International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (4), pp. 001-009, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Review

Advances in microbial heterologous production of flavonoids

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Accepted 21 February, 2019

Flavonoids are important plant specific secondary metabolites that are synthesized from the general phenylpropanoid pathway and have many pharmaceutical and nutraceutical functions. Separation of pure flavonoids from plants material is very difficult and chemical syntheses of flavonoids require extreme reaction conditions and toxic chemicals. However, in the past decades, engineered microbes are becoming increasingly important as recombinant production platforms. Cultivation of the recombinant strains in rich medium offers another choice for the production of flavonoids. In the present review, we cover the main achievements that the multi-gene pathway of phenylpropanoid is introduced as microorganisms to heterologously produce flavonoids, analyze the key factors affecting heterologous production of flavonoids in microbes, and discuss the new research prospect.

Key words: Flavonoids, metabolic engineering, natural products, synthetic biology.

INTRODUCTION

Flavonoids, containing a 15-carbon phenylpropanoid core (Figure 1), are plant secondary metabolites that occur in a large variety of plants, fruits and vegetables (Turnbull et al., 2004). Flavonoids have significant properties and applications, such as antioxidant activity that confers beneficial effects on coronary heart disease, cancer, and allergies (Shaik et al., 2006; Steinmetz and Potter, 1996). Flavonoid-derived compounds have drawn much attention from scientists and researchers, who are keen on their native nutraceutical properties or who use them as starting formulations for market pharmaceuticals (Fowler and Koffas, 2009).

Till date, the low yield of flavonoids yet remains a bottleneck for its large scale applications. When these compounds are separated from plant materials, the availability of plant flavonoids is first limited by seasonal and regional variations. Moreover, it continues to be a major challenge for separating pure flavonoid compounds from plants material because of the low concentrations of certain flavonoids and numerous similar natural products in plants (Du et al., 2010).

In addition, the chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals (Park et al., 2009). To date, mass production of flavonoids from plant cell or tissue cultures have been reported for a few species, while the economic feasibility has not been established due to engineering challenges in large-scale cultivation and difficulties to maintain the cell viability during long period (Kobayashi et al., 1993; Zhong et al., 1991).

Combinatorial biosynthesis is a new tool for production of rare and expensive natural products (Chang and Keasling, 2006; Hutchinson, 1994), and can be used in both simple and complex transformations without the tedious blocking and deblocking steps that are common in enantio and regioselective organic synthesis (Wang et al., 2010). These methods in flavonoids biosynthetic pathway are attractive targets for metabolic engineering processes to enhance the production of flavonoids.

This article reviews the main works done on the microbial synthetic flavonoids in the decades, including the optimization of synthetic route, carrier, and culture conditions, the selection of strains, and synthetic biology

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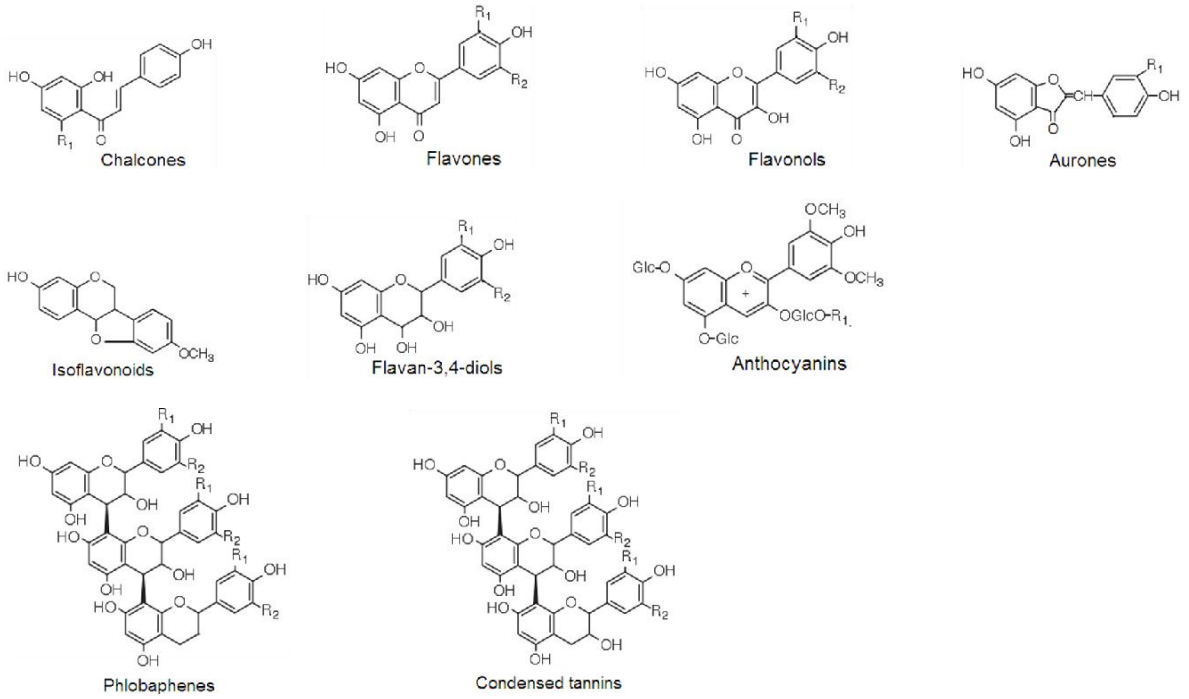

