RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering

Jian-Qiang Kong*

Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (State Key Laboratory of Bioactive Substance and Function of Natural Medicines & Ministry of Health Key Laboratory of Biosynthesis of Natural Products), Beijing, 100050, China

*Author to whom correspondence should be addressed;

E-Mail: jianqiangk@imm.ac.cn; TEL: (86-10)-63165169

Abstract

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) catalyzes the deamination of phenylalanine to cinnamate and ammonia, the first step of phenylpropanoid pathway. PALs are ubiquitous in plants and also commonly found in fungi, but have not yet been detected in animals. Typically, PAL is encoded by a small multigene family and the presence of PAL isoforms is a common observation. PAL belongs to the 3,5-dihydro-5-methylidene-4H-imidazol-4-one-containing ammonia-lyase family and has been shown to exist as a tetramer. Both the forward and reverse reactions catalyzed by PALs were of great interest and had potential industrial and medical applications. This review, therefore, covers the recent developments related to the PAL genes distribution, phenylalanine ammonia-lyase gene family, structure and function study of PALs, as well as the several potential applications of PALs. As a key gateway enzyme linking the phenylpropanoid secondary pathway to primary metabolism, PALs were extensively applied in heterologous hosts to produce phenylpropanoids. The review thereby highlights the synthetic potentials of PALs as a key component used in metabolic engineering and synthetic biology. Moreover, the other potential PALs applications, like enzyme replacement therapy of phenylketonuria, therapeutic enzyme in cancer treatment and microbial production of L-phenylalanine were also discussed in detail. Together these results provide a synopsis of a more global view of potential applications of PALs than previously available.

Key words Phenylalanine ammonia-lyase; phenylpropanoids; metabolic

engineering; synthetic biology

Abbreviations: **accABCD**, acetyl-CoA carboxylase complex; **CADC**, *trans*-cinnamic acid decarboxylase; **CHS**, chalcone synthase; **CHI**, chalcone isomerase; **C4H**, cinnamic acid 4-hydroxylase; **4CL**, 4-coumarate-CoA ligase; **CUS**, curcuminoid synthase; **CPR**, cytochrome P450 reductase; **FabA,** 3-hydroxydecanoyl-ACP dehydrase; **FabB**, β-ketoacyl-ACP synthase I; **FabD**, malonyl-CoA:ACP transacylase; **FabF**, β-ketoacyl-ACP synthase II; **FabG**, β-ketoacyl-ACP reductase; **FabH**, β-ketoacyl-ACP synthase III; **FabI**, enoyl-ACP reductase; **FabZ,** β-hydroxyacyl-ACP dehydratase; **F3H**, flavanone 3-hydroxylase; **F3'H**, flavonoid 3'- hydroxylase; **FLS**, flavonol synthase; **FNS1**, flavone synthase I; **IFS**, isoflavone synthase; **matB**, malonyl-CoA synthetase; **matC**, malonate carrier protein; **PAL**, phenylalanine ammonia lyase; **PAL/TAL**,PAL also has tyrosine ammonia-lyase (TAL) activity; **PDC**, *p*-coumarate decarboxylase; **PMT**, pinosylvin methyltransferase; **RS**, resveratrol synthase; **STS**, stilbene synthase; **TAL**, tyrosine ammonia-lyase activity.

1. Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) catalyzes the conversion of L-phenylalanine(L-Phe) to *trans*-cinnamic acid(*t*-CA) by a non-oxidative deamination(Fig.1).[1] This reaction is the first step in the phenylpropanoid pathway and ultimately leads to various phenylpropanoids and their derivatives, such as flavonoids, isoflavonoids, resveratrol, coumarins, stilbenes, anthocyanins, lignin, and other phenolic compounds. In addition to serving as cell wall components (lignin)[2] or signaling molecules (acetosyringone) [3] in plants, many of these compounds have also displayed promising biological activities, including flower and fruit pigments (anthocyanins),[4] UV protectants of plants (apigenin),[5] antitumor activities(luteolin, apigenin),[6-9] antimicrobial actions(1-methoxyficifolinol, licorisoflavan A, and 6,8-diprenylgenistein),[10] as well as anti-inflammatory and neuroprotective effects(rutin, pinocembrin).[11,12] Hence, PAL is considered to be the key entry point into the phenylpropanoid pathway, channeling the carbon flow from primary metabolism to secondary metabolism. PAL enzyme is thereby of particular interest to researchers and extensively studied.

At present, many PAL genes have been isolated and characterized from plants,[13-15] fungi [16-18] and prokaryotes.[19-21] The structure and mechanism of PAL have been well-characterized.[22-24] The activity of PAL is induced dramatically in response to biotic and abiotic stresses.[25-30] Moreover, PAL has recently been studied extensively for the aim of potential applications in clinical,[19,31,32] industrial,[33-36] and biotechnological areas.[37-40] As a key gateway enzyme linking the phenylpropanoid

secondary pathway to primary metabolism, PAL was therefore an attractive target for metabolic engineering and synthetic biology process.[33,37-39,41-43] Versatile artificial gene clusters carrying PAL were transferred into a variety of heterologous microbial cell factories for the fermentative production phenylpropanoid-derived natural products.[44,45] Furthermore, varied PALs may serve in therapeutic applications for treating phenylketonuria (PKU) [31,32,46] and cancer.[47,48] In addition to medical applications, PAL has synthetic potentials as a biocatalyst for L-phenylalanine production.[49-51] Diverse strategies were applied to enhance activity, stability and yield of PALs with the aim of improvement its applications in industrial and medical potentials. There are some reviews focusing on biotechnological production of PALs.[52-55] These reviews, however, did not cover the detailed strategies used for functional improvement of PALs in industrial and medical applications. This review, therefore, highlighted the breakthroughs of PALs as a key component used in metabolic engineering and synthetic biology. Furthermore, the advance in medical applications as a therapeutic enzyme for treating PKU and cancer and industrial applications as a biocatalyst for L-phenylalanine production was also discussed in detailed in the present review. Together these introductions provide a synopsis of a more global view of potential applications of PALs than previously available.

2. PAL genes distribution

Due to its central role in phenylpropanoid metabolism, PAL was studied extensively. PAL was first purified from *Hordeum vulgare* in 1961, [1] but was shown to widely

exist in plants(Table 1), [13-15] fungi [16-18] and prokaryotes [19-21] afterwards. In plants, PAL occurs widely, including monocots, [56-59] dicots, [13,14,60,61] gymnosperms,[62-65] ferns,[66] lycopods,[66] liverworts,[66] and algae.[67] The PALs from plants account for more than 90% of known PALs. PALs are also commonly found in fungi, mainly distributing in Basidiomycetes,[16,68-71] Ascomycete, [71] and yeast.[17,18,29,72-77] The ubiquitous PALs largely reflect the phenylpropanoids are common natural products in plants and fungi, serving diverse physiological functions. In bacteria, PALs are rare. To date, few bacterial counterparts have been identified in *Rubrobacter xylanophilus*,[19] *Streptomyces maritimus*,[78-80] *Streptomyces verticillatus*,[81,82] *Photorhabdus luminescens*,[20,83] and some species of cyanobacteria like *Synechocystis* sp., [84] *Leptolyngbya* sp.,[84] *Oscillatoria* sp.,[84] *Anabaena variabilis*,[21,31,85] and *Nostoc punctiforme*.[21] The rarity of PALs apparently mirrors the dearth of phenylpropanoids in prokaryotes. In these bacteria, the PAL product *trans*-cinnamic acid serves as an intermediate in the biosynthesis of antibiotic or antifungal compounds, like enterocin,[80,86-88] 5-dihydroxy-4-isopropyl-stilbene,[89,90] soraphen A[91,92] and cinnamamide.[82] So far, there are no reports for animal PALs.

3. Phenylalanine ammonia-lyase gene families

PAL proteins are encoded by a multi-gene family in almost all of the plant species studied (Table 1). The number of PAL genes varied greatly in different plant species, ranging from 2 to a dozen or more copies (Table 1). For example, there are two genes in lemon balm (*Melissa officinalis* L.),[93] *Ornithogalum caudatum*[94] and raspberry

(*Rubus idaeus*).[95] 20 PAL genes, however, were found in in maize(*Zea mays* L.).[96,97] Even in potato (*Solanum tuberosum* L. cv. Datura), the number of PALs may reach to approximately 40-50.[98] Of course, the definite gene number of *bona fide* PAL in several plant species was uncertain due to prediction result without functional characterization. [13,14,99,100]

To my knowledge, there are no reports about fungi and bacterial PAL with multi-isoenzymes. The presence of PAL isoforms is a common observation, but the significance of this diversity is still questioned.[101]

The PAL gene organization in the same gene family exists some variations.[14] For example, nine of twelve PAL genes in *Citrullus lanatus* contained no introns in their ORFs. The ORFs of the other three PAL genes, however, were interrupted by a single intron.[14]

The isoenzymes of the same PAL gene family are usually expressed differentially in various tissues. Three PAL members from *Salvia miltiorrhiza* showed differential expression in different tissues. SmPAL1 showed the highest expression in roots, followed by leaves, stems and flowers. SmPAL2 was predominately expressed in stems and flowers. Its expression levels in roots and leaves were very low. SmPAL3 had the highest expression in leaves, less in roots, stems and flowers.[13]

The individual PAL gene in the same family may respond differentially to biotic or abiotic stresses. The results reported by Hou *et al* indicated that all three PALs in *Salvia miltiorrhiza* were drought- and MeJA-responsive, but the time and degree of reaction differed from one another.[13] Although all induced by mechanical wounding,

three PAL genes of artichoke showed a gene-specific behaviour with respect to response time, level and duration of expression.[102]

4. Structure and function of PAL

PAL has the catalytic prosthetic 3,5-dihydro-5-methylidine-4H-imidazol-4-one group(formerly 4-methylideneimidazole-5-one; MIO, Fig. 2),[103,104] which is produced by the posttranslational and autocatalytic cyclization of the tripeptide of alanine, serine and glycine (Ala-Ser-Gly triad; ASG motif, Fig. 2) and acts as the electrophile in the reaction mechanism.[23,24,104,105] Deamination of L-Phe is dependent upon the MIO prosthetic group in the enzyme. MIO group is conserved in both ammonia-lyases, including PALs, TALs (Tyrosine ammonia-lyases) and HALs (Histidine ammonia-lyases), and aminomutases, that is L-phenylalanine and L-tyrosine 2,3-aminomutases.[52-55] The amino acid residues involved in formation of the MIO moiety were conserved and important to enzyme activity of PALs. Mutation of serine-202, a component of ASG motif, in PAL from parsley (*Petroselinum crispum*) to alanine resulted in the lack of catalytic activity. [106] The same conclusion can be drawn by several reports.[19,60,107-109] All of these observations showed the active site domain containing ASG motif played a crucial role in enzyme activity of PALs.

