Biotechnological production of flavonoids: an update on plant metabolic engineering, microbial host selection and genetically encoded biosensors

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Abstract

Flavonoids represent a diversified family of phenylpropanoid-derived plant secondary metabolites. They are widely found in fruits, vegetables and medicinal herbs and plants. There has been increasing interest on flavonoids because of their proven bioactivity associated with anti-obesity, anti-cancer, anti-inflammatory, anti-diabetic activity and the prevention of aging-related chronic conditions, such as nervous and cardiovascular disease. Low bioavailability of flavonoids is a major challenge restricting their wide applications. Due to safety and economic issues, traditional plant extraction or chemical synthesis could not provide a scalable route for large-scale production of flavonoids. Alternatively, reconstruction of biosynthetic gene clusters in plants and industrially relevant microbes offer significant promise for discovery and scalable synthesis of flavonoids. This review provides an update on biotechnological production of flavonoids. We summarized the recent advances on plant metabolic engineering, microbial host and genetically encoded biosensors. Plant metabolic engineering holds the promise to improve the yield of specific flavonoids and expand the chemical space of novel flavonoids. The choice of microbial host provides the cellular chassis that could be tailored for various stereo- or regio-selective chemistries that are crucial for their bioactivities. When coupled with transcriptional biosensing, genetically encoded biosensors could be welded into cellular metabolism to achieve high throughput screening or dynamic carbon flux re-allocation to deliver efficient and robust microbial workhorse. The convergence of these technologies will translate the vast majority of plant genetic resources into valuable flavonoids with pharmaceutical/nutraceutical values in the foreseeable future.

Keywords: Flavonoids, plant metabolic engineering, biosensor, microbial host, synthetic biology

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1. Introduction

Flavonoid are a major group of plant secondary metabolites having a benzo-γ-pyrone structure which fulfill a multitude of functions during plant development such as antioxidant activity, resistance to various biotic and abiotic stress, visual signals and control of auxin transport (Mol, Grotewold et al. 1998, Winkel-Shirley 2002, Bradshaw Jr and Schemske 2003). They have been used as valuable nutraceuticals for the treatment of an assortment of human maladies (Patil, Jain et al. 2014). This major group are sub-classified into chalcones, flavones, flavonols, flavanonols, flavanones, flavanols or catechins, anthocyanins, and proanthocyanidins. Condensed tannins and aurones are the seventh subgroup found in only some species, according to the structure and modifications to the A, B and C rings that varies in degree of hydroxylation, substitutions and conjugations, and degree of polymerization (Winkel-Shirley 2001, Heim, Tagliaferro et al. 2002, Winkel 2006). The biosynthetic pathway of some important flavonoids which have been widely used for human health is summarized in **Fig. 1**.

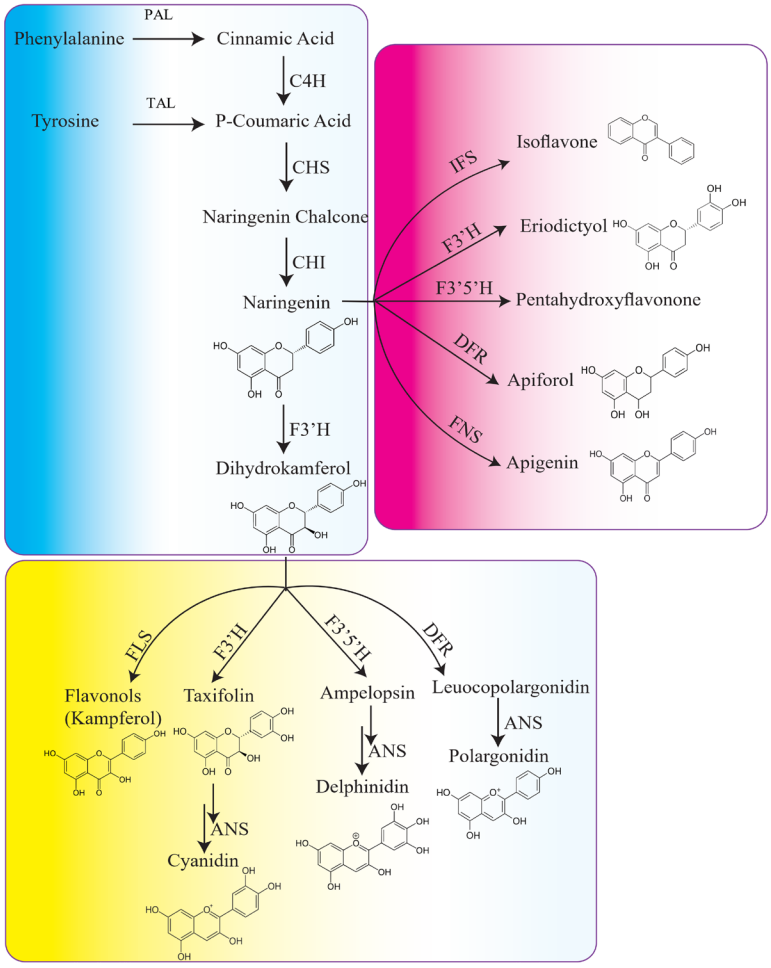


Fig. 1. Detailed biosynthetic steps for flavonoids biosynthetic pathway. This pathway starts with the general phenylpropanoid metabolism in plastid and subsequent steps are catalyzed by a series of structural enzymes from endoplasmic reticulum and cytosol leading to the biosynthesis of final end-products. Abbreviations are as follows: PAL Phenylalanine ammonia-lyase, TAL Tyrosine ammonia-lyase, C4H Cinnamic acid 4-

hydroxylase, CHS Chalcone synthase, CHI Chalcone isomerase, F3'H Flavonoid 3'-hydroxylase, F3'5'H Flavonoid 3',5'-hydroxylase, DFR Dihydroflavanone reductase, IFS Isoflavonoid synthase, FNS Flavone synthase, FLS Flavonol synthase, ANS anthocyanidin synthase.

As a large family of secondary metabolites, flavonoid accumulation in plant is highly tissue-specific and regulated at transcription level. The amounts of flavonoids in plant are also subject to developmental stage or environmental conditions. In the past decades, various plant species have been modified to produce transgenic varieties with different level of flavonoids. These genetic modifications for some extent are predictable but it is not always associated with the amount of flavonoids accumulated (Tanaka, Sasaki et al. 2008). Improvement of flavonoid accumulation in plant can be achieved by modifying biosynthetic steps and/or regulatory genes. In higher organisms like plants, some transgenes are susceptible to methylation and silencing, they can make unpredictable changes at the transcriptional level, which even results in global phenotypic changes and consequently leads to unpredictable flavonoid accumulation (Elomaa, Helariutta et al. 1995).

