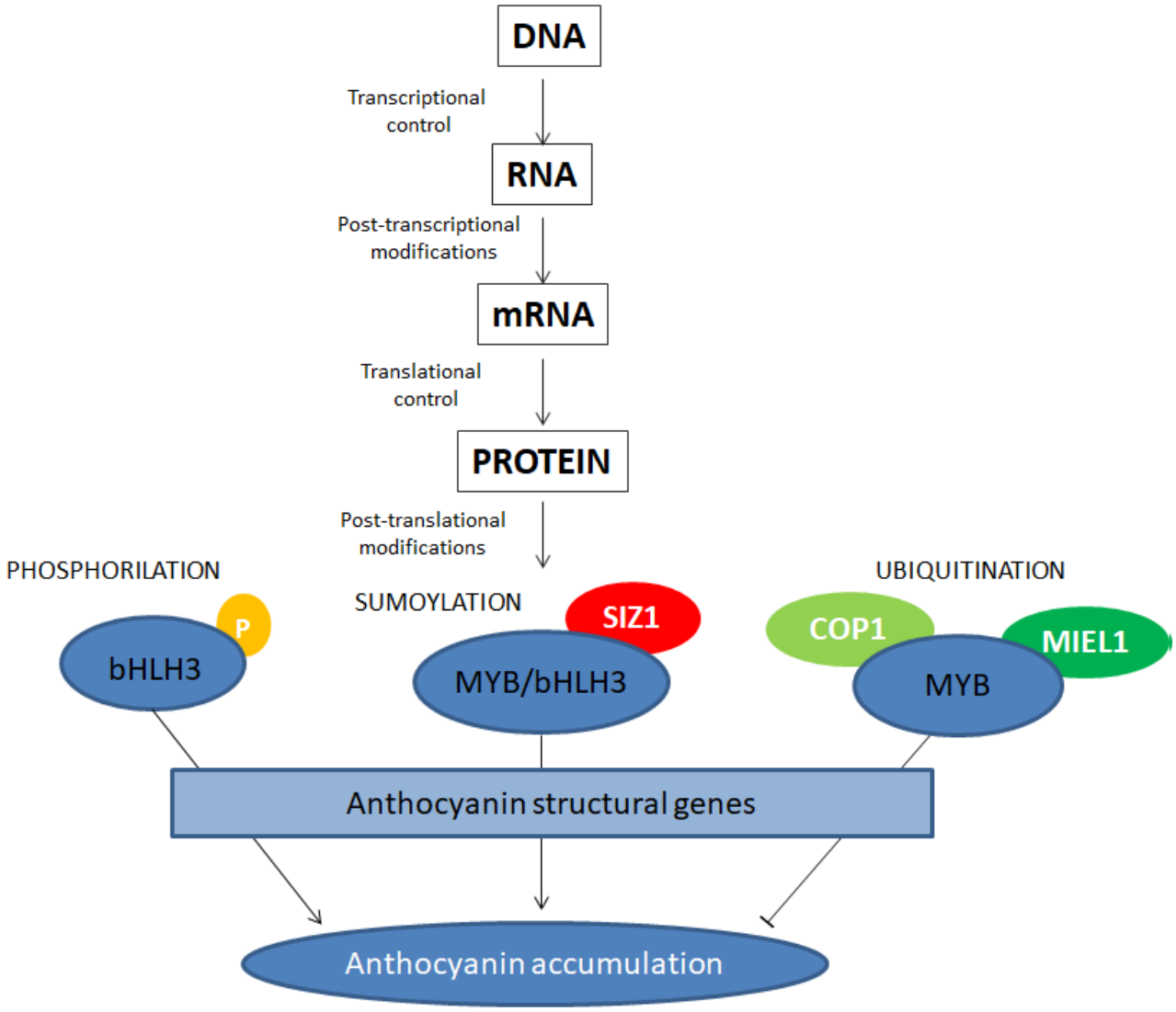


Table 1 Completed trial studies on flavonoids registered on the database Clinical Trials.gov database

Number	Study Title	Conditions	Number Identifier
1	Efficacy of Brooke Bond Black Tea Extract on Flow Mediated Dilation in Indian Males	Healthy	NCT01561300
2	Role of Flavanols In Cardiovascular Function in Healthy Aging	Aging	NCT01395277
3	Advancing Niacin by Inhibiting Flushing (ANTI-FLUSH)	Flushing	NCT00913081
4	A Comparison Chocolate With and Without High Cocoa Solids in Patients With Type 2 Diabetes in a Randomised Clinical Trial	Type 2 Diabetes	NCT01617603
5	Purple Grape Juice in Improving Vascular Health in Childhood Cancer Survivors	Cardiovascular Disease	NCT01043939
6	Effects of Epigallocatechin Gallate (EGCG) in Healthy, Young Adults	Cognitive Function, Mood	NCT00981292
7	Flow Mediated Dilation in Response to Black Tea	Vascular Function	NCT02273323
8	Beneficial Effects of Quercetin in Chronic Obstructive Pulmonary Disease (COPD)	Chronic Obstructive Pulmonary Disease	NCT01708278

Table 2. Examples of flavonoid engineering in floricultural plants.

Plants	Original colour	Transgenic plant colour	Modification	Reference
forsythia (Forsythia x intermedia cv ‘Spring Glory)	yellow	bronze-orange petal color due to the cyanidin-derived anthocyanins	expression of dihydroflavonol 4-reductase from snapdragon and anthocyanidin synthase from hoary stock	Rosati et al., (2003)
petuania (*Petunia hybrida*)	purple	white	F3H and DFR gene suppression	Tsuda et al., 2004
	purple	red	down regulation of F3´5´H	
	red	orange	suppression of the F3´H and expression of the rose DFR gene	
	violet	pale violet	expression of rose FLS gene	
rose (*Rosa hybrida*)	white to red	bluedue to the delphinidin accumulation	expression of the viola F3´5´H gene	Katsumoto et al., 2007
gentian (Gentian sp.)	vivid-blue	light-coloured (white)	downregulation of the 5/3´AT gene	Nakatsuka et al., 2010
chrysanthemums (Chrysanthemum × morifolium Ramat.)	pink to red	bluish due to the delphinidin accumulation	utilization of F3′5′H sequences from pansy and down-regulation of the endogenous F3′H	Brugliera et al., 2013