PALs had been shown to occur as a homotetramer *in vivo*[110,111] or *in vitro*.[112-114] When various PALs were co-expressed in the single heterologous host, however, the mixed purified proteins formed heterotetrameric enzyme exclusively and preferentially.[115] Many studies have shown that the PAL of monocotyledonous plants [96,114] and some

dicotyledons [116] catalyzed the deamination of both L-phenylalanine (PAL activity) and L-tyrosine (TAL activity), which indicated PAL and TAL activities reside in the same polypeptide. This activity is valuable for an alternative route avoiding the reaction catalyzed by cinnamate 4-hydroxylase(C4H).[37,117-119] The mechanism of how these bifunctional PALs are able to use both substrates is confirmed experimentally to be existence of substrate selectivity switch.[99] Phe is the key residue in the substrate selectivity switch. When mutated to His, this single amino acid functions as a highly efficient substrate selectivity switch, converting the enzyme from exclusively PAL activity to exclusively TAL activity.[99] When Phe144 of PAL1 from *Arabidopsis* was replaced by His, the F144H mutant displayed a marked reduction (30-fold) in affinity for the substrate phenylalanine.[99] The observation was supported by different experiments.[60,112-114,120,121]

5. A key component used in metabolic engineering and synthetic biology process

Up to date, PALs were introduced into different hosts, like *Escherichia coli*, [39,40,42,43,122-124] *Saccharomyces cerevisiae*,[37,38,42,125-127] *Pseudomonas putida*[33,128,129] and *Streptomyces*[80,130-132] to construct varied artificial biosynthetic gene clusters for the fermentative production of flavonoid and flavonoid-related compounds(Fig. 3-4). The reconstructed phenylpropanoid pathway in engineered microbes leads to the biosynthesis of a wide range of phenylpropanoid -derived compounds, including phenylpropanoid acids (*trans*-cinnamic acid (**1**)[33,41] and *p*-coumaric acid(**2**)[123,124]), flavanones ((*2RS*)-pinocembrin(**3**),[123,124] (*2RS*)-naringenin(**4**),[123,124]

(*2S*)-pinocembrin(**5**),[40,43,150] (*2S*)-naringenin(**6**)[43,86,122,126] and eriodictyol(**19**) [133]), flavones (chrysin(**7**)[122] and apigenin(**8**)[122]), flavonols (galangin(**9**),[122] kaempferol(**10**) [122,127] and quercetin(**28**) [127]), curcuminoids (dicinnamoylmethane(**11**),[134,135] 6-fluoro-dicinnamoylmethane(**11a**),[135] 6,6'-difluorodicinnamoylmethane(**11b**), [135] cinnamoyl-*p*-coumaroylmethane(**12**)[134] and bisdemethoxycurcumin(**13**)[134]), triketide pyrone(**14,15**),[134] stilbenoids (*trans*-resveratrol(**18**),[136,137] pinosylvin(**20**),[137,138] pinosylvin monomethyl ether(**21**),[163] pinostilbene(**22**), [163] pinosylvin dimethyl ether(**23**) [163] and pterostilbene(**24**) [163]), chalcone (2',4',6'-trihydroxydihydrochalcone(**25**) [37]and phloretin (**26**)[37]) and isoflavone (genistein(**27**) [42,155]) (Fig. 3-4, Table 2) , which collectively have diverse biological functions as anticancer activity,[79,80,139] neuroprotection effect, [140] antiviral potency,[132] nephroprotective activity,[141] antimicrobial effect, [142,143] anti-inflammatory and antioxidant activities.[97,134] Moreover, some engineered organisms can produce commodity petrochemical with diverse commercial applications, like styrene(**16**) [37,39,125] and *p*-hydroxystyrene(**17**)(Fig.4, Table 2).[33,144]

Although various complex chemicals of therapeutic and industrial importance were produced by these engineering microorganisms containing PALs, the yield is in very small amount.[37,123,124,126] It is necessary to improve the yield of flavonoids produced by engineered cell factories. Common methods used to improve production from engineered biosynthetic pathways include selection of appropriate hosts,[33,38,130,136] enhancing production of pathway enzymes,[43,123-125] yield enhancement of the intracellular pool of precursors,[43,122] balancing multi-gene expression to optimize

flux,[40,126,136] alleviating the metabolic burden,[33,40,136,145] and optimization of the culture conditions.[41,43,136]

5.1 Selection of appropriate hosts

According to the characteristic of the pathway enzymes, varied hosts such as *E.coli*, [39-43,86,117,122-124,133,134,136,137,144-147] *S.cerevisiae*,[37,38,125,126] *P. putida* [33,128,129] and *Streptomyces* [80,130-132] were selected as production platforms for the synthesis of flavonoids.

E.coli is a preferred host for flavonoids production due to its fine characteristics like fast growth rate, inexpensive fermentation media and well understood genetics. Even more important, almost all *E.coli* strains and vectors can be commercially available, facilitating most extensive application in synthetic biology. Up to date, engineered *E.coli* can be used to produce various flavonoid molecules, such as flavanones ((*2S*)-pinocembrin(**5**), (*2S*)-naringenin(**6**), eriodictyol(**19**)), flavones (chrysin(**7**), apigenin(**8**)), flavonols(galangin(**9**), kaempferol(**10**)), isoflavones(genistein(**27**)), stilbenes (*trans*-resveratrol(**18**), pinosylvin(**20**)) and so on. Varied *E.coli* strains were selected to express heterologous proteins based on quality of engineered pathways. For example, when multi-gene pathway under regulations of the same promoter was introduced into *E.coli*, to prevent a deletion of the repeats as a result of possible homologous recombination, a recA mutant *E.coli* BLR (DE3) was used to be a host.[43,122,134] Moreover, to make more precursors enter the target metabolic flux, precursor-overproducing *E.coli* strains were usually used as chassises for increased yields.[86,145]

It is generally accepted that *E.coli* has a disadvantage in cytochrome P450 expression.[117] As the biochemical knowledge regarding cytochrome P450s increases, so does the efficiency of functional P450 protein expression in *E.coli* through various modifications including N-terminal modifications,[133] and using optimized genes.[133] Used alone or in combination, these methods can improve protein expression in *E.coli*, which will undoubtedly extend the application area of *E.coli* as a host in synthetic biology.[148]

Although extensively applied in metabolic engineering, *E.coli* is not always the ideal host due to inability to perform post-translational modifications and formation of inclusion bodies. Other organisms, including *S.cerevisiae***,** are thus selected to produce flavonoids. *S. cerevisiae* has many inherent properties as a metabolic engineering platform for flavonoid production. Firstly, as a single cell eukaryotic organism, *S.cerevisiae* has many similar mechanisms to higher eukaryotes, facilitating efficient expression and posttranslational modification of plant-derived flavonoid-biosynthetic proteins.[37,38,125,126] Second, *S. cerevisiae* has long served as a host organism for heterologous expression of proteins. Its genetics and physiology are well documented. Thus, genetic tools of *S. cerevisiae* for metabolic pathway manipulation are more abundant.[149] Third, *S.cerevisiae*, classified as a GRAS(generally recognized as safe) organism, is easily cultivated in chemically defined medium and exhibits fast growth rates, thus facilitating subsequent application for scaling-up production of flavonoids. Finally, *S.cerevisiae* can be tolerant to a wide range of physiological stresses such as

low pH and high osmotic stress. Collectively, these advantageous traits support the industrial use of yeast for chemicals including flavonoids production.

Normally, *P.putida* strains may be applied in metabolic engineering and synthetic biology in the presence of toxic chemicals due to its solvent-tolerant nature (Table 2). One of the underlying mechanisms of solvent-tolerance is the presence of solvent efflux pump, which can export hydrophobic molecules, including solvents and toxic products from the interior of whole cells. This mechanism not only protects the cell from toxic effects, but also offers better production by exporting a desired molecule from the cell into the medium.[140] A variety of solvent tolerant *P. putida* strains including S12,[33,128,129] DOT-T1E, and IH-2000 have been isolated and used as hosts for the production of toxic end-products. These toxic chemicals mainly refer to aromatic compounds, like *p*-hydroxystyrene (**16**, 4-vinyl phenol),[33] *trans*-cinnamic acid(**1**) [129] and *p*-coumaric acid(**2**, also known as *p*-coumarate).[128]

Streptomyces are well-known antibiotic producer with well-characterized genetic backgrounds. Because *Streptomyces* have high tolerance towards aromatic compounds [150] and powerful biomass-assimilating ability,[130,131] they were mainly used for building-block compounds production from biomass nowadays. These *Streptomyces* strains comprised *Streptomyces lividans*[130-132] and *Streptomyces coelicolor*. [80] These resulting aromatic building blocks contained *trans*-cinnamic acid(**1**),[80,131,132] *p*-coumarate(**2**).[130]

5.2 Enhancing production of pathway enzymes

As precise control of enzyme expression levels is often critical for the efficient activity of metabolic pathways, the introduction of transcriptional (promoters), translational (ribosome binding site (RBS)) and enzyme (codon-optimized gene) variability to modulate pathway expression levels is essential for generating balanced metabolic pathways and maximizing the productivity of an engineered host. Increased protein production was achieved by addition of T7 promoter and RBS in front of each gene of reconstructed pathway, therefore in turn resulting in yield enhancement of desired products.[43,123,124] This, however, is not always the case. The results presented by Kim *et al* revealed coordinated expression control of 4CL(4-coumarate-CoA ligase) and CHS(chalcone synthase) by one T7 promoter is better than independent expression regulated by two T7 promoters.[146] These authors further attributed the contradictory observations to the order of related genes. This may be a reasonable explanation because more than one paper referred to the phenomenon.[146,151]

Besides regulators, optimal performance of a heterologous flavonoids pathway is also dictated by the kinetic properties of its enzymatic components. Variation in these kinetic properties may be achieved either by mutagenic experiments to create the desired attributes of an enzyme[40,86,126,133,136,145] or through selection of variant enzymes deposited in public databases with differing kinetic properties.

Typically, codon optimization is used to improve the efficiency of heterologous protein production, especially in the context of synthetic biology and metabolic engineering. Approaches normally used to optimize codons include targeted

mutagenesis to remove rare codons[40,86,126,133,136,145] or the addition of rare codon tRNAs in specific hosts.[145]

Screening various target enzymes with desired attributes from the public databases can increase the likelihood of assembling a functional pathway. By screening several prokaryotic and eukaryotic microorganisms, Vannelli *et al* acquired a bifunctional enzyme possessing both the highest TAL specific activity and the lowest PAL/TAL catalytic activity ratio. When the resulting bifunctional enzyme was introduced to *S.cerevisiae*, as anticipated, high levels of *p*-coumarate(**2**) were observed.[41] To obtain an appropriate *p*-coumarate decarboxylase (PDC) used for the generation of *p*-hydroxystyrene(**16**), various microorganisms like *Bacillus. subtilis*, *Pseudomonasfluorescens*, *P. putida*, *S. cerevisiae*, *Rhodotorula rubra* and *Lactobacillus plantarum* were tested for their ability to decarboxylate *p*-coumarate(**2**) to *p*-hydroxystyrene(**16**) by Qi *et al*. [144] The appropriate genes encoding for PDC with highest activity were then amplified from *L. plantarum* and *B.subtilis* genome and used to co-express with bifunctional PAL/TAL gene in *E.coli* to obtain *p*-hydroxystyrene(**16**).[144] The same strategy was also found in a paper reported by McKenna *et al*.[39] Prior to rebuilding of styrene biosynthetic pathway, candidate isoenzymes for each step were screened from bacterial, yeast, and plant genetic sources by these authors. PAL2 from *Arabidopsis thaliana* and FDC1 from *S.cescerevisiae* were then selected to co-express in *E.coli* due to their higher activity. The resulting strain led to the styrene accumulation of up to 260mg/L in shake flask cultures.[39] In another paper, Santos *et al* also screened enzyme variants for each

successive step in engineered naringenin biosynthetic pathway. The isoenzymes with higher activity were selected to further assemble a functional pathway.[145] Additionally, to choose the best candidate CPR (cytochrome P450 reductase) gene, two *A. thaliana* CPR variants (CPR1 or CPR2) were selected to test activity. Results revealed CPR1 has higher activity and therefore is used in further experiments.[126]

The kinetic characteristics of the enzyme variants were characterized either individually as standalone enzymes[41,144] or in the context of the entire flavonoid biosynthetic pathway.[125,126]

5.3 Yield enhancement of the intracellular pool of precursors

There are varied precursors in different metabolic pathways reconstructed in chassises. The low level of intracellular precursors is becoming one of the largest obstacles to efficient microbial biosynthesis of flavonoids. Several strategies were applied to enhance the availability of precursors in engineered hosts of flavonoids. These strategies include up-regulated expression of key genes of precursor biosynthesis and down-regulation or deletion of the precursor competition pathways.