Although chemical synthesis is available for some flavonoid compounds, but the use of toxic chemical solvents and extreme reaction conditions often limit the yield and scalability of these high-value compounds (Chemler and Koffas 2008, Xu, Bhan et al. 2013). Essential modifications that form truly active molecules, such as glycosylation, prenylation and chiral synthesis, are also crucial challenges in chemical syntheses. With many of genes associated with flavonoids biosynthesis pathway have been identified, microbial production of flavonoids has attracted significant interests in both industry and academia. This was generally achieved by reconstructing or introducing the flavonoid biosynthetic pathways as well as exploring the power of fermentation to produce specific compounds under controlled conditions. Most of the existing fermentation facility and strain engineering systems can be adapted to produce plant-derived flavonoid compounds (Wang, Wang et al. 2011, Ochoa-Villarreal, Howat et al. 2015). In recent years, other than *E. coli*, a number of different host strains are emerging, including *S. cerevisiae*, *Y. lipolytica* and *L. lactis*. When coupled with transcriptional biosensing, genetically encoded biosensors could be welded into cellular metabolism to achieve high throughput screening or dynamic carbon flux re-allocation to deliver efficient microbial workhorse. This review provides an update on biotechnological production of flavonoids; we summarized the recent advances on plant metabolic engineering, microbial host and genetically encoded biosensors to improve flavonoids production. The convergence of these technologies will largely transform the vast majority of plant genetic resources into valuable flavonoids with pharmaceuticals values in the foreseeable future.

2. Biotechnological production of flavonoids in plants

In recent years, multitude studies have been carried out to engineering of flavonoids pathway in plants. These studies follow four main goals: i) engineering of model plants to identify key factors in the general flavonoid biosynthetic pathways, ii) engineering of ornamental plants for decorative purposes (i.e. flavonoids with strong pigment), iii) engineering of plants for tolerance improvement towards biotic and abiotic stress and iv) engineering of crop plants to increase flavonoids

accumulation (Nabavi, Šamec et al. 2018). In this section, we summarized the recently applied strategies to improve the biotechnological application of flavonoids in planta.

2.1. Overexpression of transcriptional factors: a forward approach

Environmental or developmental regulation of flavonoid biosynthesis mostly rely on the coordinated expression of Early Biosynthetic Genes (EBGs) and Late Biosynthetic Genes (LBGs). Specific combinations of R2R3-MYB transcription factor with bHLH and WD40 will form ternary complexes MYB-bHLH-WD40 (named as MBW) to regulate genes encoding enzymes in the final steps of flavonoid biosynthesis (Jones 2004, Hichri, Barrieu et al. 2011, Czemmel, Heppel et al. 2012). This interaction involved the R3 repeat of the MYB and the N-terminal MYB-interacting region (MIR) of the bHLH with the WD40- repeat of WDR families (Baudry, Heim et al. 2004, Hichri, Barrieu et al. 2011). R2R3-MYB factors play a specific function for trait regulation while the bHLH factors redundantly regulate different traits (Zhang and Schrader 2017). MBW complexes also control various functions such as the development of trichomes and root hairs (Wang, Barron et al. 2010). It was suggested that the activity of MYB genes represent a class of natural variation in anthocyanin pigmentation in plants (Singh, Low et al. 2014). Regulation and manipulation of transcription factor involved in flavonoids biosynthesis could remove the rate-limiting steps in various plants and provide an excellent opportunity to uncover the regulatory control of flavonoids biosynthesis.

It was revealed, activation of the R2R3-MYB transcription factor anthocyanin1 (CsAN1) could specifically upregulate the bHLH transcription factor CsGL3 and anthocyanin LBGs to confer ectopic accumulation of pigment in purple tea (*Camellia sinensis* L.) spices. In this study, it was confirmed CsAN1 could interact with bHLH and recruits a WD-repeat protein CsTTG1 to form the MYB-bHLH-WDR (MBW) ternary complex which regulates and enhances anthocyanin accumulation. CsAN1 and anthocyanin LBG expression levels were highly correlated with anthocyanin accumulation (Sun, Zhu et al. 2016).

It was recently reported the co-expression of *ROSEA1* (*ROS1*, a MYB-type) and *DELILA* (*DEL*, a bHLH-type) transcription factors from snapdragon origin improved the anthocyanin accumulation (delphinidin and cyanidin) and abiotic stress tolerance in tobacco flowers and *Lilium* tepals (Naing, Ai et al. 2018, Fatihah, Moñino López et al. 2019). The overexpression of the MYB transcription factor Anthocyanin2 in tomato (LeAN2) enhanced anthocyanin accumulation and improve chilling and oxidative stress (Meng, Yin et al. 2014). Introducing of *Arabidopsis thaliana* L. regulatory factor PAP1/AtMYB75 into hop plants by *Agrobacterium*-mediated genetic transformation led to higher levels of anthocyanins, rutin, isoquercitin, kaempferol-glucoside, kaempferol-glucoside-malonate, desmethylxanthohumol, xanthohumol, a-acids and b-acids, which consequently caused the change of color of female flowers and cones from reddish to pink in transgenic plants compared to wild type plants (Gatica-Arias, Farag et al. 2012). There are some other investigations which used MYB transcription factors to produce colorful plants with enhanced anthocyanin content such as purple tomatoes (Butelli, Titta et al. 2008), purple cauliflower (Chiu, Zhou et al. 2010), red

apples (Espley, Brendolise et al. 2009) and purple rice (Zhu, Yu et al. 2017), demonstrating the economical value of using transgenic plants to produce functionalized flavonoids.

2.2. Reverse genetic engineering in plants

Reverse genetics is considered as a powerful tool for assessing gene function and identifying genetic targets to improve flavonoid production in plant (Li, Jiang et al. 2011, Guo, Liu et al. 2016). Reverse genetics, required strategy for functional genomics, relate a certain genetic sequence with a specific phenotype on the organism. This strategy includes gene silencing (RNA interference or RNAi), identification and screening of mutated populations such as insertional mutagenesis, knock-out and point mutation or TILLING (Target Induced Local Lesions in Genome), as well as gene targeting and using transgenics for ectopic expression. Each strategy has its own strengths and weaknesses. Through analyzing the phenotypic effects of specific engineered gene, endeavor to probe gene function sequences and assign related phenotypes has moved rapidly in recent years (Ben-Amar, Daldoul et al. 2016).

RNA silencing and insertional mutagenesis are the most common strategies for producing functional mutants (McCallum, Comai et al. 2000). Significant endeavor for certain target genes is required for RNAi even before knowing whether it will work. In RNAi technology, the introduction of double-stranded RNAs (dsRNAs) into cells could silence the expression of an endogenous target gene at transcriptional and posttranscriptional levels without deleting or altering its gene structure. These two approaches could also overcome gene redundancy issues by expressing various homologous genes (Li, Jiang et al. 2011)..