Figure 1. The biosynthesis of nine major classes of flavonoid derivatives starting with general phenylpropanoid metabolism. R_1= H or OH; R_2= H or OH.

used for production of flavonoids is also discussed.

Synthetic pathway and diversity of flavonoids

In the plant phenylpropanoid pathway (Figure 2), phenylalanine ammonia lyase (PAL) is the first enzyme in the general phenylpropanoid pathway, which catalyzes phenylalanine to yield cinnamic acid; Cinnamic acid is hydroxylated by the action of cinnamate-4-hydroxylase (C4H) to 4-coumaric acid. Then 4-coumaric acid is activated to 4-coumaroyl-CoA by 4-coumarate:coenzyme A ligase (4CL); Chalcone synthase(CHS) is a plant-specific polyketide synthase that uses a starter CoA-ester, which catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone, the precursor of a large number of flavonoids. In the last stages of the biosynthesis of flavonoids, naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) (Weisshaar and Jenkins, 1998).

Compared with the plant pathway, the heterologous expression of phenylpropanoid biosynthetic pathway (Figure 3) has many characteristics as follows: the PAL from *Rhodotorula rubra* can use both phenylalanine and tyrosine as substrates to synthesize flavonoids, which increases the selectivity of substrates (Kyndt et al., 2002; Rosler et al., 1997; Scott et al., 1992). Simultaneously, the combinatorial biosynthesis of flavonoids bypasses the C4H step. Because the C4H is a membrane-bound cytochrome P-450 hydroxylase, its activation requires molecular oxygen and a reducing equivalent from NADPH delivered via cytochrome P-450 reductase (Hotze et al., 1995; Pompon et al., 1996). Some microorganisms lack the cytochrome P-450 hydroxylase.

Additionally, some researchers reported the ScCCL that encodes a cinnamate/coumarate: CoA ligase from the actinomycete *Streptomyces coelicolor A3(2)*, which can directly attach CoA to both cinnamic acid and 4-coumaric acid (Hwang et al., 2003). By increasing the selectivity of substrates and shortening the reaction steps, the hetero-logous large-scale production of targeted flavonoids has been improved. These strategies also provide reference for the heterologous synthesis of other natural products in microorganisms.

Based on the synthesis of the precursor of flavonoids in the above flavonoids synthetic route, the structural diversities of flavonoids could originate from various modification reactions. Typical flavonoids modification reactions are mediated by O-methyltransferases (OMT), glycosyltransferases (GT), and cytochrome P450 etc (Ibrahim et al., 1998; Kim et al., 2006b; Schuler and Werck-Reichhart, 2003; Winkel-Shirley, 2001). Kim et al., 2006a) have reported the characterization and expression of SaOMT-2 from *Streptomyces avermitilis* MA-4680; SaOMT-2 transfers the methyl onto the 7-hydroxyl group of isoflavones, daidzein and genistein, and the flavones, kaempferol, apigenin, and quercetin, as well as the flavanone naringenin, which make various compounds only by means of using one gene.