5.3.1 Increased metabolic flux to precursor pools

Malonyl-CoA is one of the flavonoids precursors (Fig.5). The concentration of malonyl-CoA in *E.coli* cells was calculated to be only 4-90 μM (0.01–0.23 nmol/mg dry weight).[152] The low content of intracellular malonyl-CoA is becoming a bottleneck of flavonoid yields in engineered *E.coli*.[43] To increase the supply of malonyl-CoA, varied modifications, which focused on overexpression of pathway

enzymes related to malonyl-CoA biosynthesis, were applied to the engineered hosts. The metabolic regulation included reconstruction of malonate assimilation pathway containing two components of matB (encoding malonyl-CoA synthetase) and matC(encoding malonate carrier protein)(Fig.5),[40,136,145] or overexpression of multisubunit complex of acetyl-CoA carboxylase (ACC) (Fig.5),[42,43,122,133,134,137,146] or genetic modification in acetate assimilation pathways (Fig.5, Table 3).[133]

Malonate assimilation pathway was utilized for both the transport of supplemented malonate into the cell and its subsequent conversion to malonyl-CoA. Upon introduction of this recombinant pathway into engineered *E.coli*, the strain showed a dramatic increase in desired production over the control.[40,136,145]

The acetyl-CoA carboxylase can catalyze the conversion of acetyl-CoA to malonyl-CoA, leading to great enhancement of a pool of malonyl-CoA. Therefore, an increase in the amount of malonyl-CoA by overexpressing the acetyl-CoA carboxylase gene will result in enhancement of the flavanone yields.[42,43,122,133,134,137,146]

Acetyl-CoA synthetase[19] can catalyze an irreversible reaction that converts acetate to acetyl-CoA via two enzymatic steps. To increase the capacity of intracellular acetyl-CoA poor, both overexpression of acetyl-CoA synthetase and inhibition of acetate kinase(AK), a rate-limiting enzyme of acetate competition pathway, were employed in host cells.[133]

L-phenylalanine is also a flavonoid precursor. By overexpressing two rate-limiting enzymes of L-phenylalanine biosynthesis,

3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and chorismate mutase/prephenate dehydratase, strains exhibiting an enhanced capacity for L-phenylalanine synthesis was accomplished.[40] Moreover, the deregulation of DAHP synthase by substitution of DAHP encoding gene with a tyrosine insensitive allele was also proved to alleviate tyrosine feedback inhibition, leading to an increase of naringenin yields.[126] When PAL enzyme was transferred into an L-phenylalanine overproducing *E. coli* strain, there was a substantial increase in the level of desired products accumulated.[41,129,144]

Many PALs have tyrosine ammonia-lyase activity, by which L-tyrosine can be catalyzed to form *p*-coumarate(**2**). In the engineered strains harboring PAL/TAL, L-tyrosine was thus used as a precursor to produce the L-tyrosine-derived aromatic products. To enhance capacity for L-tyrosine synthesis, some of rate-limiting enzymes in L-tyrosine biosynthesis were usually overexpressed within the recombinant cells.[86] Moreover, to direct more L-tyrosine to naringenin synthesis, increased expression of a TAL independent of reconstituted pathway resulted in an enhancemen of naringenin yield.[126]

5.3.2 Reducing or inhibiting metabolic flux to precursor competition pathways

Competing pathways are native pathways present in a metabolic network of engineered cells. These pathways competed with reconstructed pathways that share the same precursor. The competing consumption of precursors usually diverged metabolic flux away from producing a required chemical, resulting in decreased or no

detection of desired product. To diminish or delete adverse effect brought to target pathways by competing pathways, several approaches were explored in developing engineered cells. To limit the amount of malonyl-CoA lost to the synthesis of fatty acids, fatty acid pathway inhibitor cerulenin was added to repress both fabB(β-ketoacyl-ACP synthases I) and fabF(β-ketoacyl-ACP synthases Ⅱ), which led to a precipitous increase of over190% naringenin (Fig.5).[145] Furthermore, elimination of competing phenylpyruvate decarboxylase activity can cause an improvement in naringenin production.[126] Additionally, to increase the supply of *p*-coumarate(**2**), a direct precursor for *p*-hydroxystyrene, the first gene of the *p*-coumarate degradation pathway, fcs (encoding feruloyl-coenzyme A synthetase), was inactivated by homologous recombination in *P.putida* S12 427. The resulting *P. putida* S12 construct showed a higher p-hydroxystyrene yield.[33] In another paper reported by Nijkamp *et al*, fcs was also inactivated to hamper product degradation.[128]

5.3.3 Balancing multi-gene expression to optimize metabolic flux

In the expression of a multi-gene heterologous pathway, the activity of a single enzyme may be out of balance with that of the other enzymes in the pathway, leading to unbalanced carbon flux and the accumulation of an intermediate. Different strategies were employed to balance the overall pathway.[40]

A modular metabolic strategy is one of the efficient methods. The entire pathway was divided into different modules. The modular expression levels could be estimated according to promoter strengths and copy numbers. A most optimized performance

for each biological system can be achieved by balancing the expression levels of multiple modules.[40,86,136,145]

Expression correlation analysis is another strategy to balance the protein-protein interactions in one specific pathway. According to the expression correlation analysis, the best set of isoenzymes for naringenin production was identified and introduced into yeast to produce desired end-product with high titre.[126]

5.4 Alleviating the metabolic burden

When multiple genes were introduced into a non-native host to reconstruct a artificial pathway, significant decreases in growth rate and biomass yield were commonly observed in the recombinant strain. These effects are thought to result from the metabolic load imposed on the production host by the expression of foreign proteins[40,145] or the toxicity of the product of interest.[144]

The heterologous expression of multi-protein pathway in an engineered host cell often utilizes a significant amount of the host cell's resources, removing those resources away from host cell metabolism and placing a metabolic burden on the chassis. As one of the most commonly observed consequence of the imposed metabolic load, the growth rate of the host cell decreased dramatically or was halted. To overcome or reduce the adverse effect, efficient solutions, like minimizing the size of foreign plasmids,[145] reducing the copy number of introduced plasmids,[86,136] and delaying IPTG induction,[40] were proposed in many papers.[40,145]

The physiological toxicity of hydrophobic flavonoids made the yield far below their maximum potential due to growth arrest of engineered strains. In recent years, a variety of approaches have been proposed to overcome or circumvent the toxicity-related adverse effects. The first way to cope with this product toxicity is to deploy solvent-tolerant microorganisms, like *P.putida* S12.[33]

Additionally, product toxicity was averted by the application of two-phase fermentation during fed-batch cultivation.[33]Moreover, protein complex formation is likely to alleviate toxic effect of intermediates.[125,126] It has been postulated that PAL can form multienzyme complexes (MECs) with other downstream enzymes such as C4H (cinnamic acid 4-hydroxylase), CPR, 4CL, CHS and CHI (chalcone isomerase), facilitating local retention and then sequential conversion of toxic intermediates.[125,126] Alternatively, feedback inhibition by intermediates had also been reported for quite a few metabolic pathways.[40,86,136,145] The inhibitory effect can be reversed by overproduction of downstream enzymes, which can lower the accumulation of intermediates and then alleviate potential inhibition within diverse host cell.[40,86,126,136,146]

5.5 Optimization of the culture conditions

Besides the genetic manipulation strategies, optimization of the culture conditions, such as medium screening,[40] optimization of protein induction factors [133] and so on, can also enhance the yields of target compounds produced by engineered constructs harboring PALs.

Medium was used to culture engineering hosts and exhibited varied effect on flavonoid production. When transferred to fresh media for fermentation, only the strains resuspended in MOPS medium can yield desired product.[40] Vannelli *et al* also reported that different production titres were obtained from distinct medium.[41] Also, the composition of mediums, such as nitrogen or carbon source has an effect on production of target products. To reduce the consumption of malonyl-CoA used for fatty acid synthesis, engineered *E.coli* was concentrated (50 g/l wet weight) to re-incubated in fresh M9 medium. Under these cultural conditions, almost no cell growth was observed, which meant no fatty acid synthesis is necessary. The accumulated malonyl-CoA will be directed to metabolic flux of flavanones biosynthesis to result in yield enhancement.[43]

Induction factors such as IPTG concentration,[133] induction time, induction temperature[133] and IPTG induction at different OD_{600} values, [40,133] also have effect on protein expression.

In addition, different culture methods can also cause yield variance in engineered organisms. Koopman *et al* revealed the the naringenin production in batch bioreactor cultivation is more than two times than that of equivalent shake flasks cultivations. [126] The similar phenomenon was observed by Verhoef *et al*.[33]

6. Enzyme replacement therapy of phenylketonuria

PAL also has been employed in medicine as an enzyme substitution therapy for the treatment of phenylketonuria (PKU, OMIM 261600), an inherited metabolic disorder

caused by mutations in phenylalanine hydroxylase (PAH; EC 1.14.16.1) gene. Patients with PKU suffer from hyperphenylalaninemia,[153] which ultimately leads to seizures and mental retardation. The established treatment for PKU resides in a low-phenylalanine diet, which has known deficiencies of several nutrients and unsatisfactory organoleptic properties, making long-term full compliance very laborious and requiring a great deal of social support. An alternative independent of dietary manipulation is thus desirable.

PAL is a robust autocatalytic protein, which is anticipated to reverse HPA by converting excess systemic L-Phe to nontoxic *trans*-cinnamic acid and metabolically insignificant levels of ammonia in the absence of additional cofactors. Also, PAL, unlike PAH, is inherently stable with prolonged life-time. Moreover, the broad pH range tolerance of PAL is 4-12, a feature compatible with an enteral route for PAL therapy.[85] All of these properties have instigated investigations into the use of PAL as an obvious lead candidate for the development of an enzyme substitution therapy for PKU. Preliminary studies showed oral or subcutaneous administration of PAL led to substantial lowering of plasma L-Phe levels.[32,154-156]

To develop a most therapeutically effective form of PAL with higher availability and stability, longer half-life, less toxicity and immunogenicity, there are many problems need to be resolved. First, sufficient amounts of purified PAL with high specific activity need to be available. At present, the production of therapeutic PAL mainly utilizes *P. crispum*,[157] *Anabaena variabilis*,[32,158] and *Rhodosporidium toruloides*.[22,32,154,155] When these PAL genes were heterologously expressed in *E.coli,*

the yields of recombinant enzyme obtained were disappointingly low. The insufficient availability of recombinant PAL proteins curtailed the human and even the animal studies. A recombinant strain capable of producing a large amount of PAL is therefore highly desirable. Up to date, many efforts, like using a high-expression promoter,[155] co-expression with molecular chaperone in *E.coli*,[157,159] and optimization of culture conditions[158,160] have been made to improve substantially recombinant PAL production. Inclusion body formation is a regular phenomenon when PAL was expressed in *E.coli*, which leads to significant reduction of soluble PAL. In order to increase the cytosolic expression, PAL and chaperone were co-expressed in *E.coli*. The resulting system can yield high titre of PAL protein, reaching 70mg/L.[159]

Second, therapeutically effective PAL needs to be tolerated by the immune system of PKU patients. Otherwise, the repeated administration of PAL will elicit immune response towards PAL, leading to clearance of the enzyme through a neutralizing immune response and to harmful allergic reactions.[157] In order to reduce immunogenicity, different strategies, mainly including chemical modification with polyethylene glycol (PEG) and site-directed mutagenesis, have been developed based on protein structural information. Protein pegylation is the routine strategy to protect enzymes from immune system access.[22,32,154,155,157,161-163] PEG-PAL conjugates were produced by covalent coupling of activated PEG molecules to PAL protein, which had been shown to reduce immunogenicity, prolong circulation half-life and retain catalytic activity in vivo.[22,154,157] PEG-PAL conjugates at lower ratios did not lead to significant lowering of the immune response. The immunoreactivities of PEG-PAL

turned to decrease as the degree of modification increased.[22] PEG-PAL formulations with high ratios (like 1:8 and 1:16) almost completely attenuate immunoreactivity.[22] Reduced immunogenicity depends on the number of PEG derivatized amino groups, indicating that the decreased immune response of pegylated PAL is a consequence of masking certain specific immunogenic PAL epitopes by polymer modification of these sites. [154] There are at least 10 fully surface-exposed lysine residues distributed throughout the PAL protein surface.[22] Pegylation derivatization of PALs can be achieved through amine coupling of these lysines with PEG derivatives, thereby resulting in improved PAL with reduced immunogenicity. Therefore, substitution of the residues near antigenic regions with lysines, such as R91K mutant in RT-PAL of *Rhodosporidium toruloides*, can generate more favorable PAL with reduced immunogenicity and increased activity.[22,162,163]

Up to date, the PALs used for pegylation modification were mainly from *Rhodotorula glutinis*,[164] *R.toruloides*,[22,32,154,162,163] *P.crispum*,[157,162] *A.variabilis*,[32,161,162] *Nostoc punctiforme*.[162]