Here we also summarized some RNA silencing strategies to identify important genes and improve flavonoids biosynthesis in various plant species. RNA interference silencing of chalcone synthase (CHS) was used to produce seedless tomato which resulted in altered distribution of auxin and the reduced level of flavonoids (Schijlen, de Vos et al. 2007). In a similar study, hpRNA construct was introduced into *Torenia* plant and changed its original blue flower color into white and pale colors (Fukusaki, Kawasaki et al. 2004). Suppression of chalcone isomerase gene (*CHI*) in transgenic tobacco is correlated with reduced pigmentation and changed flavonoid components (Nishihara, Nakatsuka et al. 2005). Transgenic tomatoes expressing an hpRNA suppressing *DET1,* a photo-morphogenesis regulatory gene which represses several signaling pathways controlled by light, showed significant increase in the level of both flavonoid and carotenoid. This research confirmed that manipulation of plant regulatory pathway could simultaneously alter multiple independent phytonutrients biosynthetic pathways (Davuluri, van Tuinen et al. 2005). Down-regulation of endogenous dihydroflavonol 4-reductase (DFR) using RNAi strategy along with overexpression of exogenous DFR and flavonoid 3′,5′- hydroxylase (F3′5′H) increase the accumulation of delphinidin (Katsumoto, Fukuchi-Mizutani et al. 2007).

TILLING uses the high density of point mutations caused by chemical mutagenesis and sensitive mutational screening instrument to identify and discover induced mutations. Specifically, TILLING strategy does not rely on genetic transformation techniques and involves some simple steps (a) Induction of mutation by ethyl methanesulfonate (EMS); (b) Preparation of DNA and

pooling the individual samples; (c) PCR amplification of a region of interest; (d) formation of heteroduplexes by denaturation and annealing; (e) Running denaturing HPLC (DHPLC) to identify mismatches in heteroduplexes; (f) screening mutant individual; and (g) sequencing of the mutant PCR product (McCallum, Comai et al. 2000).

TILLING strategy has been used in *Arabidopsis thaliana* to identify the pollen-specific flavonols biosynthetic genes. Analysis of flavonoid accumulation in organs pistils, stamens, petals and calyxes organs along with flowers of wild-type and *male sterility* mutants confirmed kaempferol/quercetin 3-*O*-β-d-glucopyranosyl-(1→2)-β-d-glucopyranosides are the pollen specific flavonols. Microarray results from comparing the wild type and *male sterility* confirmed UDP-glycosyltransferases 79B6 (UGT79B6) is a regulation enzyme which determine pollen-specific flavonols (Yonekura-Sakakibara, Nakabayashi et al. 2014). In *Brassica rapa* subsp. *rapa* cv. Tsuda TILLING line indicated the insertion of stop codon in R2R3- MYB transcription factor gene BrMYB4 and loosing of C-terminal in its corresponding protein. In mutant line, it was indicated that anthocyanin accumulated in the below-ground part which encourage the researcher to analyze the expression of anthocyanin biosynthesis genes such as tree homologs of CHS, DFR, ANS1 and ANS2 as well as C4H in the peels of storage roots and in response to sunlight and a UV-B treatment. The expression levels of anthocyanin biosynthesis genes were markedly increased in mutant lines compare to wide type in the dark condition, while the expression of BrMYB4 was similar between both of wild type and mutant lines (Zhang, Wang et al. 2014).

2.3. Plant Suspension culture

Production of complex secondary metabolites in plant cell cultures has been one of the most widely investigated areas in recent years. For multiplication and extraction of secondary metabolites in the sterile condition like bioreactor, different type of explants such as plant leaves, stems, roots, and meristems have been used. Plant cell culture (PCC) is a well-known platform to improve the amount of plant natural products (NPs) for the food, cosmetic and drug industries. Recently, PCC with feeding of more abundant natural precursors demonstrate the advantages of producing large quantify of complex NPs over their natural harvest from plant materials or semi-synthesis (Ochoa-Villarreal, Howat et al. 2016).

Plant cell suspension cultures offer a reliable and productive system to generate polyphenols and flavonoids. In one study, the effect of methyl jasmonate (MeJA) on cell growth and flavonoid biosynthesis in the *Hypericum perforatum* cell suspension culture was investigated in small batches. Some parameters, such as elicitation time and MeJA concentration, on biomass and flavonoid production were studied. The activities of key enzymes (catalase and phenylalanine ammonia lyase) related to plant stress responses and secondary metabolite biosynthesis were investigated as well. The result showed MeJA influenced the cells growth and improved flavonoid production up to 280 mg/L (2.7 times more than control cultures). With MeJA treatment, flavonoid production was enhanced through the inhibitory effect of MeJA on catalase (CAT) and its inducer effect on phenylalanine ammonia lyase (PAL) (Wang, Qian et al. 2015). *Silybum marianum* cell cultures with *Vitis vinifera* stilbene synthase was used to produce t-resveratrol in the extracellular

medium after elicitation with MeJA or methylated β-cyclodextrins. In this report, production of silymarin was less affected in the transgenic cultures, possibly due to an intrinsic precursor bottleneck from the monolignol branch limits silymarin synthesis. The fact is that overexpressed STS gene will react with the excessively produced precursors of non-bioactive compounds such as coniferyl alcohol, while the metabolic flux toward the silymarin production was kept unchanged, this may provide a route to extend the applications of plant cell cultures for both constitutive and foreign valuable metabolites production (Hidalgo, Martínez-Márquez et al. 2017). Similarity, effect of subculture on anthraquinones, flavonoids and phenolics production were established from leaf, fruit and root explants of Indian Mulberry (*Morinda citrifolia*). Based on the results, tissue specific expression of anthraquinones, flavonoids and phenolics was mapped to the leaf, fruit and root suspension cultures. Whereas the phenolic contents show less difference at high extents in leaf, fruit and root suspension cultures. For example, root suspension culture produced 198 and 204 percent and fruit suspension culture produced 146 and 161 percent higher production of anthraquinones and flavonoids respectively than widely used leaf suspension culture (Deshmukh, Wadegaonkar et al. 2011).

While bioreactor-based systems are suggested to facilitate high-yield production of flavonoids with plant cell cultures in a few species, to date economic feasibility has not been established due to engineering challenges in large-scale cultivation. As a major challenge, culture productivity is impaired by the plant cells' tendency to form aggregates, due to that fact that cells within aggregates are not sufficiently exposed to the lighting or oxygen necessary to induce the biosynthesis of flavonoid. Even though the amounts of synthesized flavonoids from transgenic plants were low due to competing pathways, diversion of metabolic flux and hereditary stability in plant cell suspension cultures are the most important reasons which have made these systems not be successful for the commercial, large-scale production of flavonoids despite of years effort in this field (Fowler and Koffas 2009). Furthermore, it is possible to establish *in vitro* cultures for several plant species using an appropriate culture medium and optimal amount phytohormones. Simply by adding successfully formed callus into appropriate liquid medium, cell suspension cultures can subsequently be generated. The produced cultures commonly have meaningful scale-up capacity for growth in industrially relevant bioreactors designed to maximize levels of NPs biosynthesis (Ochoa-Villarreal, Howat et al. 2016).