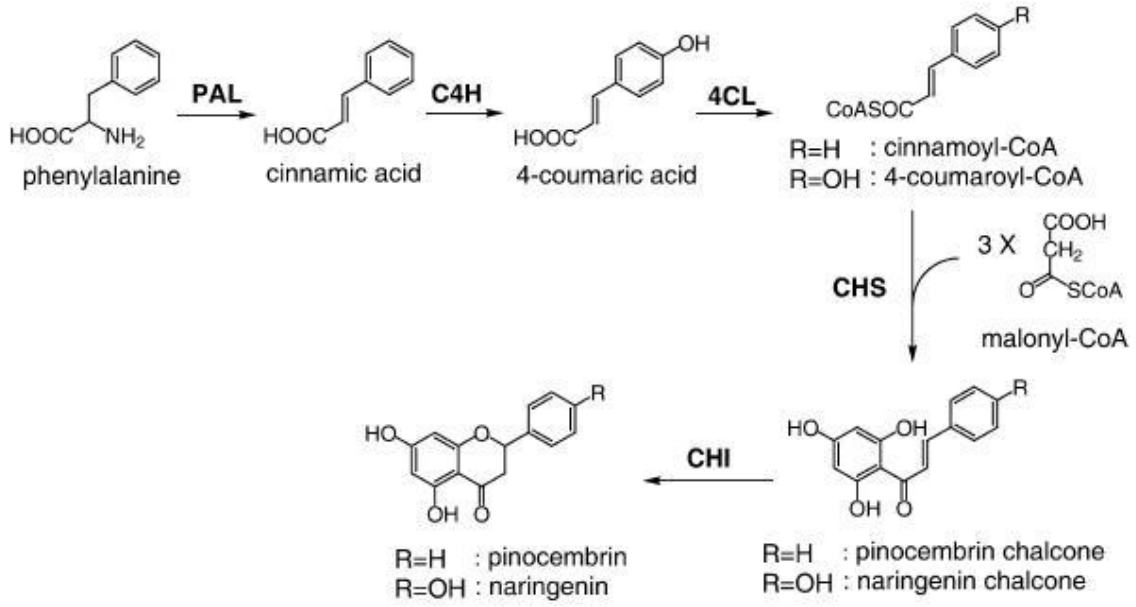

Figure 2. The schematic in the complete synthetic pathway of flavonoids in plant. Enzyme names are abbreviated as follows: Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl : CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI).

