METABOLIC ENGINEERING OF *Escherichia coli* TO PRODUCE PLANT SPECIFIC SECONDARY METABOLITES FROM AROMATIC AMINO ACIDS

by

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(Under the Direction of YAJUN YAN)

ABSTRACT

Plants are known for producing diversified metabolites that are exclusively identified from plant sources. These plant-specific metabolites have been demonstrated to perform actively in signal transmission to assist plants growth under exogenous stress. This category of natural compounds attracts more attention beyond their roles in plants for their health promoting capabilities and medicinal potentials. However, commercialization of synthetic technology towards these therapeutic candidates is yet to be developed for their insufficient availability from native origins and uneconomical chemical approaches. In order to engineer more cost-effective alternatives of yielding these valuable metabolites, microbial production has been established by exploiting plant genes and enzymes to construct artificial synthetic pathway towards end products in categories of alkaloids, phenylpropanoids and isoprenoids. Caffeic acid, as a target compound in research, was produced in metabolically engineered *E. coli*. Highest titers from *de novo* production of caffeic acid and one-step conversion were achieved in this work.

INDEX WORDS: Aromatic amino acids; *Escherichia coli*; Tyrosine; Phenylalanine; Tryptophan; Alkaloid; Phenylpropanoid; Isoprenoid; Caffeic acid

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CHAPTER 1

INTRODUCTION

Plant-specific metabolites include alkaloids, phenylpropanoids, isoprenoids and other diversely-structured compounds. These compounds, in plants, are naturally synthesized through complicated networks of pathways derived from aromatic amino acids and are ubiquitously involved in stress-induced mechanism indirectly related to plant growth through signal transmission(Pavarini et al., 2012).

Plant-specific metabolites have drawn great attention for their potent pharmaceutical capabilities, estimated therapeutic potentials and promising economic values. Even though plant kingdom provides abundant source for medicinal research and applications, further research and employment have been throttled by limited availability of this class of natural compounds for lack of efficient synthetic approaches. In plants, they accumulate at deficient amount for economic manufacturing. When plant cell suspension culture, like hair root cells (Hai-Chao et al., 2009), in bioreactors have been applied for improvement of titer, slow growth of plant cells, delicate growth conditions and relatively low yield are still preventing the technology to be commercially feasible. On the other hand, chemical synthesis strategies for these complex-structured compounds are faced with poor conversion efficiency due to high energy demand during reactions, separation and purifications of end products. Waste handling and environmental impact also raise concern over implementation of chemical synthesis (Hussain et al., 2012).

Therefore, more competent and green paths are in great need to accelerate commercialization of plant secondary metabolites. Microbial production of plant-specific metabolites is one of attractive alternatives to achieve advances towards production scales adequate for industry manufacture. To begin with, microbial synthetic mechanism and regulations of aromatic amino acids are well-understood than their plant counterparts. Boosting carbon flux towards aromatic amino acids is more feasible in microbial systems. Research targeting at microbial overproduction of aromatic amino acids have progressed to gram per liter level of titer over the past decades. As the availability of aromatic amino acids increases, theoretical yield of foreign plant-specific pathways expressed in microbes will rise accordingly. So far, some progress have been reported in metabolic engineered bacteria and yeast to produce alkaloids, especially benzylisoquinoline (Schäfer and Wink, 2009, Nosov, 2010), phenylpropanoids (Trantas et al., 2009) and isoprenoids. Among these microbial platforms, metabolically engineered *Escherichia coli* strains have performed well to produce plant secondary metabolites from aromatic amino acids.

In following chapters, pathway and regulations of aromatic amino acids synthesis in microbe were explained. Recent work of improvement of aromatic amino acids production efficiency in microbial system, including my submitted manuscript on caffeic acid biosynthesis is listed with progress and opportunities in realization of microbial production of these valuable compounds in *E. coli*.

CHAPTER 2

MICROBIAL AAA PATHWAY

In *Escherichia coli*, the first committed step for aromatic amino acids synthesis is the conversion to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) from erythrose 4-phosphate and phosphoenolpyruvate catalysed by DAHP synthase. One phosphate group is released from this reaction. This reversible reaction is realized through three subunits of DAHP synthase encoded by *aroF*, *aroH* and *aroG*. Another phosphate group is lost through the further conversion from DAHP to 3-dehydroquinate by 3-dehydroquinate synthase which is encoded by *aroB*. And one molecule of water will be released after a reversible reaction from 3-dehydroquinate to 3-dehydroshikimate by 3-dehydroquinate dehydratase encoded by *aroD*.

One of the most important intermediates in aromatic amino acid pathway is shikimate. And shikimate is synthesized by shikimate dehydrogenase from 3-dehydroshikimate consuming one molecule of NADPH. The dehydrogenase is encoded by *aroE* and *ydiB*. One molecule of ATP is consumed to add a phosphate group onto shikimate by shikimate kinase resulting in shikimate-3-phosphate, which then reacts with phosphoenolpyruvate again to form 5-enolpyruvyl-shikimate-3-phosphate. The latter reaction is reversible.

The second key intermediate, chorismate is produced from the dephosphorylation of 5-enolpyruvyl-shikimate-3-phosphate. In aromatic amino acid pathway, chorismate serves as a branch point towards either -tryptophan or phenylalanine and tyrosine.