3. Microbial host selection for engineering flavonoids biosynthesis

The accumulation of flavonoids in plants without developmental or environmental stimuli is always limited. Some of the important flavonoid products are extracted from plant species that are difficult to culture or require long growing seasons, further increasing the cost of production (Wang, Chen et al. 2011). Additionally, extraction and purification processes add cost and result in product loss and degradation. For example, the concentration of flavonoids in different varieties and sources of fruits oscillate between 30 and 4000 mg / kg of dry weight. This means that for the production of 1 kg of flavonoids, it is required to process 0.25–33 Tons of dry weight of fruits or vegetables (Rodriguez, Strucko et al. 2017). In addition, plant extraction also yields a mixture of

various substituted flavonoids; thus, generating an extract containing only one class with a single substitution pattern, requires countless purification steps that would eventually add to the economic cost and negatively impact the environment (Lim, Jung et al. 2001, Mavel, Dikic et al. 2006). Furthermore, these processes are time-consuming, expensive regarding natural resource consumption and, sometimes, environmentally unfriendly due to the usage of solvents during the isolation and purification procedures. It has been attracting the attention of scientists to develop alternative platforms, with the aim to significantly reduce the costs related with isolation and purification steps and increase the yield and recovery (Trantas, Koffas et al. 2015).

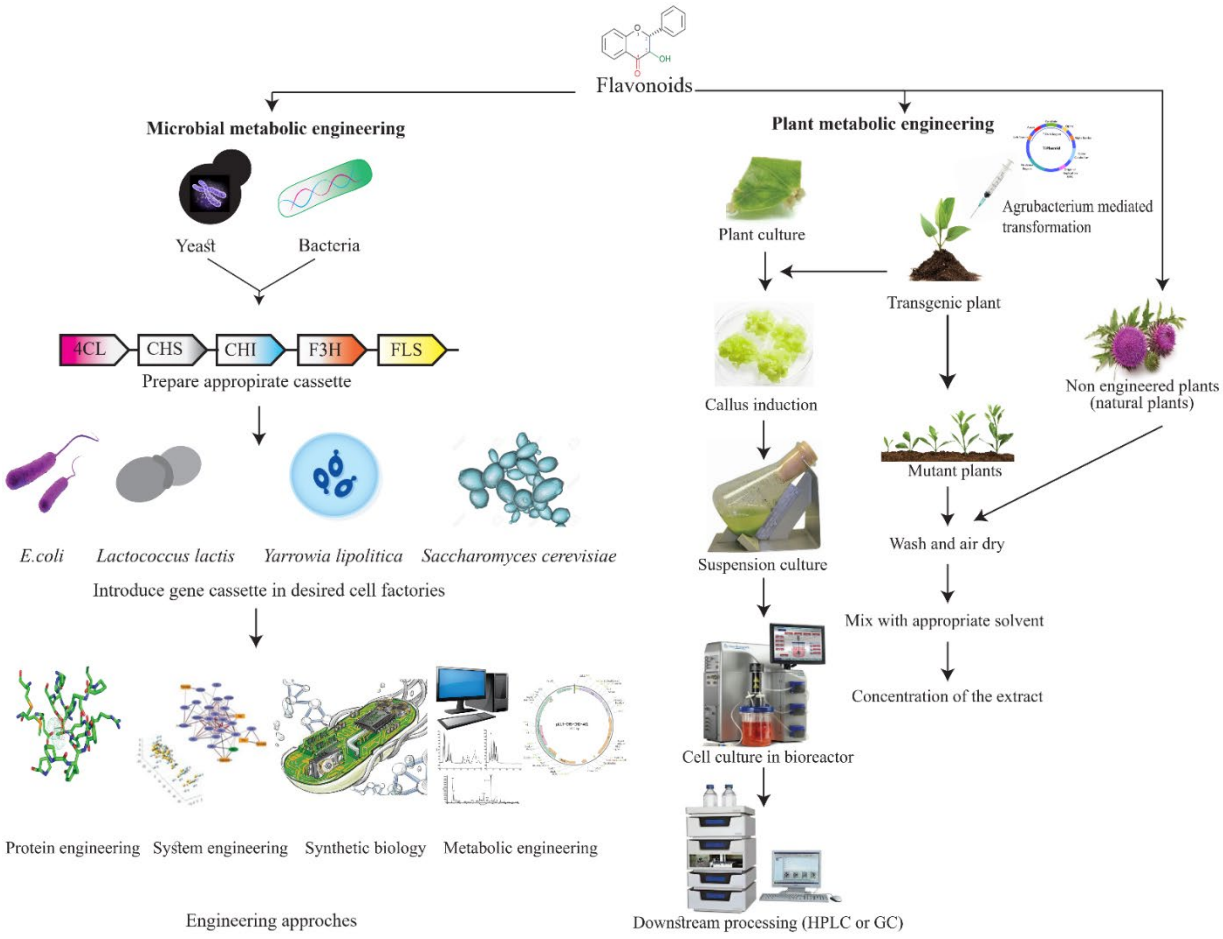


Fig. 2. Biotechnological production of flavonoids from plant, suspension culture and microbial metabolic engineering. Plant extraction uses toxic solvent which is not environmentally-friendly. Plant suspension culture is an alternative strategy with drawbacks including economic feasibility, scalability and culture stability. Microbial metabolic engineering may overcome some of the limitations. Biosynthetic gene clusters encoding flavonoid pathways could be assembled in a number of hosts including *E. coli, S. cerevisiae, Y. lipolitica* or *L. lactis*. Specific modification of pathways will enhance the rate of substrate uptake; reduce undesirable by-products accumulation and improve precursor and cofactor flux *et al*.

Alternatively, microbial synthesis of flavonoid in heterologous hosts has attracted significant interests for industry and academia. The engineering of microbes by reconstructing the flavonoid biosynthetic pathways explores the power of fermentation to produce specific compounds under

controlled conditions. Recent advances toward improvement of flavonoid production using synthetic microbial factory and bioreactor-based systems have significantly improved the expression of single gene or entire metabolic pathways, which allowed the biosynthesis of high value products in a relatively short time and at large quantity. Microbial factories pose many advantages over plant extraction and cell cultures or chemical synthesis such as eco-friendliness, rapid growth rate, streamlined cultivation facility and availability of advanced genetic manipulation techniques. In this part, we review the current metabolic engineering approaches to produce flavonoids in various host strains. Some applicable strategies which have been used for production of flavonoids are summarized in Fig. 2.

3.1 Microbial metabolic engineering and the host criteria

In order to effectively implement metabolic engineering strategies, the following factors should be considered: I) using a robust host organism with genetic and physiological advantages for the high production of target product; II) a thorough understanding of the metabolic pathways with an emphasis on the cofactor balances and regulatory networks; III) selection of most important enzymes and genetic factors in the metabolic pathway of the desired compound; IV) analysis of stoichiometry and thermodynamics for native or introduced pathway; V) identification and availability of endogenous pathways with strong precursor flux; VI) efficient transformation method/s; and VII) existing strategies for reducing toxic intermediates and sequestrating or separating end-products. For most of the metabolic engineering projects, model organisms such as *Escherichia coli* and *Saccharomyces* cerevisiae were mostly used chassis for production of high value natural products (Zhu and Jackson 2015).