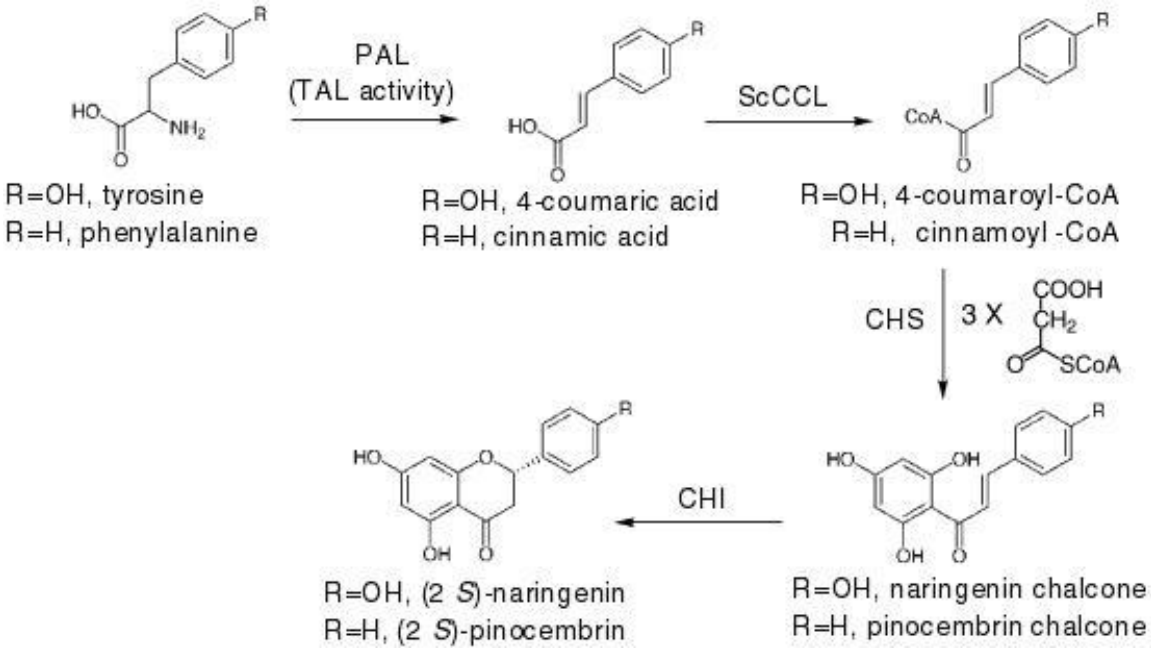

Figure 3. The heterologous biosynthetic flavonoids pathway in microorganism. PAL can also use tyrosine as a substrate; ScCCL attaches CoA to both cinnamic acid and 4-coumaric acid at the same efficiency.

In a previous study, genes representing enzymes of flavonoids pathway were individuallly cloned into yeast expression cassettes, then these cassettes were randomly combined on yeast artificial chromosomes. So these new combinatorial chromosomes create a variety of flavonoids producing pathways in a single transformation of yeast, which also presents a novel strategy for synthesis of diverse flavonoids *in vivo* (Michael et al., 2009). Only recently, construction of a multiplasmid approach for producing unnatural plant polyketides in *Escherichia coli* is very useful; incubation of the recombinant *E. coli* with exogenously supplied carboxylic

acids led to production of 36 unnatural flavonoids and stilbenes (Katsuyama et al., 2007b).

Strains

Escherichia coli

E. coli is widely used as model systems and considered as the primary prokaryotic host for the expression of heterologous genes due to its extensive genetic characterization (Krings and Berger, 1998). Moreover, most of its biological processes are well understood and there are extensive genetic tools readily available for its gene manipulation (Rodriguez et al., 2003). It is also firstly chosen as host and to heterologously produce the flavonoids by designing and constructing the artificial phenylpropanoid biosynthetic pathways.

Now about 0.75 mg/L of pinocembrin and 0.45 mg/L of naringenin could be produced with *E. coli* (Hwang et al., 2003). However, the yields of flavonoids were too low for large-scale production. This possibly resulted from the inefficient carbon flux from glucose, the amino acid precursors toward the phenylpropanoid biosynthetic pathway and the low amount of malonyl-CoA in *E. coli* cell.

Saccharomyces cerevisiae

Jiang et al. (2005) chose *S. cerevisiae* as the eukaryotic heterologous host to successfully produce the flavonoids after Ro and Douglas began to reconstitute the early steps of the phenylpropanoid pathway in *S. cerevisiae* (Ro and Douglas, 2004). In the *S. cerevisiae* AH22 strain that coexpressed PAL, 4CL, and CHS, approximately 7 mg/L of naringenin and 0.8 mg/L of pinocembrin could be produced. The yield in *S. cerevisiae* was higher than in *E. coli* which the phenylpropanoid pathway was firstly chosen to express.

The key factor is that *S. cerevisiae* has some advantages over *E. coli* for expressing certain eukaryotic heterologous proteins. Yeast system is not only capable of performing posttranslational modifications of the eukaryotic proteins but also has many similar intracellular compartments to plant cells. In addition, yeast has been shown to be an excellent host for CYP activity *in vivo* (Bayoumi et al., 2008; Humphreys et al., 1999; Jiang and Morgan, 2004; Pompon et al., 1996; Szczebara et al., 2003).

Other strains

Streptomyces venezuelae has a rapid growth, relative ease of genetic manipulation, abundant supply of substrates (Jung et al., 2006; Park et al., 2008; Yoon et al., 2002) and produces a wide range of important secondary metabolites (Pfeifer and Khosla, 2001), so it is also used as a robust hoterologous host for plant flavonoids production (Table 1).