At least five PEG-PAL formations have been clinical trials in the USA since 2008 as a potential injectable therapeutic for PKU. [31,32,161] Given that PKU patients require lifelong therapy, oral formation of PAL is also investigated to provide a non-invasive substitution therapy. *In vivo* studies revealed that orally administered PAL yielded statistically significant and therapeutically relevant reduction in plasma L-Phe levels in a dose- and loading-dependent manner.[32]

Finally, the sensitivity of PAL to proteolysis hampered its application as a more

efficacious molecule. Various strategies, such as chemical modification and protein engineering, have been exploited so far to reach enzymes with higher stability. These modified PALs are thus to be more stable in the circulation to ensure the therapeutic effects over a long period. Besides restricting access to epitopes, PEGylation of PAL through the ε-amino group of lysine's side chain presumably block potential trypsin cleavage sites, leading to increased stability and prolonged in vivo protein half-life.[161] Another chemical modification strategy explored to further improve the protease resistance of therapeutic PALs utilized the silica sol-gel matrix for entrapping the enzyme.[161] The encapsulated PAL-silica particles exhibited improved resistance against intestinal proteases in the protease resistance studies.[161]

Moreover, various strategies in the protein engineering were performed to increase PALs stability. Among them, introduction of new disulfide bonds,[31] reducing protein aggregation,[31] improving protease resistance by site-directed mutagenesis of required sites[22,161] are the best exemplified ones. Disulfide bonds, in the form of a covalent linkage between the thiol groups of pairs of cysteine residues, are present in most extracellular proteins, where they presumably stabilize the native conformation by lowering the entropy of the denatured state. This stabilizing property makes introduction of new disulfide bonds an attractive strategy for engineering additional conformational stability into proteins by site directed mutagenesis. To improve stability of *A.variabilis* PAL(*Av*PAL), Gln292 of AvPAL was mutated to cysteine residue, and a new disulfide bond between G292S and Cys503 residue was consequently was introduced to the mutant enzyme.[31]

Cysteines-503 and -565 are two harmful surface cysteines in AvPAL of *A. variabilis*, taking part in aggregating the enzyme molecules and thereby resulting in decreased stability. To remove the two cysteines, mutation of both these residues to serine (C503S/C565S) was performed. The resulting C503S/C565S double mutation reduced aggregation properties of PAL, without significantly altered enzyme activity. [85,162] There are many candidate sites identified to be susceptible for proteases cleavages.[22] When these sites were substituted, the resultant mutant, like R123H, R123A, R123Q, Y110H, Y110A and Y110L mutants of *R.toruloides* PAL, [22] F18A variant of *A.variabilis* PAL,[161] exhibited modest improvements in resistance against protease inactivation.

Combination of different strategies in the chemical modification and protein engineering may also result in increased stability in a synergistic manner. When triple mutant C503S/C506S/F18A of AvPAL was further modified by PEG or silica matrix, the chemically modified PAL preparations displayed improvement in resistance against chymotrypsin and trypsin, two prevalent intestinal enzymes.[161] Also, the observation was confirmed by further proofs. In a recent work presented by Jaliani *et al*, Q292C mutation was selected to form a new disulfide bond with Cys503, along with C565S mutation. The resulting double mutation Q292C/C565S remove the free sulfhydryl groups on the PAL surface (Cys503 and 565), and thereby displayed more kinetic stability and chemical denaturation resistance.[31]

Some of PAL formulations have been clinical trials as a potential therapeutic for PKU. [32]

7. A therapeutic enzyme in cancer treatment

In addition to applications in enzyme replacement therapy of phenylketonuria, PALs may be used as a potential therapeutic enzyme in cancer treatment.[47,48,165-169] In the primary study performed by Babich *et al*, a recombinant PAL from *R.toruloides* was observed to exhibit a significant cytotoxic effect toward varied cells.[47]

8. A novel biocatalyst for synthesis of L-phenylalanine

In addition to medical applications, PAL was investigated as a biocatalyst for the preparative synthesis of L-phenylalanine.[49,50,170-172] L-Phe is a key precursor of artificial sweetener aspartame. The great demand on aspartame stimulated considerable researches on the enzymatic production of L-Phe catalyzed by PALs.[35,36,49-51,170-173] Besides the forward reaction of spontaneous, nonoxidative deamination of L-Phe to *t*-CA and ammonia, PALs can also catalyze the reverse reaction, namely formation of L-Phe under conditions of high concentration of *trans*-cinnamic acids and ammonia at an elevated pH.[174] The biotransformation approach catalyzed by PALs has the advantage of asymmetric synthesis of an optically pure product, L-Phe.[50] Although PAL has disadvantages of low specific activity and decreased stability, significant strides have been made recently in the application of PAL for the synthesis of L-Phe. [170-172] In order to improve PAL activity, a combine promoter was used to regulate the expression of PAL. The resulting specific activity of PAL reached a high level of 123U/g(dry cells weight, DCW), 8-12

fold higher than that of wild-type.[51] To overcome the instability of PAL, the preparation of cross-linked enzyme aggregates of PAL was described in several reports.[49,170-172] Compared to the free enzyme, the aggregates exhibited the higher thermal stability, denaturant stability and storage stability, retaining 30% initial activity even after 11 cycles of reuse.[170] Overall, with the improvement of PAL activity and stability, PALs might be used as an efficient biocatalyst for enzymatic production of L-Phe.

9. Conclusions and prospects

Great strides have been made recently in the potential application of PALs, including gene component of engineered strains producing phenylpropanoids, enzyme replacement therapy of phenylketonuria, therapeutic enzyme in cancer treatment and biocatalysts for synthesis of L-phenylalanine. There are, however, several problems to be solved.

In recent years, a wide variety of additional, novel synthetic routes harboring PALs had been proposed and engineered in microorganisms for the production of phenylpropanoids with pharma- and nutraceutical properties.[40,126,145] The final titers of phenylpropanoids, however, are still low, thereby impeding subsequential industrialization of metabolites from engineered microorganisms. As such, several obstacles, like low enzyme efficiency, coordinated expression of multiple-enzyme pathway, toxic intermediates accumulation and inadequate substrates supply, will be overcome to improve microbial yield of interest compounds.

Although PEG-PAL conjugates (PAL conjugated with polyethylene glycol) have been introduced in clinical trials for the treatment of PKU,[31,32,46,85,161] the PALs with highest stability and reduced immunogenicity are still the most therapeutically effective molecules.

Using enzymes as biocatalysts is a useful tool for the synthesis of L-phenylalanine on a large scale. However, many PALs are sensitive and fragile to reaction conditions. Therefore, the more detailed investigations improving PALs activity and stability should be strengthened. Overall, if all these challenges were overcome, more and more robust and competitive PALs will be appeared in industrial and medical applications.

Acknowledgements

The author gratefully acknowledges the support of the Independent Subject of Key Project of State Key Laboratory of Bioactive Substance and Function of Natural Medicines (GTZA201404) in producing this review.

References

1.J. Koukol and E. E. Conn, *J Biol Chem,* 1961, **236**, 2692-2698.

2.C. J. Liu, *Mol Plant,* 2012, **5**, 304-317.

3.C. J. Baker, N. M. Mock, B. D. Whitaker, D. P. Roberts, C. P. Rice, K. L. Deahl and A. A. Aver'yanov, *Biochem Biophys Res Commun,* 2005, **328**, 130-136.

4.L. Jaakola, *Trends Plant Sci,* 2013, **18**, 477-483.

5.S. Das, J. Das, A. Paul, A. Samadder and A. R. Khuda-Bukhsh, *J Acupunct Meridian Stud,* 2013, **6**, 252-262.

6.M. J. Tuorkey, *Eur J Cancer Prev,* 2015, **Epub ahead of print**,

7.S. Shukla and S. Gupta, *Pharm Res,* 2010, **27**, 962-978.

8.Y. Lin, R. Shi, X. Wang and H. M. Shen, *Curr Cancer Drug Targets,* 2008, **8**, 634-646.

9.D. Patel, S. Shukla and S. Gupta, *Int J Oncol,* 2007, **30**, 233-245.

10.S. J. Ahn, S. N. Park, Y. J. Lee, E. J. Cho, Y. K. Lim, X. M. Li, M. H. Choi, Y. W. Seo and J. K. Kook, *Caries Res,* 2015, **49**, 78-89.

11.X. Lan, W. Wang, Q. Li and J. Wang, *Mol Neurobiol,* 2015, **Epub ahead of print**,

12.L. S. Chua, *J Ethnopharmacol,* 2013, **150**, 805-817.

13.X. Hou, F. Shao, Y. Ma and S. Lu, *Mol Biol Rep,* 2013, **40**, 4301-4310.

14.C. J. Dong and Q. M. Shang, *Planta,* 2013, **238**, 35-49.

15.S. Giberti, C. M. Bertea, R. Narayana, M. E. Maffei and G. Forlani, *J Plant Physiol,* 2012, **169**, 249-254.

16.H. Yoon, Y. H. You, Y. E. Kim, Y. J. Kim, W. S. Kong and J. G. Kim, *J Microbiol Biotechnol,* 2013, **23**, 1055-1059.

17.C. A. Vaslet, R. L. Strausberg, A. Sykes, A. Levy and D. Filpula, *Nucleic Acids Res,* 1988, **16**, 11382.

18.C. W. Abell and R. S. Shen, *Methods Enzymol,* 1987, **142**, 242-253.

19.K. Kovacs, G. Banoczi, A. Varga, I. Szabo, A. Holczinger, G. Hornyanszky, I. Zagyva, C. Paizs, B. G. Vertessy and L. Poppe, *PLoS One,* 2014, **9**, e85943.

20.J. S. Williams, M. Thomas and D. J. Clarke, *Microbiology,* 2005, **151**, 2543-2550.

21.M. C. Moffitt, G. V. Louie, M. E. Bowman, J. Pence, J. P. Noel and B. S. Moore, *Biochemistry,* 2007, **46**, 1004-1012.

22.L. Wang, A. Gamez, C. N. Sarkissian, M. Straub, M. G. Patch, G. W. Han, S. Striepeke, P. Fitzpatrick, C. R. Scriver and R. C. Stevens, *Mol Genet Metab,* 2005, **86**, 134-140.

23.H. Ritter and G. E. Schulz, *Plant Cell,* 2004, **16**, 3426-3436.

24.J. C. Calabrese, D. B. Jordan, A. Boodhoo, S. Sariaslani and T. Vannelli, *Biochemistry,* 2004, **43**, 11403-11416.

25.N. M. Shaw, G. P. Bolwell and C. Smith, *Biochem J,* 1990, **267**, 163-170.

26.J. Jorrin and R. A. Dixon, *Plant Physiol,* 1990, **92**, 447-455.

27.Y. Tanaka, M. Matsuoka, N. Yamanoto, Y. Ohashi, Y. Kano-Murakami and Y. Ozeki, *Plant Physiol,* 1989, **90**, 1403-1407.

28.H. Habereder, G. Schroder and J. Ebel, *Planta,* 1989, **177**, 58-65.

29.J. F. Kane and M. J. Fiske, *J Bacteriol,* 1985, **161**, 963-966.

30.M. A. Lawton, R. A. Dixon, K. Hahlbrock and C. Lamb, *Eur J Biochem,* 1983, **129**, 593-601.

31.H. Z. Jaliani, S. Farajnia, S. A. Mohammadi, A. Barzegar and S. Talebi, *Appl Biochem Biotechnol,* 2013, **171**, 1805-1818.