3.2 *E coli* as flavonoid production host

In the past 20 years, metabolic engineering has focused rather extensively on diversifying chemical production in the bacterial system *E. coli* due to simplified genome structure and robust, yet centralized regulatory systems (Table 1). It has been widely adopted as the production host for the biosynthesis of phytochemicals, especially flavonoids. Simple genetic modification for semi-synthesis or *de novo* synthesis have been performed by introducing flavonoid biosynthetic gene clusters into *E. coli*. There is also an advantage to introducing simple chemical modifications into flavonoids through combinatorial biosynthesis (Chemler, Yan et al. 2007, Zhang and Tang 2008, Kim, Yang et al. 2015).

3.3 *S. cerevisiae* as flavonoid production host

E. coli is not always the ideal host due to relatively low stress tolerance, a lack of post-translational modifications, difficulty in expressing complex enzymes like P450s, and a lack of subcellular compartments. In contrast, yeasts often possess spatially-separated organelles and also have favorable bioprocessing traits such as a larger cell size (thus enabling an easier separation), a lower growth temperature, lower pH and high by-product tolerance, a lack of potential phage contamination. The specialized organelles in yeast supports functional expression of membrane-bound cytochrome P450 enzymes that they are often absent in simple prokaryotic microbes such

as *E. coli*. P450 enzymes require the attachment to the eukaryotic cell's endoplasmic reticulum (ER) membrane and a redox partner typically in the form of a P450 reductase that transports electrons from the NADPH donor to the heme-core of the P450 complex (Lv, Edwards et al. 2019, Lv, Marsafari et al. 2019). Some of the flavonoid biosynthetic enzymes, including the C4H hydroxylase or phenol monooxygenase, belong to the P450 enzyme class. This feature, together with the expectation of better expression of the plant-derived enzymes due to the capability of posttranslational modifications and its phylogenetical similarity to plants, makes yeast an attractive host platform for flavonoid biosynthesis (Table 1). Moreover, yeast mating allows for improved cellular engineering and can lead to diploids with robust growth and increased adaptation. Collectively, these advantageous traits support the industrial use of yeast for chemical and fuel production.

For example, the subcellular compartmentalization of yeasts allows for pathway insulation and increased fluxes towards heterologous product formation. Furthermore, the yeast kingdom is quite broad and while *S. cerevisiae* is conventionally used for metabolic engineering, robust nonconventional yeasts such as *Yarrowia lipolytica* and *Pichia ciferrii* are increasingly being recognized as promising hosts to produce unique and valuable compounds. Thus, interest has begun to switch from *E. coli* to yeasts as production hosts, but it is important to mention that both systems have been developed concurrently and have highlighted some important characteristics of phenylpropanoid biosynthesis in various hosts (Fowler and Koffas 2009, Rywińska and Rymowicz 2010, Liu, Redden et al. 2013, Liu, Liu et al. 2013, Paddon, Westfall et al. 2013).

3.4 *Yarrowia lipolytica* as a novel host for flavonoid production

E. coli and *S. cerevisiae* have long been established as host strains to manufacture a large variety of plant natural products (Xu, Bhan et al. 2013). The recent emergence of oleaginous yeast platform offers a number of advantages over the *E. coli* and *S. cerevisiae* host (Table 1). *Yarrowia lipolytica,* as an oleaginous yeast cell factory for production of heterologous proteins and triacylglycerides, has attracted much of the industrial and academic interest with its high secretion capacity, a simple glycosylation pattern, a large range of genetic markers and molecular tools, and the status of "generally regarded as safe" (Liu, Liu et al. 2013). Due to its unique physiological, metabolic and genomic characteristics, *Y. lipolitica* has been widely used as a microbial host in metabolic engineering. The recent progress of manipulating internal pathways and introducing new pathways to this host have demonstrated that *Y. lipolytica* can be used as a potential platform for industrial production of chemicals and fuels derived from fatty acids, lipids and acetyl-CoA in a short time at low cost (Zhu and Jackson 2015, Xu, Qiao et al. 2016, Qiao, Wasylenko et al. 2017, Xu, Qiao et al. 2017). In addition, *Y. lipolytica* is known to internalize substantial portion of carbon feedstock as lipids and fatty acids (Blazeck, Hill et al. 2014, Xu, Qiao et al. 2017), which provides the ideal environment for nonaqueous catalysis and regioselectivity for many enzymes. Coupled with its ability to degrade a wide range of substrates, including hexose/pentose, glycerol, alkanes, alcohols and volatile fatty acids (VFAs), its low pH tolerance and strictly aerobic nature (Abghari

and Chen 2014, Ledesma-Amaro, Lazar et al. 2016, Liu, Marsafari et al. 2019), making this yeast an attractive candidate for industrial applications.

Table 1. **Comparison of *E. coli*, *L. lactis*, *S. cerevisiae* and *Y. lipolytica* as chassis to produce specialized flavonoids.**

Expression platform	***E. coli***	***L. lactis***	***S. cerevisiae***	***Y. lipolytica***
Genetic tools	+++++	+	+++++	+++
Genome annotation	+++++	++++	++++	++++
Acetyl-CoA/Malonyl-CoA flux	++	++	+++	+++++
Substrate flexibility	++++	+++	++	++++
Acid tolerance	+++	+++++	++	++++
FDA safety	++	+++++	+++++	+++++
Specific Glycosylation	++++	+++	++	++
P450 hydroxylation	++	++	++++	+++++
Prenylation	++	++	+++	++++

Flavonoid biosynthesis starts with the condensation of multiple malonyl-CoAs by the type III polyketide synthase (Fowler and Koffas 2009). Considering the high acetyl/malonyl-CoA/HMG-CoA flux demand and the regioselectivity requirement of side-chain modification in most flavonoids (Table 1), oleaginous yeast will be a superior host to produce pharmaceutically-relevant flavonoids including hydroxylated and prenylated flavonoids. Compared to *S. cerevisiae*, *Y. lipolytica* lacks Crabtree effects, which doesn't produce ethanol under high-glucose or respiration-limited conditions (Lv, Marsafari et al. 2019). The high precursor acetyl-CoA and malonyl-CoA flux along with the hydrophobic environment within the cell make oleaginous yeast a promising host to produce highly functionalized natural products. Oleaginous yeast is rich in membrane structure and subcellular compartment (i.e. lipid bodies or oleosome), which provides the hydrophobic environment that is critical for regioselectivity and stereoselectivity in hydroxylation and prenylation reactions (Aoyama, Korzekwa et al. 1989, Eichmann, Kumari et al. 2012, Negretti, Narayan et al. 2014).