Tryptophan branch initiates from the reaction of chorimate and l-glutamine to anthranilate, pyruvate and l-glutamate. An anthranilate synthase encoded by *trpE* and *trpD* is

responsible for the conversion. Anthranilate is then transferred with a phosphoribosyl group from 5-phospho-α-D-ribose 1-diphosphate. The product of N-(5'-phosphoribosyl) - anthranilate is converted to (1s, 2R)-1-C-(indol-3-yl) glycerol 3-phosphate by a bifunctional phosphoribosylanthranilte isomerase/ indole-3-glycerol phosphate synthase encoded by *trpC*. The phosphate reversibly disintegrates into indole and D-glyceroldehyde 3-phosphate.

The last step towards tryptophan is realized by the reaction between indole and l-serine catalyzed by *trpB*, a subunit of tryptophan synthase. When chorismate is converted to prephenate by chorismate mutase/prephenate dehydrogenase (CM-PDH), the carbon flux drives into phenylalanine and tyrosine synthesis.Prephenate is dehydrogenated to keto-phenylpyruvate and 4-hydroxyphenylpyruvate. The previous reaction is enabled by gene *pheA* while the latter is enabled by *tyrA*. Keto-phenylpyruvate converts to Phenylalanine by phenylalanine transaminase and aminotransferase while tyrosine is converted from 4-hydroxyphenylpyruvate by tyrosine aminotransferase.

CHAPTER 3

MICROBIAL AAA PATHWAY REGULATIONS AND ENGINEERING

Despite the functional mechanisms of AAA biosynthesis in *Escherichia coli* system, various feedback regulations modulate the expression level of genes involved in the pathway, balancing the synthesis of tryptophan, phenylalanine and tyrosine. Understanding of bottlenecks and key regulation steps in AAA pathway provides opportunities for metabolic engineering to achieve overproduction of aromatic amino acids and their derivatives. Most of yield enhancing approaches follow a stepwise improvement logistic, beginning with increasing the availability of DAHP. E4P from pentose phosphate pathway and PEP from glycolysis pathway condense to draw carbon flux into the AAA biosynthesis. Therefore, work has been done to balance both pools for optimal DAHP production. Studies have shown that E4P is the limiting metabolite than PEP, but transketolase (Tkt) towards E4P is over-expressed together with PEP synthase (Pps) to reach a higher generating rate towards DAHP from glucose comparing to over-express Tkt or Pps alone(Rizk and Liao, 2009). By co-expressing *ppsA* and *tktA*, the yield of DAHP can nearly reach theoretical limit (Li et al., 2003), tryptophan yield can be improved by 11.9% to 40.2g/L in fed-batch cultivation(Shen et al., 2012), and tyrosine production was raised by 80% in 50ml batch culture (Lütke-Eversloh and Stephanopoulos, 2007). To elevate PEP production, another general regulator, carbon storage regulator (Csr) has been deleted to tune up gluconeogenesis and tune down glycolysis, therefore diverts more carbon flow towards PEP to be incorporated into AAA pathway twice. And *csrA* mutant was able to double Phenylalanine production over its

parent, a phenylalanine-overproducing strain *E. coli* K-12 NST74 (ATCC31884) (Tatarko and Romeo, 2001).

Within AAA common pathway, the first step serves as a major controlling point, for three isozymes of DAHP synthase, *aroF*, *aroG* and *aroH* are sensitive to tyrosine, phenylalanine and tryptophan, respectively. Most research has been focused on developing and applying *aroG* feedback inhibition resistant mutant *E. coli* strains (Doroshenko et al., 2010). A minor substitution of Asp-146-Asn yielded an $AroG^{fbr}$ enzyme and achieved 71mg/L tyrosine production from *E. coli* harboring this mutant enzyme in shake flasks study (Lütke-Eversloh and Stephanopoulos, 2007).

Another DAHP synthase isozyme subject to tyrosine repression, AroF, has been studied for its feedback inhibition mechanism. And a single modification on its N-terminal (N8K) resulted in a tyrosine insensitive mutant (Jossek et al., 2001). However, tyrosine feedback resistant $aroF^{fbr}$ strain didn't perform as well as $aroF^{wt}$ did for Phenylalanine production, while the latter achieved 34g/L titer over 28g/L from the previous strain, both under tyrosine control (Gerigk et al., 2002).

Proceeding with the carbon flow, another major regulating step is the branch point of chorismate to prephenate which phenylalanine and tyrosine are derived from by PheA and TyrA, respectively. Feedback inhibition mechanism of TyrA has been analyzed that Tyrosine binds to the PDH domain and tunes down the carbon flux towards tyrosine. And $tyrA^{fbr}$ mutants are constructed accordingly(Lütke-Eversloh and Stephanopoulos, 2005).

On the other hand, *trpEDCAB* operon generates another branch from chorismate to tryptophan (Bongaerts et al., 2001). Among this gene cluster, *trpE* went through site-directed mutation to eliminate inhibition by tryptophan,

The repression mechanism of TyrR proteins to bind to multiple genes along AAA pathway is subject to metabolic engineering as well(Pittard et al., 2005). TyrR can be activated by any one of the three AAAs (Khodursky et al., 2000). Therefore, *tyrR* knock-out *E. coli* strains were used as parent to host over-expression systems for AAA production and their derivatives (Muñoz et al., 2011, Polen et al., 2005, Lütke-Eversloh and Stephanopoulos, 2007).