32.C. N. Sarkissian, T. S. Kang, A. Gamez, C. R. Scriver and R. C. Stevens, *Mol Genet Metab,* 2011, **104**, 249-254.
33.S. Verhoef, N. Wierckx, R. G. Westerhof, J. H. de Winde and H. J. Ruijssenaars, *Appl Environ Microbiol,* 2009, **75**, 931-936.
34.S. Verhoef, H. J. Ruijssenaars, J. A. de Bont and J. Wery, *J Biotechnol,* 2007, **132**, 49-56.
35.K. Shetty, D. L. Crawford and A. L. Pometto, *Appl Environ Microbiol,* 1986, **52**, 637-643.
36.S. Yamada, K. Nabe, N. Izuo, K. Nakamichi and I. Chibata, *Appl Environ Microbiol,* 1981, **42**, 773-778.
37.H. Jiang, K. V. Wood and J. A. Morgan, *Appl Environ Microbiol,* 2005, **71**, 2962-2969.
38.S. Y. Shin, S. M. Jung, M. D. Kim, N. S. Han and J. H. Seo, *Enzyme Microb Technol,* 2012, **51**, 211-216.
39.R. McKenna and D. R. Nielsen, *Metab Eng,* 2011, **13**, 544-554.
40.J. Wu, G. Du, J. Zhou and J. Chen, *Metab Eng,* 2013, **16**, 48-55.
41.T. Vannelli, W. Wei Qi, J. Sweigard, A. A. Gatenby and F. S. Sariaslani, *Metab Eng,* 2007, **9**, 142-151.
42.Y. Katsuyama, I. Miyahisa, N. Funa and S. Horinouchi, *Appl Microbiol Biotechnol,* 2007, **73**, 1143-1149.
43.I. Miyahisa, M. Kaneko, N. Funa, H. Kawasaki, H. Kojima, Y. Ohnishi and S. Horinouchi, *Appl Microbiol Biotechnol,* 2005, **68**, 498-504.
44.S. Horinouchi, *Curr Opin Chem Biol,* 2009, **13**, 197-204.
45.Z. L. Fowler and M. A. Koffas, *Appl Microbiol Biotechnol,* 2009, **83**, 799-808.
46.N. Longo, C. O. Harding, B. K. Burton, D. K. Grange, J. Vockley, M. Wasserstein, G. M. Rice, A. Dorenbaum, J. K. Neuenburg, D. G. Musson, Z. Gu and S. Sile, *Lancet,* 2014, **384**, 37-44.
47.O. O. Babich, V. S. Pokrovsky, N. Y. Anisimova, N. N. Sokolov and A. Y. Prosekov, *Biotechnol Appl Biochem,* 2013, **60**, 316-322.
48.R. S. Shen, R. R. Fritz and C. W. Abell, *Cancer Treat Rep,* 1979, **63**, 1063-1068.
49.J. D. Cui, S. Zhang and L. M. Sun, *Appl Biochem Biotechnol,* 2012, **167**, 835-844.
50.N. J. Turner, *Curr Opin Chem Biol,* 2011, **15**, 234-240.
51.S. R. Jia, J. D. Cui, Y. Li and A. Y. Sun, *Biochem Eng J,* 2008, **42**, 193-197.
52.M. M. Heberling, B. Wu, S. Bartsch and D. B. Janssen, *Curr Opin Chem Biol,* 2013, **17**, 250-260.
53.J. D. Cui, J. Q. Qiu, X. W. Fan, S. R. Jia and Z. L. Tan, *Crit Rev Biotechnol,* 2014, **34**, 258-268.
54.M. W. Hyun, Y. H. Yun, J. Y. Kim and S. H. Kim, *Mycobiology,* 2011, **39**, 257-265.
55.M. J. MacDonald and G. B. D'Cunha, *Biochem Cell Biol,* 2007, **85**, 273-282.
56.X. H. Wang, M. Gong, L. Tang, S. Zheng, J. D. Lou, L. Ou, J. Gomes-Laranjo and C. Zhang, *Mol Biol Rep,* 2013, **40**, 97-107.
57.Q. Jin, Y. Yao, Y. Cai and Y. Lin, *PLoS One,* 2013, **8**, e62352.
58.Y. Jiang, B. Xia, L. Liang, X. Li, S. Xu, F. Peng and R. Wang, *Mol Biol Rep,* 2013, **40**, 2293-2300.
59.C. Fang, Y. Zhuang, T. Xu, Y. Li and W. Lin, *J Chem Ecol,* 2013,
60.W. Ma, M. Wu, Y. Wu, Z. Ren and Y. Zhong, *Plant Cell Rep,* 2013, **32**, 1179-1190.
61.F. Xu, G. Deng, S. Cheng, W. Zhang, X. Huang, L. Li, H. Cheng, X. Rong and J. Li, *Molecules,* 2012, **17**, 7810-7823.
62.U. R. Bagal, J. H. Leebens-Mack, W. W. Lorenz and J. F. Dean, *BMC Genomics,* 2012, **13 Suppl 3**, S1.
63.W. H. Xiao, J. S. Cheng and Y. J. Yuan, *J Biotechnol,* 2009, **139**, 222-228.
64.R. W. Whetten and R. R. Sederoff, *Plant Physiol,* 1992, **98**, 380-386.
65.G. Hao, X. Du, F. Zhao and H. Ji, *Biotechnol Lett,* 2010, **32**, 305-314.

66.M. R. Young, G. H. N. Towers and A. C. Neish, *Can J Bot,* 1966, **44**, 341-349.
67.U. Czichi and H. Kindi, *Hoppe Seylers Z Physiol Chem,* 1975, **356**, 475-485.
68.D. M. Power, G. H. N. Towers and A. C. Neish, *Can J Biochem,* 1965, **43**, 1397-1407.
69.A. Goldson-Barnaby and C. H. Scaman, *Enzyme Res,* 2013, **2013**, 670702.
70.R. J. Bandoni, K. Moore, P. V. Subba Rao and G. H. N. Towers, *Phytochemistry,* 1968, **7**, 205-207.
71.C. P. Vance, R. J. Bandoni and G. H. N. Towers, *Phytochemistry,* 1975, **14**, 1513-1514.
72.R. R. Fritz, D. S. Hodgins and C. W. Abell, *J Biol Chem,* 1976, **251**, 4646-4650.
73.J. R. Parkhurst and D. S. Hodgins, *Arch Biochem Biophys,* 1972, **152**, 597-605.
74.D. S. Hodgins, *Arch Biochem Biophys,* 1972, **149**, 91-96.
75.D. S. Hodgins, *J Biol Chem,* 1971, **246**, 2977-2985.
76.L. Zhu, W. Cui, Y. Fang, Y. Liu, X. Gao and Z. Zhou, *Biotechnol Lett,* 2013, **35**, 751-756.
77.H. J. Gilbert and G. W. Jack, *Biochem J,* 1981, **199**, 715-723.
78.L. Xiang and B. S. Moore, *J Bacteriol,* 2005, **187**, 4286-4289.
79.L. Xiang and B. S. Moore, *J Bacteriol,* 2003, **185**, 399-404.
80.L. Xiang and B. S. Moore, *J Biol Chem,* 2002, **277**, 32505-32509.
81.A. V. Emes and L. C. Vining, *Can J Biochem,* 1970, **48**, 613-622.
82.G. S. Bezanson, D. Desaty, A. V. Emes and L. C. Vining, *Can J Microbiol,* 1970, **16**, 147-151.
83.S. Chalabaev, E. Turlin, S. Bay, C. Ganneau, E. Brito-Fravallo, J. F. Charles, A. Danchin and F. Biville, *Appl Environ Microbiol,* 2008, **74**, 1717-1725.
84.S. Babaoğlu Aydaş, S. Ozturk and B. Aslım, *Food Chem,* 2013, **136**, 164-169.
85.L. Wang, A. Gamez, H. Archer, E. E. Abola, C. N. Sarkissian, P. Fitzpatrick, D. Wendt, Y. Zhang, M. Vellard, J. Bliesath, S. M. Bell, J. F. Lemontt, C. R. Scriver and R. C. Stevens, *J Mol Biol,* 2008, **380**, 623-635.
86.B. S. Moore, C. Hertweck, J. N. Hopke, M. Izumikawa, J. A. Kalaitzis, G. Nilsen, T. O'Hare, J. Piel, P. R. Shipley, L. Xiang, M. B. Austin and J. P. Noel, *J Nat Prod,* 2002, **65**, 1956-1962.
87.J. Piel, K. Hoang and B. S. Moore, *Journal of the American Chemical Society,* 2000, **122**, 5415-5416.
88.J. Piel, C. Hertweck, P. R. Shipley, D. M. Hunt, M. S. Newman and B. S. Moore, *Chem Biol,* 2000, **7**, 943-955.
89.Y. Zhang, J. Gao, H. Zheng, R. Zhang and Y. Han, *Int J Nanomedicine,* 2011, **6**, 649-657.
90.V. J. Paul, S. Frautschy, W. Fenical and K. H. Nealson, *J Chem Ecol,* 1981, **7**, 589-597.
91.R. Zirkle, J. M. Ligon and I. Molnar, *Microbiology,* 2004, **150**, 2761-2774.
92.R. Zirkle, J. M. Ligon and I. Molnar, *J Biosci Bioeng,* 2004, **97**, 267-274.
93.C. Weitzel and M. Petersen, *Planta,* 2010, **232**, 731-742.
94.Z.-B. Wang, X. Chen, W. Wang, K.-D. Cheng and J.-Q. Kong, *RSC Adv,* 2014, **4**, 27159-27175.
95.A. Kumar and B. E. Ellis, *Plant Physiol,* 2001, **127**, 230-239.
96.J. Rosler, F. Krekel, N. Amrhein and J. Schmid, *Plant Physiol,* 1997, **113**, 175-179.
97.H. C. Rawal, N. K. Singh and T. R. Sharma, *Int J Genomics,* 2013, **2013**, 678969.
98.H. J. Joos and K. Hahlbrock, *Eur J Biochem,* 1992, **204**, 621-629.
99.K. T. Watts, B. N. Mijts, P. C. Lee, A. J. Manning and C. Schmidt-Dannert, *Chem Biol,* 2006, **13**, 1317-1326.
100.A. Chang, M. H. Lim, S. W. Lee, E. J. Robb and R. N. Nazar, *J Biol Chem,* 2008, **283**, 33591-33601.
101.F. C. Cochrane, L. B. Davin and N. G. Lewis, *Phytochemistry,* 2004, **65**, 1557-1564.
102.A. De Paolis, D. Pignone, A. Morgese and G. Sonnante, *Physiol Plant,* 2008, **132**, 33-43.
103.H. Oufedjikh, M. Mahrouz, M. J. Amiot and M. Lacroix, *J Agric Food Chem,* 2000, **48**, 559-565.