In order to synthesize homoeriodictyol through transferring one methyl group of S-adenosyl-l-methionine (SAM) to eriodictyol and using flavone 3′-O-methyltransferase ROMT-9, a research team used recombinant *Y. lipolytica* with a growth phase-dependent constitutive promoter hp4d. The highest ROMT-9 activity reached 5.53 U/L after 4 days of culture in shake flask. The purified ROMT-9 was used to synthesize homoeriodictyol, and the maximal transformation ratio reached 52.4% at 16 h (Liu, Liu et al. 2013). In a recent study, Lv *et al* have developed an iterative gene integration and marker curation method to explore flavonoid biosynthesis in *Y. lipolytica* (Lv, Edwards et al. 2019). The authors further confirmed that (i) the bottlenecks of the hydroxylated flavonoid production in *Y. lipolytica* were CHS and CPR; ii) the optimal gene copy number of CHS and CPR was 5 and 2, respectively; iii) enhancing chorismate and malonyl-CoA pathways is critical to improve flavonoid production; iv) the optimal pH conditions and nutritional factors, i.e. C/N ratio were identified (Lv, Marsafari et al. 2019). *Gu* et al have refactored the Ehrlich pathway

for high-yield aromatic production in *Y. lipolytica* (Gu, Ma et al. 2020), indicating the strong shikimate flux could be harnessed for *de novo* synthesis of flavonoids. A recent study has expanded *Y. lipolytica*'s polyketide-producing capability to include 4-coumaroyl-CoA derived products, including resveratrol and bisdemethoxycurcumin (Palmer and Alper 2018, Palmer, Miller et al. 2020).

3.5 Probiotic bacteria *L. lactis* as flavonoid production host

Lactococcus lactis, a gram-positive probiotic bacterium, has a relatively simple metabolism, compact genome structure (~2.5 Mb) and a long history of safe use in food industry. The generally-regarded as safe (GRAS) status of this organism, as well as robustness, stress tolerance, and genetic accessibility make this species an attractive candidate for production of food and pharmaceutical ingredients (Gaspar, Carvalho et al. 2013), including enzymes, protein or peptide ingredients and therapeutic proteins (de Vos and Hugenholtz 2004). Arguably the most valuable genetic tools is the nisin-controlled gene expression system (NICE); this system allows for the inducible expression of a gene of interest when placed under the control of the inducible promoter P_{nisA} episomally or chromosomally, in the presence of nisin (Mierau and Kleerebezem 2005). Recent metabolic engineering of *L. lactis* has been foused on: (1) introduction and over-expression of homologous and/or heterologous genes and metabolic engineering of spesific pathway to obtain large quantities of interested natural products; (2) expression of prokaryotic and eukaryotic membrane proteins; (3) protein secretion and anchoring in the cell envelope; (4) expression of genes with toxic products and analysis of essential genes and (Mierau and Kleerebezem 2005).

A recent study reported that *L. lactis* can be engineered as an efficient cell factory for flavonoids production. Specifically, the heterologous pathway for stilbene resveratrol was assembled. The strain was further optimized in order to enhance the production efficiency. Analysis of the phenolic content in culture supernatants revealed that the assembled biosynthetic pathway is functional, and the best producer strain yielded 3.0 ± 0.7 μM of resveratrol and 0.7 ± 0.1 μM of *p*-coumaric acid. In order to test whether malonyl-CoA is a bottleneck in *L. lactis*, the production strain was treated with cerulenin, an inhibitor of fatty acid synthase that is known to increase malonyl-CoA levels in bacteria. Addition of cerulenin 2 hours post-induction resulted in about 4-fold increase of resveratrol production, proving that the malonyl-CoA pool is indeed the limiting factor here. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA into malonyl-CoA, when ACC genes of plant and fungal origins were integrated into the genome of *L. lactis*, resveratrol production was increased by about threefold (Gaspar, Dudnik et al. 2016).

4. Biosensors and their application in flavonoids production

Polyphenols and flavonoids are typically measured with low through-put liquid or gas chromatography methods (Litescu, Eremia et al. 2010). One of the major challenges for screening mutant strains or libraries is the lack of highly specific, rapid and sensitive analytical methods. One cannot rely on the time-consuming or trivial HPLC or LC-MS to analyze the samples. Biosensors rely on the molecular interaction of DNA, RNA or protein and metabolites, capable of

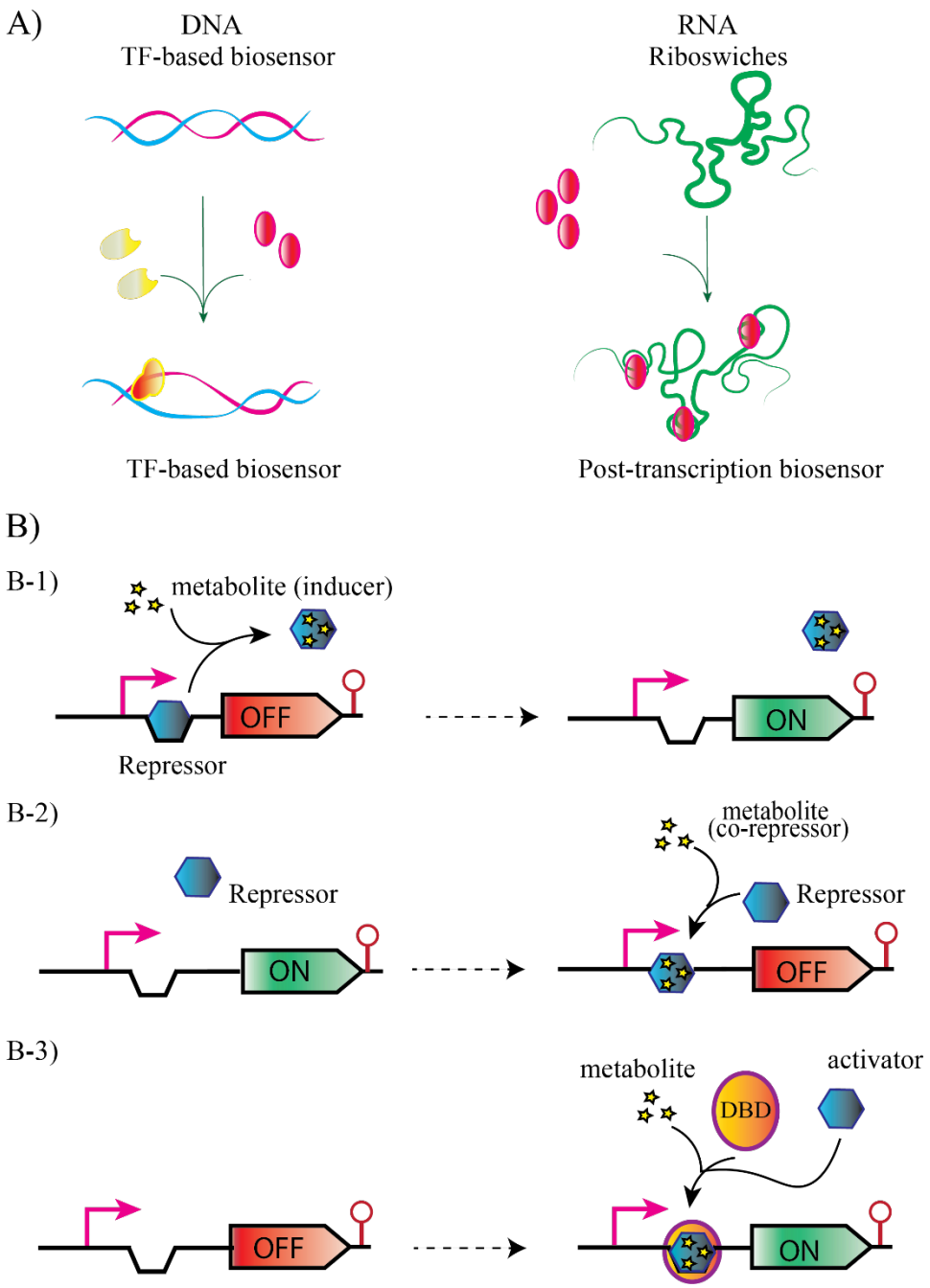
providing specific quantitative or semi-quantitative information based on biological reporter output. For protein-based biosensors, the receptor domain will specifically interact with a target molecule and a transducer domain will propagate a conformational change leading to altered DNA-binding activity or enzyme activity. Compartmentalized biosensors may be used as a tool to explore tissue-specific metabolic heterogeneity and discovery of novel metabolic pathway regulators. Some of the hallmark biomolecules can be perceived by a wide range of natural sensors/actuators such as riboswitches, transcription factors (TF) or enzymes. We will summarize two major categories of genetically encoded biosensors for flavonoids: TF-based biosensors and RNA-based (riboswitches) biosensor (Fig. 3A).