In addition to engineering native pathway and enzymes in *E. coli* to enhance AAA production, foreign enzymes have also been introduced to extend the potential for specific steps of reactions. A phenylalanine dehydrogenase gene (*phedh*) from *Bacillus lentus* was cloned and expressed in *E. coli* BL21 (DE3) to overproduce Phenylalanine at 366 mg/l (Thongchuang et al., 2012). In this study, *E. coli* genes *yddG* and *glpF* were coexpressed, encoding an AAA exporter and a glycerol transport facilitator, to further assist phenylalanine overproduction from glycerol.

CHAPTER 4

PLANT-SPECIFIC METABOLITE SYNTHESIS IN PLANTS

In plants, AAAs are not only utilized as components for protein synthesis, but also as precursors for secondary metabolism towards a wide range of complex natural compounds named as plant-specific metabolites. AAA biosynthesis in plants follow shikimate pathway and divide at the branch point where chorismate is an important intermediate as in *E. coli*. However, the regulation and coordination of plant AAA metabolism is not yet to be thoroughly proven(Tzin and Galili, 2010). Though some natural compounds are synthesized in certain species, for example morphinan alkaloids naturally occur in the genus *Papaver* only(Allen et al., 2008), plant-specific metabolites are found ubiquitously throughout all plants. Mechanisms and regulations of these plant-specific metabolites biosynthesis are not yet elucidated. Therefore, native metabolic engineering approaches in plants are still opportunistic than systematic. In this review, recent progress will be discussed in three categories, alkaloids, phenylpropanoids and isoprenoids.

4.1 Alkaloids

Alkaloids have been characterized into various subclasses based on different structures, yet most of them exhibit promising human disease-curing and health-promoting features because of their toxicity and biological activity. Among all alkaloids, isoquinoline alkaloid biosynthesis pathway is one of the best-characterized systems with genes and enzymes along the pathway isolated.

As the only commercial source of benzylisoquinoline alkaloids (BIAs), opium poppy has been studied for its biosynthetic pathway of codein, morphine and noscapine. In Opium Poppy, tyrosine is transaminated with α-ketoglutarate by tyrosine aminotransferase (TyrAT), and then decarboxylated to 4-hydroxyphenylacetaldehyde (4-HPAA), as one of its occurrences(Lee and Facchini, 2011). One of the most important intermediates in BIA biosynthetic pathway, (s)-reticuline is a heat spot around which BIA researches are conducted (Han et al., 2010, Winkler et al., 2009, Nanae et al., 2007). Downstream from (s)-reticuline, berberine bridge enzyme (BBE) expression has been knocked down in California poppy cells, (s)-reticuline accumulation in transgenic California poppy cell was observed at 6 mg/20 mL in culture and end-products of isoquinoline alkaloids were considerably reduced(Nanae et al., 2007). Once (s)-scoulerine is converted by BBE, scoulerine o-methyltransferases (SOMTs) have been studied using virus-induced gene silencing, and noscapine and papaverine level was monitored, confirming a metabolic step in alkaloids biosynthesis in Opium poppy(Dang and Facchini, 2012). (S)-reticuline is also branched to synthesize thebaine, codeine, and morphine via salutaridine and salutaridinol. Over-expression and suppression of salutaridinol 7-o-acetyltransferase (SalAT) resulted in corresponding variations of alkaloid products that accumulate in transgenic opium poppy cells(Allen et al., 2008).

Another AAA, L-tryptophan is decarboxylated to tryptamine and then paves the way towards monoterpene indole alkaloids. An interesting fact is that the downstream pathway from tryptamine is not affected when tryptophan decarboxylase is silenced by RNAi, inspiring feeding of tryptamine analogs to produce unnatural compounds with similar structures of native metabolites in *Catharanthus roseus* hair root culture(Runguphan et al., 2009).

4.2 Phenylpropanoids

Phenylpropanoids form the largest family of plant secondary metabolites and they also possess medicinal properties as anti-inflammatory, anti-cancer, antiviral, antibacterial and wound healing agents(Korkina et al., 2011). As the starting point of general phenylpropanoid pathway, Phenylalanine is converted to cinnamic acid by phenylalanine ammonia lyase. Cinnamate 4-hydroxylase (C4H) is the second enzyme and the first cytochrome P450 in the pathway, which, recently, has been isolated and characterized from apricot and plum to study its mechanism and regulations.(Pina et al., 2012)

4-coumaroyl CoA ligase (4CL) is responsible for adding a CoA group to 4-coumaric acid and has been reported to be subjected to allosteric inhibition by downstream products of phenylpropanoid pathway such as naringenin (Alberstein et al., 2012) .

4.3 Isoprenoids

Isoprenoids, unlike alkaloids and phenylpropanoids, carry a class of both primary metabolites and secondary metabolites in plants. As the most structurally diverse group of natural product, isoprenoids are also ubiquitously found in fungi and other forms of life. Therefore, their occurrences and metabolic regulations are much more complicated. Despite the fact that isoprenoids synthesis is led by multi-step catalysis and subjected to frequent inhibitions, they still manage to stay in heat spot for metabolic and therapeutic study. Taxol, for instance, one of the most competent anticancer drugs originally extracted from the bark of the Pacific yew tree, is now produced at gram level in engineered *E. coli*. (Ajikumar et al., 2010a)

The synthesis of isoprenoids in plant cells starts with universal precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Vranová et al., 2012), following mevalonate pathway in animal cells and yeast, and nonmevalonate pathway in plant cells and *E.*

coli.(Liu and Khosla, 2010) Alternative synthesis strategies of isoprenoids have been explored in other organisms such as algae(Lohr et al., 2012).