104.L. Poppe, *Curr Opin Chem Biol,* 2001, **5**, 512-524.
105.J. Retey, *Biochim Biophys Acta,* 2003, **1647**, 179-184.
106.B. Schuster and J. Retey, *FEBS Lett,* 1994, **349**, 252-254.
107.L. Poppe and J. Rétey, *Angew Chem Int Ed Engl,* 2005, **44**, 3668-3688.
108.D. Rother, L. Poppe, G. Morlock, S. Viergutz and J. Retey, *Eur J Biochem,* 2002, **269**, 3065-3075.
109.B. Schuster and J. Retey, *Proc Natl Acad Sci U S A,* 1995, **92**, 8433-8437.
110.E. A. Havir and K. R. Hanson, *Biochemistry,* 1973, **12**, 1583-1591.
111.H. V. Marsh, Jr., E. A. Havir and K. R. Hanson, *Biochemistry,* 1968, **7**, 1915-1918.
112.L. S. Hsieh, C. S. Yeh, H. C. Pan, C. Y. Cheng, C. C. Yang and P. D. Lee, *Protein Expr Purif,* 2010, **71**, 224-230.
113.L. S. Hsieh, Y. L. Hsieh, C. S. Yeh, C. Y. Cheng, C. C. Yang and P. D. Lee, *Mol Biol Rep,* 2011, **38**, 283-290.
114.L. S. Hsieh, G. J. Ma, C. C. Yang and P. D. Lee, *Phytochemistry,* 2010, **71**, 1999-2009.
115.A. I. Reichert, X. Z. He and R. A. Dixon, *Biochem J,* 2009, **424**, 233-242.
116.J. Gao, S. Zhang, F. Cai, X. Zheng, N. Lin, X. Qin, Y. Ou, X. Gu, X. Zhu, Y. Xu and F. Chen, *Mol Biol Rep,* 2012, **39**, 3443-3452.
117.K. T. Watts, P. C. Lee and C. Schmidt-Dannert, *Chembiochem,* 2004, **5**, 500-507.
118.Y. Nishiyama, C. S. Yun, F. Matsuda, T. Sasaki, K. Saito and Y. Tozawa, *Planta,* 2010, **232**, 209-218.
119.J. Q. Kong, D. Lu and Z. B. Wang, *Molecules,* 2014, **19**, 1608-1621.
120.S. Bartsch and U. T. Bornscheuer, *Protein Eng Des Sel,* 2010, **23**, 929-933.
121.S. Bartsch and U. T. Bornscheuer, *Angew Chem Int Ed Engl,* 2009, **48**, 3362-3365.
122.I. Miyahisa, N. Funa, Y. Ohnishi, S. Martens, T. Moriguchi and S. Horinouchi, *Appl Microbiol Biotechnol,* 2006, **71**, 53-58.
123.M. Kaneko, E. I. Hwang, Y. Ohnishi and S. Horinouchi, *J Ind Microbiol Biotechnol,* 2003, **30**, 456-461.
124.E. I. Hwang, M. Kaneko, Y. Ohnishi and S. Horinouchi, *Appl Environ Microbiol,* 2003, **69**, 2699-2706.
125.D. K. Ro and C. J. Douglas, *J Biol Chem,* 2004, **279**, 2600-2607.
126.F. Koopman, J. Beekwilder, B. Crimi, A. van Houwelingen, R. D. Hall, D. Bosch, A. J. van Maris, J. T. Pronk and J. M. Daran, *Microb Cell Fact,* 2012, **11**, 155.
127.E. Trantas, N. Panopoulos and F. Ververidis, *Metab Eng,* 2009, **11**, 355-366.
128.K. Nijkamp, R. G. Westerhof, H. Ballerstedt, J. A. de Bont and J. Wery, *Appl Microbiol Biotechnol,* 2007, **74**, 617-624.
129.K. Nijkamp, N. van Luijk, J. A. de Bont and J. Wery, *Appl Microbiol Biotechnol,* 2005, **69**, 170-177.
130.Y. Kawai, S. Noda, C. Ogino, Y. Takeshima, N. Okai, T. Tanaka and A. Kondo, *Microb Cell Fact,* 2013, **12**, 45.
131.S. Noda, T. Miyazaki, T. Tanaka, C. Ogino and A. Kondo, *Bioresour Technol,* 2012, **104**, 648-651.
132.S. Noda, T. Miyazaki, T. Miyoshi, M. Miyake, N. Okai, T. Tanaka, C. Ogino and A. Kondo, *J Ind Microbiol Biotechnol,* 2011, **38**, 643-648.
133.S. Zhu, J. Wu, G. Du, J. Zhou and J. Chen, *Appl Environ Microbiol,* 2014, **80**, 3072-3080.
134.Y. Katsuyama, M. Matsuzawa, N. Funa and S. Horinouchi, *Microbiology,* 2008, **154**, 2620-2628.
135.S. Wang, S. Zhang, T. Zhou, J. Zeng and J. Zhan, *Appl Microbiol Biotechnol,* 2013, **97**, 7877-7885.
136.J. Wu, P. Liu, Y. Fan, H. Bao, G. Du, J. Zhou and J. Chen, *J Biotechnol,* 2013, **167**, 404-411.
137.Y. Katsuyama, N. Funa and S. Horinouchi, *Biotechnol J,* 2007, **2**, 1286-1293.

138.S. Wang, S. Zhang, A. Xiao, M. Rasmussen, C. Skidmore and J. Zhan, *Metab Eng,* 2015, **29**, 153-159.
139.Z. Xue, M. McCluskey, K. Cantera, F. S. Sariaslani and L. Huang, *J Ind Microbiol Biotechnol,* 2007, **34**, 599-604.
140.J. Kieboom, J. J. Dennis, J. A. de Bont and G. J. Zylstra, *J Biol Chem,* 1998, **273**, 85-91.
141.A. A. Gatenby, W. W. Qi, S. Sariaslani, X. S. Tang and T. M. Vannelli (2001). Bioproduction of para-hydroxycinnamic acid, Google Patents.
142.M. T. Gatto, S. Falcocchio, E. Grippa, G. Mazzanti, L. Battinelli, G. Nicolosi, D. Lambusta and L. Saso, *Bioorg Med Chem,* 2002, **10**, 269-272.
143.M. M. Chan, *Biochem Pharmacol,* 2002, **63**, 99-104.
144.W. W. Qi, T. Vannelli, S. Breinig, A. Ben-Bassat, A. A. Gatenby, S. L. Haynie and F. S. Sariaslani, *Metab Eng,* 2007, **9**, 268-276.
145.C. N. Santos, M. Koffas and G. Stephanopoulos, *Metab Eng,* 2011, **13**, 392-400.
146.B. G. Kim, H. Lee and J. H. Ahn, *J Microbiol Biotechnol,* 2014, **24**, 1536-1541.
147.F. S. Sariaslani, *Annu Rev Microbiol,* 2007, **61**, 51-69.
148.E. Leonard, Y. Yan and M. A. Koffas, *Metab Eng,* 2006, **8**, 172-181.
149.R. McKenna, B. Thompson, S. Pugh and D. R. Nielsen, *Microb Cell Fact,* 2014, **13**, 123.
150.G. Zafra, Á. Absalón, M. D. Cuevas and D. Cortés-Espinosa, *Water, Air, & Soil Pollution,* 2014, **225**, 1-18.
151.C. G. Lim, Z. L. Fowler, T. Hueller, S. Schaffer and M. A. Koffas, *Appl Environ Microbiol,* 2011, **77**, 3451-3460.
152.Y. Takamura and G. Nomura, *J Gen Microbiol,* 1988, **134**, 2249-2253.
153.A. S. Deshpande, K. K. Surendranathan and P. M. Nair, *Indian J Biochem Biophys,* 1993, **30**, 36-41.
154.A. Gamez, C. N. Sarkissian, L. Wang, W. Kim, M. Straub, M. G. Patch, L. Chen, S. Striepeke, P. Fitzpatrick, J. F. Lemontt, C. O'Neill, C. R. Scriver and R. C. Stevens, *Mol Ther,* 2005, **11**, 986-989.
155.C. N. Sarkissian, Z. Shao, F. Blain, R. Peevers, H. Su, R. Heft, T. M. Chang and C. R. Scriver, *Proc Natl Acad Sci U S A,* 1999, **96**, 2339-2344.
156.S. Safos and T. M. Chang, *Artif Cells Blood Substit Immobil Biotechnol,* 1995, **23**, 681-692.
157.K. Ikeda, E. Schiltz, T. Fujii, M. Takahashi, K. Mitsui, Y. Kodera, A. Matsushima, Y. Inada, G. E. Schulz and H. Nishimura, *Amino Acids,* 2005, **29**, 283-287.
158.H. Z. Jaliani, S. Farajnia, Y. Safdari, S. A. Mohammadi, A. Barzegar and S. Talebi, *Adv Pharm Bull,* 2014, **4**, 261-266.
159.M. Baedeker and G. E. Schulz, *FEBS Lett,* 1999, **457**, 57-60.
160.J.-D. Cui, *Korean J Chem Eng,,* 2010, **27**, 174-178.
161.T. S. Kang, L. Wang, C. N. Sarkissian, A. Gamez, C. R. Scriver and R. C. Stevens, *Mol Genet Metab,* 2010, **99**, 4-9.
162.C. N. Sarkissian, A. Gamez, L. Wang, M. Charbonneau, P. Fitzpatrick, J. F. Lemontt, B. Zhao, M. Vellard, S. M. Bell, C. Henschell, A. Lambert, L. Tsuruda, R. C. Stevens and C. R. Scriver, *Proc Natl Acad Sci U S A,* 2008, **105**, 20894-20899.
163.A. Gamez, L. Wang, C. N. Sarkissian, D. Wendt, P. Fitzpatrick, J. F. Lemontt, C. R. Scriver and R. C. Stevens, *Mol Genet Metab,* 2007, **91**, 325-334.
164.K. J. Wieder, N. C. Palczuk, T. van Es and F. F. Davis, *J Biol Chem,* 1979, **254**, 12579-12587.
165.R. S. Shen, R. R. Fritz and C. W. Abell, *Cancer Res,* 1977, **37**, 1051-1056.
166.J. Roberts, F. A. Schmid and K. Takai, *Cancer Treat Rep,* 1976, **60**, 261-263.

167.W. J. Stith, D. S. Hodgins and C. W. Abell, *Cancer Res,* 1973, **33**, 966-971.
168.C. W. Abell, D. S. Hodgins and W. J. Stith, *Cancer Res,* 1973, **33**, 2529-2532.
169.C. W. Abell, W. J. Stith and D. S. Hodgins, *Cancer Res,* 1972, **32**, 285-290.
170.J. Cui, L. Cui, S. Zhang, Y. Zhang, Z. Su and G. Ma, *PLoS One,* 2014, **9**, e97221.
171.J. D. Cui, L. M. Sun and L. L. Li, *Appl Biochem Biotechnol,* 2013, **170**, 1827-1837.
172.J. D. Cui, L. L. Li and H. J. Bian, *PLoS One,* 2013, **8**, e80581.
173.C. Evans, D. Conrad, K. Hanna, W. Peterson, C. Choma and M. Misawa, *Appl Microbiol Biotechnol,* 1987, **25**, 399-405.
174.E. A. Havir and K. R. Hanson, *Biochemistry,* 1968, **7**, 1904-1914.
175.X. W. Liang, M. Dron, C. L. Cramer, R. A. Dixon and C. J. Lamb, *J Biol Chem,* 1989, **264**, 14486-14492.
176.D. Shufflebottom, K. Edwards, W. Schuch and M. Bevan, *Plant J,* 1993, **3**, 835-845.
177.M. Mavandad, R. Edwards, X. Liang, C. J. Lamb and R. A. Dixon, *Plant Physiol,* 1990, **94**, 671-680.
178.H. Xu, N. I. Park, X. Li, Y. K. Kim, S. Y. Lee and S. U. Park, *Bioresour Technol,* 2010, **101**, 9715-9722.
179.P. A. Howles, T. Arioli and J. J. Weinman, *Gene,* 1994, **138**, 87-92.
180.J. Song and Z. Wang, *Mol Biol Rep,* 2009, **36**, 939-952.
181.J. Huang, M. Gu, Z. Lai, B. Fan, K. Shi, Y. H. Zhou, J. Q. Yu and Z. Chen, *Plant Physiol,* 2010, **153**, 1526-1538.
182.J. Raes, A. Rohde, J. H. Christensen, Y. Van de Peer and W. Boerjan, *Plant Physiol,* 2003, **133**, 1051-1071.
183.M. A. Costa, R. E. Collins, A. M. Anterola, F. C. Cochrane, L. B. Davin and N. G. Lewis, *Phytochemistry,* 2003, **64**, 1097-1112.
184.L. A. Wanner, G. Li, D. Ware, I. E. Somssich and K. R. Davis, *Plant Mol Biol,* 1995, **27**, 327-338.
185.T. Okada, M. Mikage and S. Sekita, *Biol Pharm Bull,* 2008, **31**, 2194-2199.
186.Y. Ozeki, Y. Ito, N. Sasaki, M. Oyanagi, H. Akimoto, Y. Chikagawa and J. Takeda, *J Plant Res,* 2000, **113**, 319-326.
187.T. Fukasawa-Akada, S. D. Kung and J. C. Watson, *Plant Mol Biol,* 1996, **30**, 711-722.
188.L. Pellegrini, O. Rohfritsch, B. Fritig and M. Legrand, *Plant Physiol,* 1994, **106**, 877-886.
189.N. Nagai, F. Kitauchi, M. Shimosaka and M. Okazaki, *Plant Physiol,* 1994, **104**, 1091-1092.
190.Y. Osakabe, K. Nanto, H. Kitamura, S. Kawai, Y. Kondo, T. Fujii, K. Takabe, Y. Katayama and N. Morohoshi, *Planta,* 1996, **200**, 13-19.
191.Y. Osakabe, K. Osakabe, S. Kawai, Y. Katayama and N. Morohoshi, *Plant Mol Biol,* 1995, **28**, 1133-1141.
192.Y. Osakabe, Y. Ohtsubo, S. Kawai, Y. Katayama and N. Morohoshi, *Plant Science,* 1995, **105**, 217-226.
193.E. Logemann, M. Parniske and K. Hahlbrock, *Proc Natl Acad Sci U S A,* 1995, **92**, 5905-5909.
194.C. Appert, E. Logemann, K. Hahlbrock, J. Schmid and N. Amrhein, *Eur J Biochem,* 1994, **225**, 491-499.
195.R. Lois and K. Hahlbrock, *Z Naturforsch C,* 1992, **47**, 90-94.
196.W. Schulz, H. G. Eiben and K. Hahlbrock, *FEBS Lett,* 1989, **258**, 335-338.
197.R. Lois, A. Dietrich, K. Hahlbrock and W. Schulz, *EMBO J,* 1989, **8**, 1641-1648.
198.R. Shi, C. M. Shuford, J. P. Wang, Y. H. Sun, Z. Yang, H. C. Chen, S. Tunlaya-Anukit, Q. Li, J. Liu, D. C. Muddiman, R. R. Sederoff and V. L. Chiang, *Planta,* 2013, **238**, 487-497.