Phellinus igniarius is a medicinal mushroom containing many bioactive compounds, and is viewed as a attractive alternative for the efficient production of secondary metabolites (Zhong, 2005). Zhu et al. (2010) have constructed an expression vector containing *Vitreoscilla* hemoglobin gene, which supplies more oxygen for the aerobic organisms growth, for the first successful and significant heterologous production of flavonoids in *P. igniarius* (Table 1).

Molecular biology technology used in the heterologous production of flavonoids

Knockout of related genes

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It is reported that, using the λ Red Recombinase/FLP system to knock out the udg gene encoding for UDP-glucose dehydrogenase, the endogenous UDP-glucose consumption pathway could be eliminated (Leonard et al., 2008). By the above molecular biological technology, UDP-glucose intracellular concentration was extremely improved, which resulted in the increment of the production of flavanones and anthocyanins to 700 and 113 mg/L, respectively.

Combination of promoter and target genes

The promoter often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used in the the synthesis of flavonoids such as T7 promoter (Kaneko et al., 2003), ermE* promoter (Park et al., 2009), GAL1 promoter (Yan et al., 2005) (Figure 4). It needs to choose the suitable promoter according to the specificity of host. In addition, every gene from the phenylpropanoid pathway is cloned under the control of the promoter, especially in *E. coli* and *S. venezuelae*. By employing a ribosomebinding sequence (rbs) in front of each gene, the yields of pinocembrin and naringenin could be enhanced strongly.

Over expression of malonyl-CoA

The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids (Davis et al., 2000). Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens*, Leonard et al. (2007) have augmented the intracellular malonyl-CoA pool and increased the production of pinocembrin, naringenin, and eriodictyol in 36 h up to 429, 119 and 52 mg/L, respectively. Moreover, the introduction of *R. trifolii* MatB and MatC genes allowed for the

Table 1. Heterologous production of flavonoids in various kinds of microbial.

End-product	Precursor molecule	Gene	Host organism	Level of production (mg/L)	Reference
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, ScCCL, CHS, RBS	*E. coli*	0.75 0.45	(Hwang et al., 2003)
Naringenin	Tyrosine	TAL, 4CL, CHS	*E. coli*	20.8	(Watts et al., 2004)
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, ScCCL, CHS, CHI, ACC	*E. coli*	58 57	(Miyahisa et al., 2005)
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, 4CL, CHS	*S. cerevisiae*	0.8 7	(Jiang et al., 2005)
Apigenin Luteolin Genkwanin	Phenylpropanoid acids	4CL, CHI, CHS, FSI, OMT	*E. coli*	0.415 0.01 0.208	(Leonard et al., 2006b)
Pinocembrin Naringenin Eriodictyol	Cinnamic acid *p*-Coumaric acid Caffeic acid	C4H, 4CL, CHI, CHS	*S. cerevisiae*	16.3 28.3 6.5	(Yan et al., 2005)
Naringenin Dihydrokaem-pferol Kaempferol	*p*-Coumaric acid	4CL, CHS, CHI, FHT, FLS	*E. coli*	2.4 2.4 0.3	(Leonard et al., 2006a)
Chrysin Galangin Apigenin Kaempferol	Phenylalanine Tyrosine 	pET-PT7-4GS(05,3) ACC, FNSI, F3H, FLS	*E. coli*	9.4 1.1 13.0 15.1	(Miyahisa et al., 2006)
Genistein	Tyrosine	PAL, ScCCL, CHS, CHI, IFS	*E. coli* and *S. cerevisiae* cells	6	(Katsuyama et al., 2007a)
Flavanone Dihydroflavonol	*p*-coumaric acid	4CL, CHS, CHI, FHT	*S. cerevisiae*	60 62.8	(Chemler et al., 2007)
Flavanone Flavone Flavonol	*p-coumaric acid*	pCDF-LE4CL-1 pRSF-ACC pET-CHS/CHI pACYCDuet-1 pACYC-FNS pACYC-F3H/FLS	*E. coli*	87 84 33	(Katsuyama et al., 2007b)

Table 1. Contd.