CHAPTER 5

MICROBIAL PRODUCTION OF PLANT-SPECIFIC METABOLITES IN *E. coli*

As a general direction towards heterologous expression of plant pathways in microbes, *E. coli* appears to be unparalleled because of its robust metabolism and researchers' profound knowledge upon its native biochemical reactions. Therefore, a great proportion of microbial production approaches was constructed in *E. coli*, and shared similarities in rationale and perspectives. A universal angle to go into this metabolic engineering subfield is always to take advantage of *E. coli*'s native pathways and metabolites and direct them to foreign pathways. Under most circumstances, plant enzymes need to be substituted with microbial isoenzymes for effective expression and catalytic activities. Once proof of concept is achieved, tuning strategies for higher productivity will be developed to adjust and harmonize individual units in engineered biosynthetic system for optimal overall performances. While primary genetic modifications are applied upon synthetic pathway construction, native *E. coli* genes also undergo different levels of engineering. These alterations include deletion and tune-down of inhibition-sensitive genes and competitive pathways, over-expression of genes for more metabolic flux and cofactors, and balancing reaction rates and drive forces.

5.1 Alkanoids

Microbial production of (s)-reticuline has been realized by combining and co-expressing microbial enzymes and plant enzymes in transgenic *E. coli* and then using crude enzyme to convert dopamine, yielding 55mg/L (s)-reticuline within 1h. In addition to it, an aporphine alkaloid, magnoflorine, or a protoberberine alkaloid, scoulerine were synthesized from dopamine

via reticuline by using different combination cultures of transgenic *E. coli* and *S. cerevisiae* cells. The final yields of magnoflorine and scoulerine were 7.2 and 8.3 mg/L in culture medium (Minarni et al., 2008). This tailored expression system was then adopted into a tyrosine overproducing platform, increasing the yield of (s)-reticuline and end products of BIA such as magnoflorine and scoulerine (Nakagawa et al., 2011). (s)-Reticuline production was also established yielding 33.9mg/L from glucose and glycerol in bioengineered *E. coli* in shake flasks at bench level (Nakagawa et al., 2012).

5.2 Phenylpropanoids

Tyrosine, other than Phenylalanine is used as precursor of phenylpropanoid pathway in *E. coli* to construct an artificial expression network towards biosynthesis of ferulic acid, naringenin and resveratrol. Tyrosine ammonia lyase and 4-coumarate 3-hydroxylase (Sam5) from S. espanaensis yielded caffeic acid via 4-coumaric acid, followed by a methylation step catalyzed by caffeic acid O-methyltransferase (COM) to form ferulic acid. Cinnamate/4-coumarate: coenzyme A ligase (ScCCL) from S. coelicolor directed 4-coumaric acid to 4-coumaroyl-CoA, which was then condensed with three molecules of malolnyl-CoA by chalcone synthase (CHS) and stilbene synthase (STS) to form naringenin chalcone and resveratrol, respectively. Naringenin was produced spontaneously from naringenin chalcone. COM and CHS were from Arabidopsis thaliana, and STS was from Arachis hypogaea.(Choi et al., 2011) Caffeic acid synthesis from Tyrosine was achieved by substitution of Sam5 for endogenous 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H) in *E. coli* at 50.2mg/L.(Lin and Yan, 2012) However, the highest titer of caffeic acid in *E. coli* was realized by expressing a F185L mutant bacterial cytochrome P450 CYP199A2, catalyzing one step reaction from p-coumaric acid to yield 2.8g/L caffeic acid.(Furuya et al., 2012) Stilbene biosynthesis in recombinant *E. coli* was

built upon a series of feeding experiments that converted 4-coumaric acid to resveratrol and caffeic acid to piceatannol by 4CL from A. thaliana and STS from A. hypogaea. (Watts et al., 2006) Trans-resveratrol was also achieved at 0.31mg/L in recombinant S. cerevisiae culture with 1mM p-coumaric acid supplement and 0.29mg/L with 10mM phenylalanine supplement. C4H and 4CL were exploited again in this recombinant construct together with phenylalanine ammonia lyase (PAL), resveratrol synthase (RS) and cytochrome P450 reductase (CPR). (Trantas et al., 2009)

As described above, flavonoids biosynthesis pathway have been adopted in both *E. coli* and S. cerevisiae to yield flavones, resulting 0.57μg/l naringenin and 0. 2μg/l pinocimbrin from 360mg/L tyrosine and 330mg/L phenylalanine, respectively (Limem et al., 2008). Using a similar recombinant *S. cerevisiae* with CHS and CHI instead of RS, naringenin was produced at 15.6mg/L after 100 hours with 1mM p-coumaric acid as well. When 10mM phenylalanine was added, 8.9mg/L naringerin was observed in yeast culture after 150 hours (Trantas et al., 2009). Upon efforts to produce naringenin, a major precursor of flavonoid, tyrosine overproducing *E. coli* was transformed with a heterologous pathway with tyrosine ammonia lyase (TAL), 4CL, CHS and chalcone isomerase (CHI). Naringenin was observed in minimal salt culture without feeding phenylpropanoic precursors. After optimization, this platform could produce naringenin at 29mg/L from glucose and 84mg/L if assisted by fatty acid enzymes inhibitor, cerulenin.