199.K. Sato, N. Nishikubo, Y. Mashino, K. Yoshitomi, J. Zhou, S. Kajita and Y. Katayama, *Tree Physiol,* 2009, **29**, 1599-1606.
200.S. L. Butland, M. L. Chow and B. E. Ellis, *Plant Mol Biol,* 1998, **37**, 15-24.
201.Y. Osakabe, K. Osakabe and V. L. Chiang, *Plant Cell Rep,* 2009, **28**, 1309-1317.
202.Q. M. Shang, L. Li and C. J. Dong, *Planta,* 2012, **236**, 1093-1105.
203.R. L. Frank and L. O. Vodkin, *DNA Seq,* 1991, **1**, 335-346.
204.S. W. Lee, J. Robb and R. N. Nazar, *J Biol Chem,* 1992, **267**, 11824-11830.
205.P. V. van Summeren-Wesenhagen and J. Marienhagen, *Appl Environ Microbiol,* 2015, **81**, 840-849.
206.A. Vargas-Tah, L. M. Martinez, G. Hernandez-Chavez, M. Rocha, A. Martinez, F. Bolivar and G. Gosset, *Microb Cell Fact,* 2015, **14**, 6.

Table 1 phenylalanine ammonia-lyase gene family in plant species

Plant species	Number of PAL genes	Reference
Lemon balm (*Melissa officinalis* L.)	2	93
Ornithogalum caudatum	2	94
Raspberry (*Rubus idaeus*)	2	95
Bean (*Phaseolus vulgaris* L.)	3	175-177
Scutellaria baicalensis	3	178
Trifolium subterraneum	3	179
Salvia miltiorrhiza	3	13,180
Arabidopsis thaliana	4	97,101,181-184
Ephedra sinica	4	185
Carrot (*Daucus carota*)	4	186
Medicago truncatula	4	97
Tobacco (*Nicotiana tabacum*)	4	115,187-189
Populus kitakamiensis	4	190-192
Parsley(*Petroselinum crispum*)	4	193-197
Artichoke(*Cynara cardunculus* var. *scolymus* L.)	4	102
Bambusa oldhamii	4	112-114
Populus trichocharpa	5	97,198,199
Pinus banksiana	5	200
Loblolly pine (*Pinus taeda*)	5	62,64,201
Cucumber (*Cucumis sativus* L.)	7	202
Soybean (*Glycine max*)	8	97,203
Sorghum bicolor	9	97
Brachypodium distachyon	9	97
Rice(*Oryza sativa* L.)	12	15,97
Watermelon (*Citrullus lanatus*)	12	14
Vivis vinifera	16	97
Tomato (*Lycopersicon esculentum*)	18	204
Maize(*Zea mays* L.)	20	96,97
Potato (*Solanum tuberosum* L. cv. Datura)	40-50	98

Table 2 Synthetic potentials of PALs in metabolic engineering

No	Hosts	End products	Pathway enzymes	Source of PAL	References
1	*E. coli*	*trans*-cinnamic acid (**1**) *p*-coumaric acid (**2**) (*2RS*)-pinocembrin(**3**) (*2RS*)-naringenin(**4**)	PAL/TAL+4CL +CHS	*R.rubra*	123,124
2	*E. coli*	*trans*-cinnamic acid(**1**)	PAL+C4H +4CL+CHS	*A. thaliana*	117
3	*E.coli*	(*2RS*)-pinocembrin(**3**)	PAL+4CL+CHS	*A. thaliana*	146
4	*E.coli*	(*2S*)-pinocembrin(**5**)	PAL+4CL+CHS +CHI	*R.glutinis*	40
5	*E. coli*	(*2S*)-pinocembrin(**5**) (*2S*)-naringenin(**6**)	PAL/TAL+4CL +CHS+CHI	*R.rubra*	43
6	*E. coli*	(*2S*)-naringenin(**6**)	PAL/TAL+4CL +CHS+CHI	*R.glutinis*	145
7	*E.coli*	(*2S*)-naringenin(**6**)	PAL/TAL+4CL +CHS+CHI	*R.glutinis*	86
8	*E. coli*	(*2S*)-pinocembrin(**5**) (*2S*)-naringenin(**6**)	PAL/TAL+4CL +CHS+CHI	*R. mucilaginosa*	122
		chrysin(**7**) apigenin(**8**)	PAL/TAL+4CL +CHS+CHI+FNS1		
		galangin(**9**) kaempferol(**10**)	PAL/TAL+4CL+ CHS+CHI+F3H+F LS		
9	*E. coli*	dicinnamoylmethane(**11**) cinnamoyl-*p*-coumaroylmethane(**12**) bisdemethoxycurcumin(**13**) triketide pyrone(**14,15**)	PAL/TAL+4CL +CUS	*R. rhodotorula*	134
10	*E.coli*	dicinnamoylmethane(**11**) 6-fluoro-dicinnamoylmethane(**11a**) 6,6'-difluoro-dicinnamoylmethane(**11b**)	PAL+4CL +CUS	*Trifolium pratense*	135
11	*E. coli*	*p*-hydroxystyrene(**16**)	PAL/TAL+PDC	*R. glutinis*	144
12	*E. coli*	styrene(**17**)	PAL+CADC	*A. thaliana*	39
13	*E.coli*	*trans*-resveratrol(**18**)	PAL/TAL+4CL+S TS	*R.glutinis*	136
14	*E.coli*	eriodictyol(**19**)	PAL/TAL+4CL+C HS+CHI+F3'H+C PR	*R.glutinis*	133
15	*E.coli*	*trans*-resveratrol(**18**) pinosylvin(**20**) pinosylvin	PAL/TAL+4CL+S TS	*R.rubra*	137

		monomethyl ether(**21**) pinostilbene(**22**) pinosylvin dimethyl ether(**23**) pterostilbene(**24**)			
16	*E.coli*	pinosylvin(**20**)	PAL+4CL+STS	*T.pratense*	138
17	*E.coli*	pinosylvin(**20**)	PAL+4CL+STS	*P. crispum* *A. thaliana*	205
18	*E.coli*	*trans*-cinnamic acid(**1**) *p*-coumarate(**2**)	PAL/TAL	*A.thaliana* *R.glutinis*	206
19	*S.cerevisiae* *E. coli*	*trans*-cinnamic acid(**1**) *p*-coumarate(**2**)	PAL+C4H+CPR	*R.glutinis*	41
20	*E.coli* *S.cerevisiae*	genistein(**27**) (*2S*)-naringenin(**4**)	PAL/TAL+4CL+CHS+CHI+IFS	*R.rubra*	42
21	*S.cerevisiae*	*trans*-cinnamic acid(**1**) *p*-coumarate(**2**) 4-vinylphenol(**16**) styrene(**17**)	PAL+C4H+CPR	*Poplar* hybrid (*Populustricho carpa* × *P. deltoids*)	125
22	*S.cerevisiae*	pinocembrin(**3**) naringenin(**4**) styrene(**17**) 2',4',6'-trihydroxydihydrochalcone(**25**) phloretin(**26**)	PAL/TAL+4CL+CHS	*R.toruloides*	37
23	*S.cerevisiae*	*p*-coumarate(**2**)	PAL+C4H+CPR	*Poplar* hybrid (*P.Trichocarpa* ×*P.deltoids*)	127
		trans-resveratrol(**18**)	PAL+C4H+ CPR+4CL+RS		
		(*2S*)-naringenin(**6**)	PAL+C4H+CPR+ 4CL CHS+CHI		
		genistein(**27**)	PAL+C4H+CPR+ 4CL +CHS+CHI +IFS		
		kaempferol(**10**)	PAL+C4H+CPR+ 4CL CHS+CHI+ FLS+F3H		
		quercetin(**28**)	PAL+C4H+4CL+ FLS+CHS+CHI+F 3H+ F3'H		
24	*S.cerevisiae*	*trans*-resveratrol(**18**) *p*-coumarate(**2**)	PAL+C4H+4CL+ STS	*R. toruloides*	38
	S.cerevisiae	styrene(**17**)	PAL+FDC	*A. thaliana*	149
25	*S.cerevisiae*	(*2S*)-naringenin(**6**)	PAL+C4H+CPR+ 4CL CHS+CHI	*A. thaliana*	126

26	*P. putida*	*trans*-cinnamic acid(**1**) *p*-coumarate(**2**)	PAL/TAL	*R. toruloides*	129
27	*P. putida*	*trans*-cinnamic acid(**1**) *p*-coumarate(**2**)	PAL/TAL	*R. toruloides*	128
28	*P. putida*	*trans*-cinnamic acid(**1**) *p*-hydroxystyrene(**16**)	PAL/TAL+PDC	*R. toruloides*	33
29	*S. coelicolor*	*trans*-cinnamic acid(**1**)	PAL	*S. maritimus*	80
30	*S. lividans*	*trans*-cinnamic acid(**1**)	PAL	*S. maritimus*	132
31	*S. lividans*	*trans*-cinnamic acid(**1**)	PAL	*S. maritimus*	131
32	*S. lividans*	*p*-coumarate(**2**)	PAL/TAL	*Rhodobacter sphaeroides*	130

Table 3 Optimization strategies improving metabolic flux to precursor pools

Optimization strategies	engineered host	Product(before optimization, postoptimality)	Fold change	Reference
Reconstruction of malonate assimilation pathway	*E.coli*	(*2S*)-pinocembrin(0.71mg/L,1.22mg/L)	1.71	40
	E.coli	(*2S*)-naringenin(29mg/L,46mg/L)	1.58	145
Overexpression of multisubunit complex of acetyl-CoA carboxylase	*E.coli*	(*2S*)-naringenin (0.31mg/L,1.01mg/L)	3	43
		(*2S*)-pinocembrin(0.17mg/L,0.71mg/L)	4	
A combination of three strategies, that is ①Overexpression of acetyl-CoA carboxylase (ACC); ②overexpression of acetyl-CoA synthase (encoded by acs); ③deletion of the acetate kinase gene.	*E.coli*	eriodictyol(42.6mg/L, 107 mg/L)	2.51	133
Overexpression of multisubunit complex of acetyl-CoA carboxylase	*E.coli*	(*2RS*)-pinocembrin(58mg/L,82mg/L)	1.41	146
Use of the fatty acid pathway inhibitor cerulenin	*E.coli*	(*2S*)-naringenin(29mg/L,84mg/L)	2.9	145
		pinosylvin(3.29mg/L, 59mg/L)	18	205
Alleviation of tyrosine feedback inhibition of yeast 3-deoxy-D-arabinose-heptulosonate-7-phosphate (DAHP) synthase	*S.cerevisiae*	(*2S*)-naringenin	2	126
Elimination of competing phenylpyruvate decarboxylase activity	*S.cerevisiae*	(*2S*)-naringenin	3	126