4.1 Transcriptional factor-based biosensors

Transcription factors (TF) are sensory elements that control transcriptional regulation based on the concentration of metabolite or environmental changes. Hence, hacking the TFs into the host transcription system and rewiring the native or synthetic promoter provide us a powerful toolset to probe the intracellular metabolite change. This is often achieved by linking the transcriptional input (ligand molecule or target molecules) to a transcriptional output (reporter protein, i.e. GFP) (Zhang, Jensen et al. 2015). The design of sensor-reporter system based on native TF is simple but due to uncharacterized crosstalk between candidate TFs and non-cognate operator sites, they suffer from poor orthogonality and background noise (Lefrançois, Euskirchen et al. 2009, Xu, Li et al. 2014, Xu, Wang et al. 2014, Becker, Beer et al. 2015). Furthermore, non-native chemicals produced may not be directly sensed by a native TF. Introducing putative metabolite-binding domain and constructing synthetic TFs through protein directed evolution, have proven as effective strategies to overcome the challenges in biosensor design. Prokaryotic transcriptional repressors are suggested as wide toolkit of potentially orthogonal metabolite-binding TFs for the applications as biosensors in eukaryotes. Despite the differences between transcriptional regulation in prokaryotes and eukaryotes, early work has suggested that prokaryotic transcriptional repressors may be functional in eukaryotes (Brent and Ptashne 1984).

As illustrated in Fig. 3B, three different type of prokaryotic sensor-reporter systems have been investigated ever since. One type of this repressor is the repressed-to-derepressed mode (i.e. LacI repressor for sensing lactose). In the absence of effector molecule, repressor binds to its cognate operator and represses gene expression. Once the effector molecules become available, it will form a complex with the repressor causing the dissociation of repressor from the operator, thus gene expression is switched from the OFF (repressed) to ON (de-repressed) state. (Fig. 3B-1) (Teo and Chang 2014, Teo and Chang 2015). Another type of this repressor system is the derepressed-to-repressed mode (i.e TrpR repressor for sensing tryptophan). In the absence of effector molecule (which also called co-repressor), gene expression is kept at ON state. Once the effector molecules become available, it will form a complex with the repressor and the TF-metabolite complex will occupy the operator, thus gene expression is switched from the ON (de-repressed) to OFF (repressed) state (Fig. 3B-2). A third type of TF-based sensor-regulator could be constructed by fusing a putative DNA binding domain (DBD) with a metabolite binding domain to produce a

hybrid activator, which has proven to be effective when a prokaryote-derived transcriptional repressor is applied to eukaryotic hosts. The hybrid activator consequently could sense the desired metabolite and output an increased gene expression in the system (Fig. 3B-3) (Moser, Horwitz et al. 2013, Umeyama, Okada et al. 2013). The application of TF-based biosensor in combination with autofluorescent proteins have been reported to improve flavonoid production in *E. coli*. A number of flavonoid-responsive transcriptional factors, like QdoR from *Bacillus subtilis* and FdeR from *Herbaspirillum seropedicae*, have been engineered to sense different types of flavonoids recently (Siedler, Stahlhut et al. 2014, Wang, Cress et al. 2019). The authors demonstrated that there was a linear correlation between the fluorescence intensity and externally added flavonoids, albeit with different specificity and dynamic response range.



458

459 Fig. 3. Schematic pictures of different type of biosensors. A) Three different biosensor models that have
460 been introduced and their mechanism of action in the presence of ligand. B) The suggested models for
461 application of TF-based biosensors in the engineering research. B-1) In the absence of ligand, prokaryotic
462 repressors bind to its operator and it may dissociate from DNA only when the ligand becomes available.
463 B-2) in this scenario, in the absence of ligand, repressor doesn't repress gene expression. Upon binding
464 with ligand (co-repressor), the TF-metabolite complex will occupy the operator, thus gene expression was
465 turned off. B-3) The fusion of putative eukaryotic activator domain to prokaryotic DNA binding domain
466 (DBD) can be used for construction of hybrid transcriptional activator, the DBD-activator complex will
467 activate gene expression upon binding with the small ligand (metabolite).

4.2 RNA-based (riboswitches) biosensor

Riboswitches are the mRNA regulatory domains that can selectively bind to a ligand. Riboswitches generally consist of a sensing domain and a regulating domain (Tucker and Breaker 2005, Wittmann and Suess 2012). Original or modified RNA, single or double stranded DNA can be utilized as sensing domain and have been engineered to bind variety of targets from small organic molecules to entire organisms (Lee, Hesselberth et al. 2004). Entrapment of a metabolite in the riboswitch stabilizes the ligand-bound conformation of the aptamer and consequently induces structural changes in the adjacent RNA/DNA region, thereby regulating transcription or translation (Serganov and Nudler 2013). Riboswitches respond faster in comparison with TF-based sensor because they do not rely on transcriptional level TF–RNAP or TF–metabolite interactions: the RNA has already been transcribed and is readily available for effector binding. Similarly, antisense RNA has the same feature and was used to dynamically regulate metabolic flux in *E. coli* (Yang, Lin et al. 2018) in a recent study.

Along with the different structures of all known riboswitches, most bacterial riboswitches regulate gene expression at both transcriptional or translational level (Fig. 4). Transcriptional termination is one of the most common mechanisms used by bacterial. The formation of a stem loop structure followed by the uridine residues leads to a transcription termination signal and release RNA polymerase from DNA template. Anti-terminator (a type of riboswitch) will block the formation of functional terminator upon binding with ligand, thus inhibiting the transcriptional termination (Fig. 4A). In the case of interfering translation initiation strategy, ligand-binding will induce structural changes that hide ribosome binding site or Shine-Dalgarno (SD) sequence from ribosome (Fig. 4B), thus inhibiting the translational initiation. In eukaryotes especially in plant and fungi which possess RNA processing mechanism, the most common riboswitches rely on alternative mRNA splicing to adjust the accessibility of splice sites in responsive to the thiamin pyrophosphate (TPP) (Sudarsan, Barrick et al. 2003). When TPP is not accessible, the riboswitch is unfolded, and complementary regions of the riboswitch interact with the adjacent sequence leading to the formation of mature mRNA. While in the presence of TPP, a secondary stem loop named as anti-splice site prohibits the splicing leading to the formation of alternate long 3′ UTR (Fig. 4C) (Breaker 2012, Serganov and Nudler 2013). Regarding the detailed mechanism in this part, please refer to the listed references (Breaker 2012, Wittmann and Suess 2012, Serganov and Nudler 2013, Groher and Suess 2014). Engineered riboswitches have been recently used for improvement of naringenin production (Jang, Xiu et al. 2017). When *E. coli* co-culture system was coupled with riboswitches, the authors obtained a significant correlation between fluorescence output of the biosensor strain and naringenin production of the metabolite-producing strain (Xiu, Jang et al. 2017)..