After consolidating isoflavone synthase (IFS) into naringenin producing yeast, genistein producing pathway was reported to be able to convert 0.5mM naringenin to 7.7mg/L genistein. Meanwhile, if flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) were assembled into naringenin-producing yeast instead of IFS, kaempferol, a flavonol accumulated at 4.6mg/L.

Another flavonol, quercetin was produced at 0.38mg/L from 0.5mM naringenin by putting in another plant-origin enzyme flavonoid 3' hydroxylase (F3'H).(Trantas et al., 2009)

5.2.1 Caffeic Acid Production Realization and Enhancement

(This work has been accepted by Biotechnology and Bioengineering at the time of submission of this thesis. First authors are Qin Huang and Yuheng Lin, Correspondent author is Yajun Yan, all from College of Engineering, University of Georgia.)

Caffeic acid (3, 4-dihydroxycinnamic acid), a secondary metabolite ubiquitously occurring in plants, has presented multiple proven and potential health-improving benefits. Among them, its wide application as antioxidant (Sato et al., 2011) and anti-inflammatory agent (Chao et al., 2010) identified itself as an industrially valuable compound. Besides, one of its derivatives, caffeic acid phenethyl ester (CAPE) has presented promises in cirrhosis amelioration (Dianat et al., 2012) and demonstrated anti-tumor effect, especially against prostate cancer (Chuu et al., 2012, Kudugunti et al., 2010, Wu et al., 2011). Other derivatives, such as caffeic acid undecyl ester (CAUE) possess potent anti-tumor activities (Tomizawa et al., 2013).

Despite its established market and hopeful expansion of applications, current production approaches of caffeic acid from plant extract have been regarded as energy-intensive and environmental unfriendly (Xing et al., 2012). And it is desirable to develop more efficient innovative producing strategies to meet the growing market and to lower the cost. In nature, a family of aromatic secondary metabolites, such as caffeic acid itself, is derived from the phenylpropanoid pathway in plants via deamination and hydroxylation of phenylalanine while the latter step is a major target for metabolic engineering (Bourgaud et al., 2006). Several attempts have been made to achieve biosynthesis of caffeic acid in transgenic microorganisms in recent years. After the identification of *sam8* and *sam5*, encoding a tyrosine-ammonia lyase

(TAL) and a 4-coumarate 3-hydroxylase (Coum3H), respectively, a native pathway starting from tyrosine instead of phenylalanine was proposed in *Saccharothrix espanaensis* (Berner et al., 2006). This pathway was then reconstituted in *E. coli* and achieved heterologous production of caffeic acid from tyrosine (Choi et al., 2011). Afterward, an artificial dual pathway adopting a promiscuous bacterial 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H) was established in metabolically engineered *E. coli* resulting in the total biosynthesis of caffeic acid at a titer of 50.2 mg/L in shake flasks (Lin and Yan, 2012). Besides, another combinatorial pathway involving Coum3H, TAL and 4-coumaroyl-CoA ligase was engineered in *E. coli* affording 106 mg/L of caffeic acid from renewable sugars in bioreactor (Zhang and Stephanopoulos, 2012). Recently, efforts have been focused on increasing the intracellular availability of the direct precursor of caffeic acid. A codon-optimized TAL was used to achieve 150 mg/L caffeic acid from glucose (Kang et al., 2012). In addition, a two-phase bioconversion process developed over a mutated cytochrome P450 hydroxylase was able to catalyze the conversion of 15 mM p-coumaric acid to caffeic acid (2.8 g/L) by highly condensed cell cultures (Furuya et al., 2012).

So far, establishment of plant-specific phenylpropanoid pathway in metabolically engineered *E. coli* has been employed to synthesize more diverse compounds such as flavonoids, isoflavonoids, and stilbenes (Choi et al., 2011, Yan et al., 2007, Leonard et al., 2006) (more refs). Therefore, diverting more metabolic flux into phenylpropanoic acid such as caffeic acid and p-coumaric acid synthesis and elevating their titers are of great significance. In this work, we further explored the biosynthetic potential of the *E. coli* endogenous 4HPA3H and achieved enhanced de novo caffeic acid production by metabolic engineering of a phenylalanine over-producing *E. coli* strain. Wild-type *E. coli* expressing 4HPA3H was able to convert 3.5 g/L p-coumaric acid to caffeic acid (3.82 g/L). De novo production of caffeic acid followed step-wise

enhancement rationale, resulting in 766.68 ± 43.11 mg/L caffeic acid within 72 hrs in shake flasks.

Materials and Methods

Strains and chemicals

E. coli strain ATCC31884 was purchased from ATCC and used as the parent strain to perform gene knock-out. The knock-out strain was for transformations and shake flask experiments. *E. coli* strain XL1-Blue (Stratagene) was used for gene cloning. *E. coli* strain BW25113 (*E. coli* Genetic Resource Center) was used for expressing enzymes in feeding experiments. The characteristics of the strains are listed in Table 1. M9 minimal salt media, Luria Broth (LB) media, and yeast extract were purchased from BD Biosciences and used following manufacture instructions. MOPS was bought from Sigma for media preparation. Modified M9 media used for shake flask studies were prepared with nutrient supplement to M9 minimal salt media (Lin and Yan, 2012). Glucose and glycerol as carbon sources were purchased from Sigma and Fisher Scientific, respectively. IPTG (Zymo Research Co.), L-arabinose (Alfa Aesar), phenylalanine (Sigma), tyrosine (Sigma), p-coumaric acid (MP Biochemicals) and caffeic acid (TCI) were used for media supplement and HPLC standards.