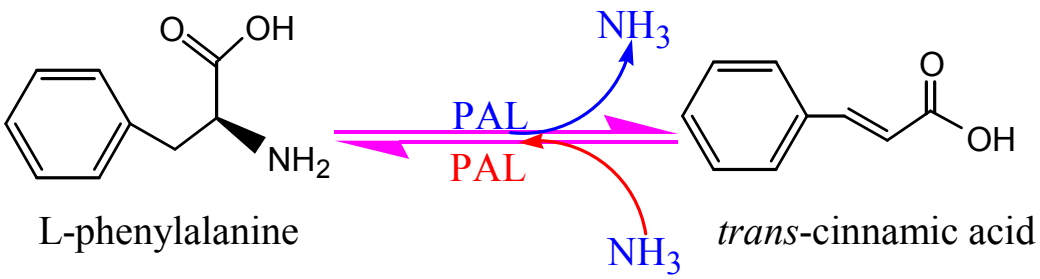


Figure 1 Forward reaction catalyzed by PALs

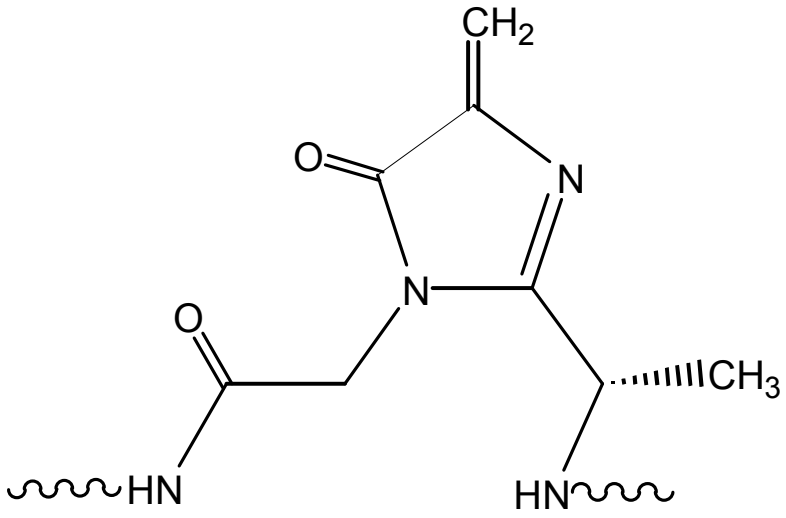


Figure 2 Chemical structure of the MIO cofactor

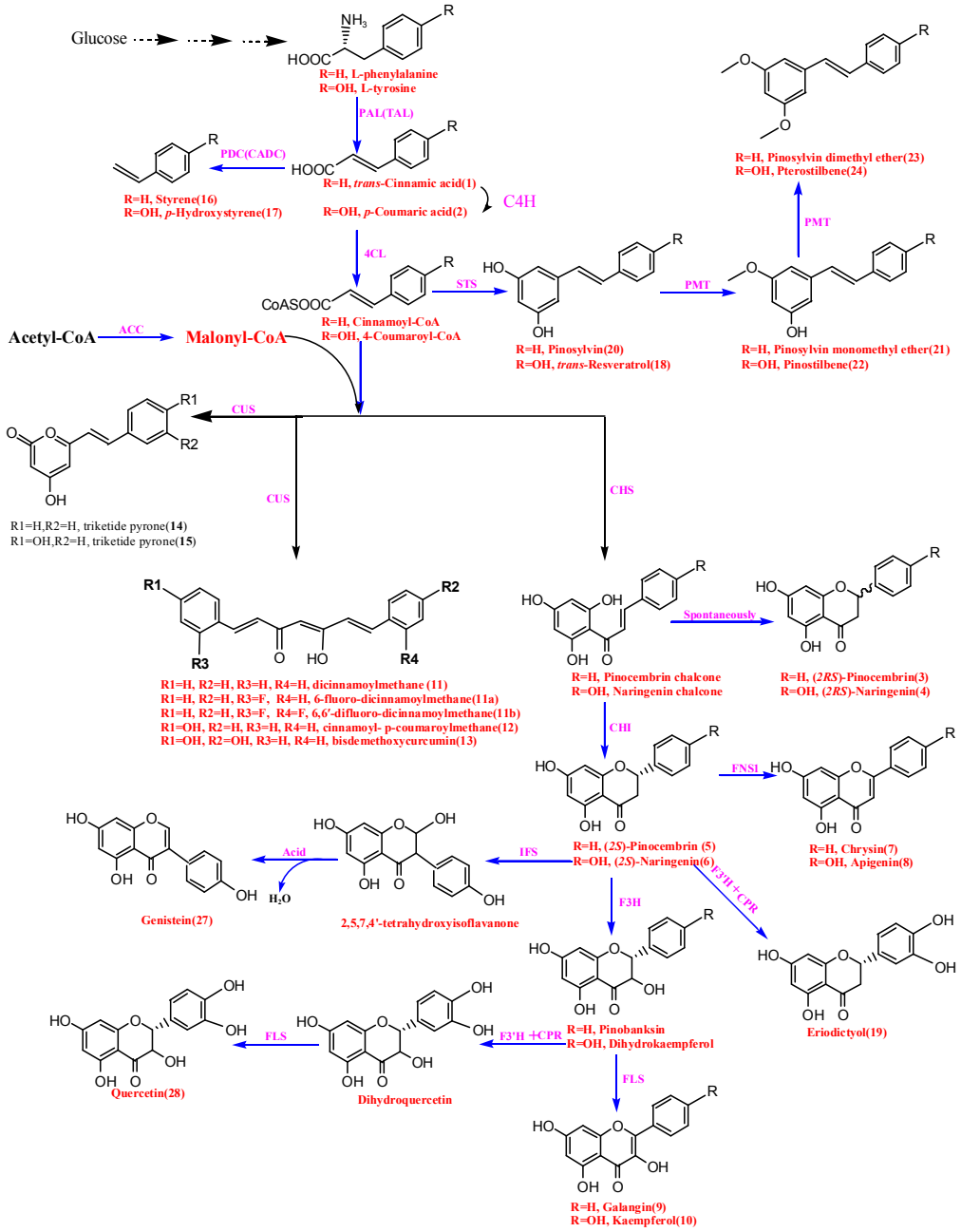


Figure 3 Schematic overview of all the engineered flavonoids biochemical pathways

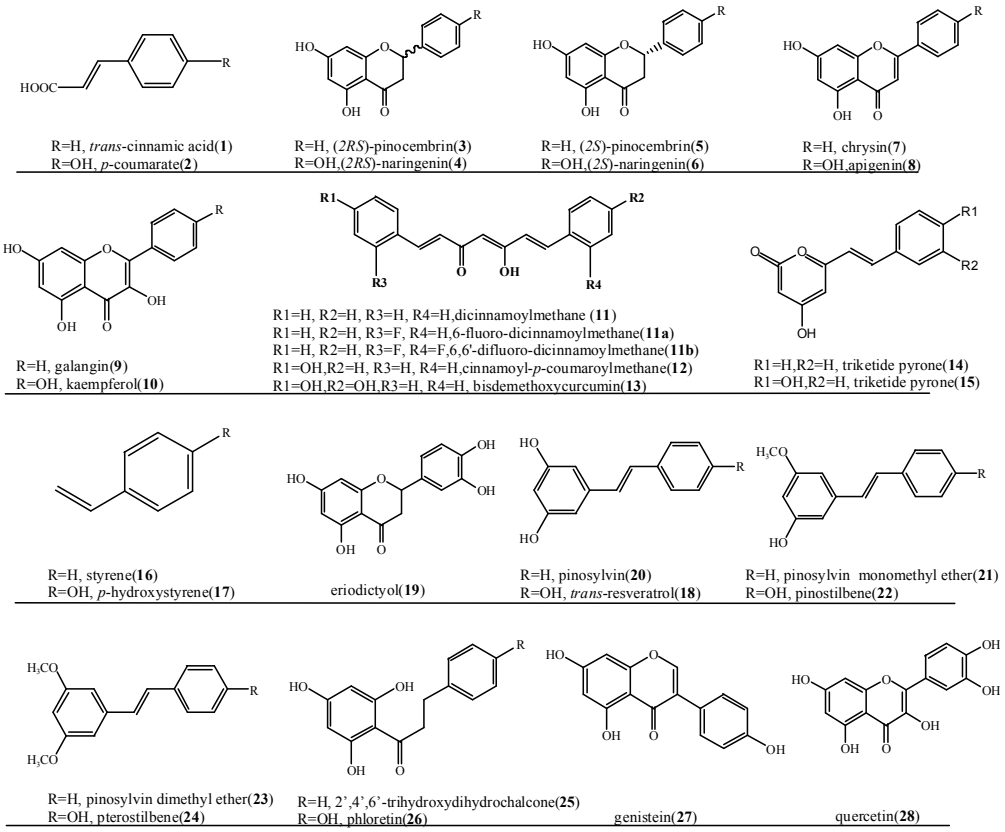


Figure 4 Phenylpropanoids produced by engineered microorganisms containing PALs

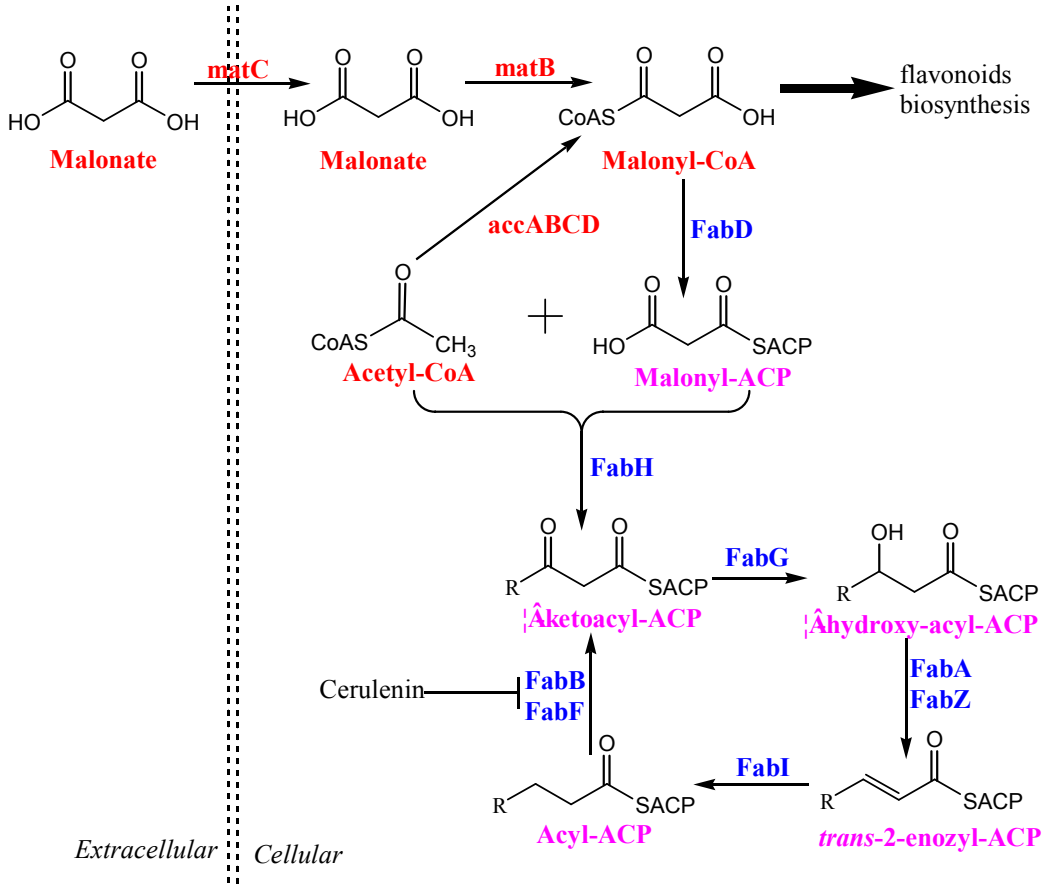
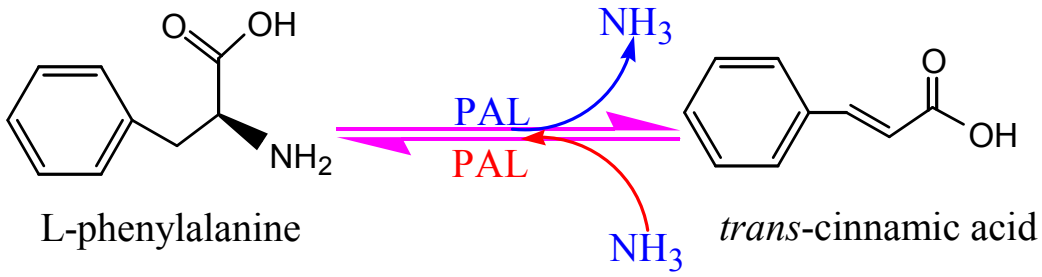


Figure 5 Metabolic crosstalk of flavonoids precursors



Phenylalanine ammonia-lyase, a versatile enzyme with industrial and medical applications.