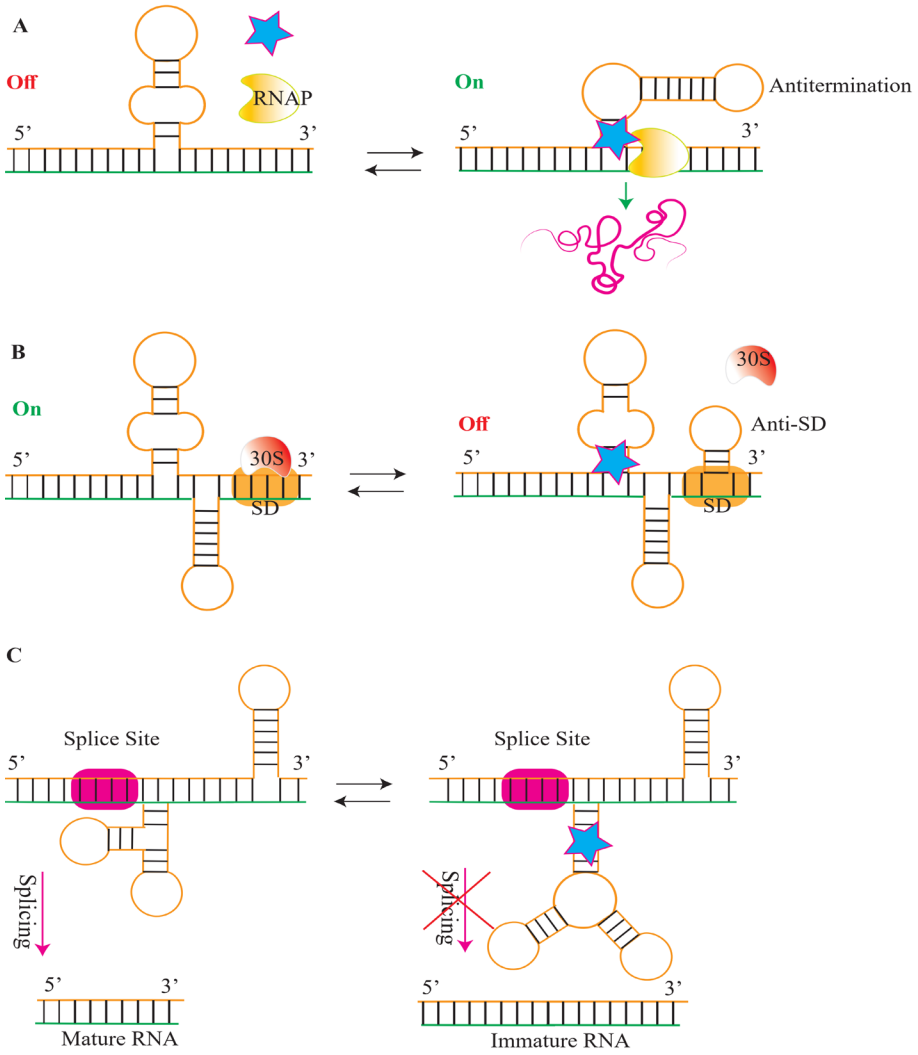


Fig. 4. The common mechanisms used by the riboswitches. A and B used for bacteria and C is the most common riboswitches type in the eukaryote. A) Regulation of transcription termination. In this mechanism and in the absence of ligand, a complementary sequence causes the RNA polymerase (RNP) dissociation. When the ligand is available, the secondary structure (Antitermination) form and RNP continue transcription. B) Regulation of translation initiation. The formation of stem loop structure establishes the access of Shine-Dalgarno (SD) sequence for small subunit of ribosome (30S). Ligand binding form folding the aptamer domain and the alternative stem loop block the access of small ribosomal subunit to the SD sequence subsequently inhibit the initiation of translation. C) In the eukaryote in the presence of thiamin pyrophosphate (TPP) coenzyme, by the formation of the anti-splice sites inhibit mRNA splicing and produce immure RNA.

5. Conclusion

Flavonoids as a large group of polyphenols are ubiquitously distributed in wide variety of plant species. These secondary metabolites are remarkable due to their valuable bioactivities in human health for treatment and prevention of most aging-related chronic diseases. There is an increasing

market demand of naturally derived flavonoids, an estimated global market at $ 200 M per year. Plant extraction could not meet this target due to complicated purification steps, low yield and scalability issues. In addition, the engineering of transgenic plants to improve flavonoid content is subjected to geographic conditions, climate change, specific developmental stage and/or species variations. After all, even if engineering of plant be successful, the plant extraction process yields a mixture of substituted flavonoids, which will require multiple purification steps adding to the cost and negatively impacting the environment.

Alternatively, heterologous production of flavonoids using microbial workhorse overcomes many bottlenecks associated with plant extraction and chemical synthesis. Microbial cell factories can use renewable and cheap feedstock to produce large quantity of flavonoids in a short period of time. Different microbial host could be tailored for specific chemistries that are important for diversifying the structure of various bioactive flavonoids. In addition, biosensor systems have been suggested as a promising tool for high throughput phenotypic screening of flavonoids. Genetically encoded biosensors can empower us to explore a larger cellular control and strain design space, which may significantly reduce our effort for strain optimization and evolution. Combined with microbial co-culture strategies (Xu, Marsafari et al. 2020), genetically-encoded biosensors have the advantage to tune the population dynamics of the engineered consortia and confer community-level metabolic performance (Xu 2018, Lv, Qian et al. 2019), with improved process economics and cost-efficiency. Novel genome-editing tools in this yeast, including CRISPR-Cas9 (Wong, Engel et al. 2017) or CRISPR-Cpf1(Yang, Edwards et al. 2020), will enable us to explore a larger number of genetic targets that may synergistically remove the pathway bottlenecks. Although microbial metabolic engineering strategies are still developing, the selection of the right host and the identification of rate-limiting steps will continue driving us to lower the cost and deliver economically-viable process for large-scale manufacturing of flavonoids. We imagine that the commercialized list of flavonoids will continue growing with new technologies contributed from novel microbial hosts, microbial co-culture and genetically encoded biosensors *et al*.

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Conflicts of interests

The authors declare that they have no competing interests.

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