Disruption of *pheLA-tyrA* genes

Red disruption method (Datsenko and Wanner, 2000) was used to knock out *pheLA-tyrA* fragment off *E. coli* strain ATCC31884. Forward and reverse primers for amplifying the kanamycin-resistant fragment from pKD13 are phe_F and phe_R, shown in Table 1. Underlined sequences are reverse-complimentary of priming sites flanking FRT and kanamycin-resistant gene on pKD13. PCR products were then transformed into *E. coli* (ATCC31884) competent cells

carrying pKD46. Single colonies were picked from the plates containing both kanamycin (50 μg/ml) and ampicillin (100 μg/ml) and inoculated into LB media with both antibiotics. Competent cells of *E. coli* ATCC31884 with the kanamycin-resistant gene replacing the *pheLA-tyrA* fragment (designated as QH-5) were prepared from overnight culture for transforming pCP20 to delete the kanamycin-resistant gene, resulting in strain QH-4. Colony PCR was performed to verify QH-4 and QH-5 using primers phe_F(v) and phe_R(v). *E. coli* strain ATCC31884 colonies were used as control.

Table 1 Primers, Plasmids and Strains

Primers	Sequence	Source or reference
phe_F	GCAACATCGGTGAAAGACGCCAACTTCGTCGAAGAAGTTGCTGTCAAACATGAGAATTAA	This Study
phe_R	CTGGCGATGGTCGGGCGACTGTTTGCTCAGGATCCGCAGCGTGTAGGCTGGAGCTGCTTC	This Study
phe_F(v)	TTGATCAACAAGCTGGAACGGC	This Study
phe_R(v)	TGGCGCTCTCTTCGCCGATTTA	This Study
RgTAL_F1 (KpnI)	GGGAAA*GGTACC*ATGGCGCCTCGCCCGACTTC	This Study
RgTAL_R1 (SphI)	GGGAAA*GCATGC*TTATGCCAGCATCTTCAGCAGAACATTG	This Study
HpaBC_F1 (SphI)	GGGAAA*GCATGC*AGGAGATATACCATGAAACCAGAAGATTTCCGCGCCAG	This Study
HpaBC_R1 (XbaI)	GGGAAA*TCTAGA*TTAAATCGCAGCTTCCATTTCCAGCATC	This Study
HpaBC_F2 (KpnI)	GGGAAA*GGTACC*ATGAAACCAGAAGATTTCCGCGC	This Study
HpaBC_R2 (SphI)	GGGAAA*GCATGC*TTAAATCGCAGCTTCCATTTCCAGC	This Study
RgTAL_F2 (SphI)	GGGAAA*GCATGC*AGGAGATATACCATGGCGCCTCGCCCGACTTC	This Study
RgTAL_R2 (XbaI)	GGGAAA*TCTAGA*TTATGCCAGCATCTTCAGCAGAACATTG	This Study
Opr1_F (SacI)	GGGAAA*GAGCTC*TCTTCACCTCGAGAATTGTGAGCG	This Study
Opr2_R (SpeI)	GGGAAA*ACTAGT*CTACTCAGGAGAGCGTTCACCG	This Study
TthpaBC_F (KpnI)	GGGAAA*GGTACC*ATGGCAAGGACCGGAGCGGA	This Study
TthpaBC_R (XbaI)	GGGAAA*TCTAGA*TCACGATGGCCATACCAACCTCC	This Study

Plasmids	Construct	Source or reference
pZE12-luc	ColE1 ori; Amp^R; $P_L$lacO1; *luc*	Lutz et al., 1997
pKD13	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *Δ(phoB-phoR)580*, *λ⁻*, *galU95*, *ΔuidA3::pir⁺*, *recA1*, *endA9*(del-ins)*::FRT*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Datsenko and Wanner, 2000
pCS27	p15A ori; Kan^R; $P_L$lacO1; MCS1	Shen et al., 2008
pCS-TPTA	From pCS27, $P_L$lacO1; *tyrA^{fbr}-ppsA-tktA-aroG^{fbr}*	Lin and Yan, 2012
pZE-EchpaBC	From pZE12, $P_L$lacO1; *EchpaBC*	Lin and Yan, 2012
pZE-TthapBC	From pZE12, $P_L$lacO1; *TthpaBC*	This Study
pZE-RgTAL	From pZE12, $P_L$lacO1; *Rg*TAL	Unpublished
pZE-TH1	From pZE12, $P_L$lacO1; *RgTAL-EchpaBC*	Unpublished
pZE-TH2	From pZE12, dual operons, both $P_L$lacO1; *RgTAL* and *EchpaBC*	This Study
pZE-TH3	From pZE12, $P_L$lacO1; *EchpaBC-RgTAL*	This Study
Strains	**Genotype**	**Source or reference**
E. coli Bw25113	F-, Δ(*araD-araB*), Δlac*Z* (::*rrnB-3*), λ-, *rph-1*, Δ(*rhaD-rhaB*), *hsdR*	Yale CGSC
E. coli XL-BLUE1	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* *lacI^{q}Z* Δ*M15 Tn10* (Tet^R)]	Stratagene
ATCC31884	*aroH367*, *tyrR366*, *tna-2*, *lacY5*, *aroF394^{fbr}*, *malT384*, *pheA101^{fbr}* ,*pheO352*, *aroG397^{fbr}*	ATCC
QH-5	*E. coli* ATCC 31884/ *ΔpheLA-tyrA::kan*	This Study
QH-4	*E. coli* ATCC 31884/ *ΔpheLA-tyrA*	This Study
QH-28	QH-4/pCS-TPTA and pZE12-luc	This Study
QH-27	QH-4/pCS-TPTA and pZE-Rgtal	This Study
QH-22	QH-4/pCS-TPTA and pZE-TH1	This Study
QH-23	QH-4/pCS-TPTA and pZE-TH3	This Study
QH-24	QH-4/pCS-TPTA and pZE-TH2	This Study
QH-31	E.coli Bw25113/pZE-TthpaBC	This Study
QH-32	*E. coli* Bw25113/pZEh-EchpaBC	This Study