Pinocembrin Naringenin Eriodictyol	Cinnamic acid *p*-Coumaric acid Caffeic acid	4CL, CHS, CHI, ACC, biotin ligase	*E. coli*	429 119 52	(Leonard et al., 2007)
Pinocembrin Naringenin Eriodictyol	Cinnamic acid *p*-Coumaric acid Caffeic acid	4CL, CHS, CHI	*E. coli*	710 186 54	(Leonard et al., 2008)
Pinocembrin Narigenin	4-coumaric acid or cinnamic acid	ScCCL , CHS, CHI	*S. venezuelae*	6.0 4.0	(Park et al., 2009)
Naringenin Genistein Kaempferol Quercetin	(*p*-coumaric acid, Phenylalanine) (Naringenin, *p*-coumaric acid, Phenylalanine)	pESC-URA-PAL-C4H pESC-HIS-4CL pESC-LEU-CHS-CHI pESC-TRP-CPR pESC-HIS-4CL-IFS pESC-HIS-4CL-FLS pESC-TRP-F3H-CPR pESC-TRP-F3H-F30H	*S. cerevisiae*	15.6, 8.9 7.7, 0.14, 0.1 4.6, 0.9, 1.3 0.38,0.26,ND	(Trantas et al., 2009)
Naringenin Eriodictyol	phenylpropanoic acid		*E. coli*	270 150	(Fowler et al., 2009)
Flavones Exopolysaccharides	ND_1	ND_2	*P. igniarius*	11.43 1.33	(Zhu et al., 2010)

ND indicates the compound was not found after extraction.
ND_1 indicates that there is no precursor molecule.
ND_2 indicates that there is not an artificial biosynthetic gene cluster of flavonoids in the *P. igniarius*.

assimilation of an exogenous carbon source to directly synthesize malonyl-CoA. This strategy bypassed the natural metabolism of malonyl-CoA from glucose, and directly increased the production of malonyl- CoA (Leonard et al., 2008).

Construction of artificial P450 enzymes

The membrane-bound cytochrome P450 enzymes preclude the use of industrially relevant prokaryotes such as *E. coli*. Koffas et al. have constructed an artificial plant cytochrome P450 enzyme for synthesis of isoflavones. The production catalyzed by P450 in vivo was improved to 20-fold higher than that achieved by the native enzyme expressed in a eukaryotic host and up to 10-fold higher than production by plants. It is an innovative method for the utilization of laboratory bacteria to robustly manufacture high-value plant P450 products (Leonard and Koffas, 2007).

Synthetic biology

Synthetic biology is a research field that designs a biological system which behaves predictably and functions superior to the natural counterpart by applying engineering tools (Liang et al., 2011). The practical applications of synthetic biology are in the

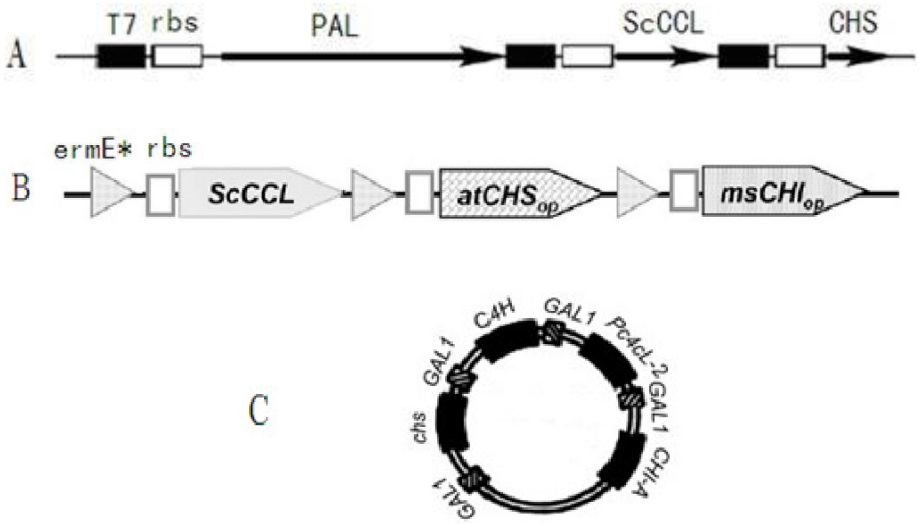

Figure 4. Schematic representation of the cloning strategy used for different assembling plasmid. A The PAL, ScCCL, CHS genes were placed under the control of the T7 promoter. B Construction of expression plasmids carrying flavanone biosynthetic genes and ermE*promoter. C The plasmids carrying four flavanone biosynthetic genes and GAL1 promoter.