Underlined sequences are designed for priming sites on pKD13. Italic sequences in primers are restriction digestion sites marked in parentheses after primer names.

DNA Manipulation

The codon-optimized RgTAL gene was synthesized for its optimal expression in *E. coli* (Eurofins MWG Operon, AL) (Santos et al., 2011). The genes *EchpaBC* and *TthpaBC* were

amplified by PCR from the genomic DNAs of *E. coli* MG1655 and *Thermus thermophilus* HB8, respectively. pZE-EcHpaBC was constructed by inserting *EchpaBC* into pZE12-luc (Lutz and Bujard, 1997) using the restriction sites *Kpn* I and *Sph* I; while *TthpaBC* was inserted to construct pZE-TtHpaBC using *Kpn* I and *Xba* I. pZE-RgTAL was constructed by inserting the RgTAL coding fragment into pZE12-luc using *Kpn* I and *Xba* I. pZE-TH1 was constructed by inserting the RgTAL cDNA and *EchpaBC* consecutively as an operon into pZE12-luc by three-piece ligation using *Kpn* I, *Sph* I, and *Xba* I; while pZE-TH3 carries *EchpaBC* and the RgTAL gene consecutively as an operon using the same restriction sites. pZE-TH2 is a plasmid constructed to express *EchpaBC* and the RgTAL gene tandemly. To construct this plasmid, the expression cassette $P_L$lac-*EchpaBC* was amplified from pZE-EchpaBC with the primers Opr-1 and Opr-2 and inserted into pZE-RgTAL using *Sac* I and *Spe* I. The configurations of pZE-TH1, pZE-TH2 and pZE-TH3 were shown in Fig.1. pCS-TPTA was constructed in our previous work (Lin and Yan, 2012). All the primers used in this study were listed in Table 1. pZE-TthpaBC and pZE-EchpaBC were transformed to *E. coli* Bw25113 yielding strains QH-31 and QH-32 for feeding experiments. The features of the used plasmids are described in Table 1.

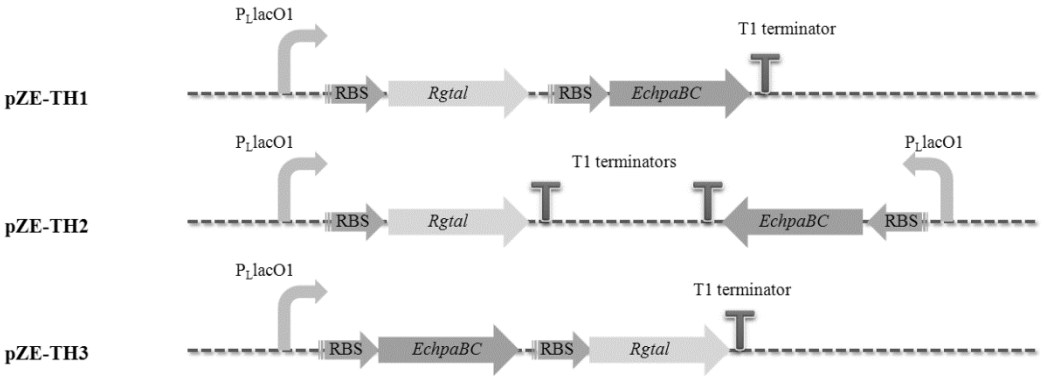

Figure 1. Plasmid constructs of artificial caffeic acid biosynthetic pathway

Feeding experiments

Feeding experiments were performed using M9 minimal salt media with 0.5% yeast extract for optimal cell growth and 20 g/L glycerol as carbon source. Overnight LB culture of wild-type *E. coli* Bw25113 carrying pZE-TthpaBC (QH-31) or pZE-EchpaBC (QH-32) was re-inoculated and induced with 0.2mM IPTG 3hrs after inoculation. All shake flasks were shaken at 250rpm with p-coumaric acid fed 3hrs after induction. Cell growth was constantly measured and bacterial cultures were sampled till no additional formation of caffeic acid or consumption of p-coumaric acid was detected by HPLC.