areas of bioremediation (Gilbert et al., 2003), biosensing (Rajendran and Ellington, 2008), and biofuel production (Steen et al., 2008; Waks and Silver, 2009) and even with potential clinical applications (Anderson et al., 2006; Khosla and Keasling, 2003; Ro et al., 2006). Although we are still very far from rationally assembling a living cell from scratch, and far from understanding all of the design principles which biological networks operate (Mukherji and Van Oudenaarden, 2009), now this emerging novel technology has been explored for use in higher production of flavonoids by the following designs.

Minimum genome factory

The Ikeda team of Kitasato University has constructed a versatile model host for the heterologous expression of secondary metabolites. They deleted a region of more than 1.4 Mb nonessential genes from the 9.02-Mb industrial microorganism *S. avermitilis* linear chromosome, which generates a series of defined deletion mutants that do not produce any of the major endogenous secondary metabolites found in the parent strain (Komatsu et al., 2010). In addition, to create a “minimum genome factory” where some nonessential genes are deleted from the bacterial genomes is also a national project in Japan. Target microorganisms are *B. subtilis, E. coli, C. glutamicum* and *yeasts* which include *S. cerevisiae* and *Schizosaccharomyces pombe* (Horinouchi, 2008). Using these model hosts, it will be beneficial for the extraction and purification of targeted products from fermentation, and will lay the foundation for large-scale synthetic flavonoids.

Multivariate-modular pathway

Multivariate-modular pathway is an innovative approach and effective strategy for assembling modules into intricate and customizable larger scale systems (Purnick and Weiss, 2009). Ajikumar et al. (2010) have reported a multivariate-modular system, which partitioned the taxadiene metabolic pathway into two modules: a native upstream methylerythritol-phosphate pathway forming isopentenyl pyrophosphate and a heterologous down-stream terpenoid–forming pathway. By the approach, they succeeded in increasing titers of taxadiene. The pathway of flavonoids synthesis may also be divided into several modules, and people can explore which step is the limiting factor in metabolic pathways such as the supply of UDP-glucose. And then unlock the potential of the phenylpropanoid pathway for higher production of flavonoids natural products.

Conclusion

The above studies clearly show that the artificial gene cluster containing enzymes of flavonoids synthesis converts phenylalanine to pinocembrin and tyrosine to naringenin in microbial. This proves that *E. coli, S. venezuelae*, *P. igniarius* and *S. cerevisiae* are excellent expression systems for reproducing the phenylpropanoid pathways of plant. To optimize the flavonoid production, some researchers have been exploring new strategies such as looking for the best flavonoids synthase gene, choosing the suitable promoter, constructing new carbon flow etc.

Nevertheless, the heterologous production of flavonoids in microorganism usually requires transferring the multi-gene of the whole pathway into the host strain, which limits its large-scale production as well as its product. Moreover, development of efficient recombinant production platforms for natural product biosynthesis is often limited by the availability of precursors and cofactors derived from the host's native metabolism. Another difficulty must also be addressed: the conditions of strains growth, the toxic and concentration of products in fermentation, byproducts also must be considered.

With the progress of microbiology and enzyme engineering technology, multi-enzyme systems would be constructed *in vitro* which contain the main enzymes of phenylpropanoid pathway, and synthesize flavonoids. This will overcome some kinds of restrictions in micro-organism fermentation production, such as the content of oxygen for aerobic organisms' growth in the fermentation, the transport of flavonoids compounds through the membrane. In addition to using enzyme catalysis, it will be more efficient and fast to produce flavonoids, and it has a high final concentration in the reaction solution. Further-more, immobilization of multi- enzymes would decrease the difficulty in the separation of enzyme and reaction mixture

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (20906016, 21076053), Technology Research and Development Program of Hangzhou (2090331N03), Special Foundation for New Researcher in Hangzhou Normal University (YS05203141), the Scientific Research Innovation Fund for Graduate Students of Hangzhou Normal University, and Zhejiang Provincial Innovative Incubation Projects for University Students (Emerging Artists Talents Scheme) (2010R421059).

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