Microbial production of caffeic acid and intermediates

Strains QH-22, QH-23 and QH-24 were generated by co-transforming pCS-TPTA with pZE-TH1, pZE-TH2, and pZE-TH3 respectively into the knock-out strain QH-4 to test caffeic acid production. As controls, strain QH-27 was constructed by co-transforming pCS-TPTA and pZE-RgTAL into strain QH-4 to test *p*-coumaric acid production and strain QH-28 was constructed by co-transforming pCS-TPTA with the blank plasmid pZE-luc to test tyrosine production. For the production, single colonies were inoculated into 3 ml LB media in test tubes and re-inoculated into 20 ml of M9 minimal salt media containing 2.5 g/L glucose and 10 g/L glycerol (Lin and Yan, 2012) in 125 ml shake flasks with phenylalanine supplement and 0.2 mM IPTG at inoculation. All shake flasks were shaken at 300 rpm under 37 ^{o}C and sampled every 24 hrs till 72 hrs after inoculation.

HPLC analysis

All samples taken from the cultures were centrifuged at 15000 rpm for 15 mins and the supernatants were used for HPLC (Dionex Ultimate 3000) with a reverse phase ZORBAX SB-C18 column and an Ultimate 3000 Photodiode Array Detector. Mobile phases used for peak

detection were 0.1% TFA in water and 100% methanol. HPLC analysis followed our previously established program (Lin and Yan, 2012), and peaks for tyrosine, *p*-coumaric acid and caffeic acid were integrated and compared to standard curves made from 0.5 mM, 1 mM and 1.5 mM samples under UV light of wavelengths 280 nm, 308 nm and 323 nm, respectively. The retention times for all three peaks were 5.58 min, 13.08 min and 10.35 min, respectively.

Results

One-step conversion to caffeic acid by 4HPA3H

In our previous work, we have demonstrated that 100 mg/L *p*-coumaric acid was completely converted to caffeic acid within in 3 hrs by the *E. coli* strain expressing endogenous 4HPA3H (Lin and Yan, 2012). It was also reported that the titer of caffeic acid converted from feeding of *p*-coumaric acid by a mutated bacterial cytochrome P450 CYP199A2 reached 15 mM (2.8 g/L) caffeic acid in condensed *E. coli* cell cultures (Furuya et al., 2012). In order to examine the catalytic capability of 4HPA3H converting *p*-coumaric acid to caffeic acid, we selected *hpaBC* genes from *E. coli* MG1655 and *T. thermophilus* HB8, and cloned them into pZE12-luc, generating pZE-EcHpaBC and pZE-TtHpaBC, respectively. The two plasmids were separately transformed into *E. coli* to yield strains QH-32 and QH-31, accordingly. We first fed 1.5 g/L *p*-coumaric acid into 20 ml QH-31 and QH-32 cultures. In 12 hrs after induction, QH-32 converted all *p*-coumaric acid to caffeic acid, while QH-31 only converted 40% of *p*-coumaric acid (0.6 g/L).

Given that the 4HPA3H from *E. coli* was predominantly more potent than its counterpart from *T. thermophilus* HB8, we continued to explore the catalytic potential of *E. coli* 4HPA3H for whole-cell bioconversion. We then tested two different concentrations of substrate feeding with 3 g/L and 5 g/L *p*-coumaric acid. In 12hrs after induction, 3 g/L *p*-coumaric acid was completely

converted while 5 g/L *p*-coumaric acid feeding was not completely consumed with 4.07 g/L substrate unconverted. Meanwhile, 5 g/L feeding had caused low cell density of the bacterial cultures with the OD_{600} value measured 12 hrs after induction as 4.95 significantly lower the OD_{600} value with 3 g/L feeding, which was 8.35. We later attempted to feed 3 g/L first and then additional 2 g/L *p*-coumaric acid when 0.54 g/L substrate was left in 20 ml bacterial cultures, which was 6 hrs after initial feeding of 3g/L substrate. When cultures were sampled 15 hrs after induction, only 2.65 g/L caffeic acid was detected, which suggested 5 g/L *p*-coumaric acid couldn't be converted even with two phase feeding.

Based on these results, a parallel of 24 hrs feeding experiments were designed and performed by feeding a total of 3 g/L and keeping adding 0.5 g/L *p*-coumaric acid afterward. The OD_{600} value of cultures and concentrations of substrate and product were constantly monitored (Fig. 2). Initial feeding of *p*-coumaric acid was 3 g/L and additional 0.5 g/L was added when the concentration of *p*-coumaric acid in the cultures decreased to around 0.5 g/L. In 18 hrs after induction, 21.32 mM (3 g/L and additional 0.5 g/L) *p*-coumaric acid was all consumed with around 21.32 mM caffeic acid detected from the QH-32 cultures with an around 100% conversion ratio (Fig. 2A). When fed additional 0.5 g/L *p*-coumaric acid twice after initial feeding of 3 g/L substrate to reach a total of 4 g/L *p*-coumaric acid, about 0.5 g/L *p*-coumaric acid remained after 18 hrs after induction and the final OD_{600} value is around 6.87 (Fig. 2B), which was lower than 8.33 of the OD_{600} value from 3.5 g/L feeding experiments. The results indicated that, endogenous 4HPA3H expressed in *E. coli* was capable of yielding 3.82 g/L (461.12mg/L/OD) caffeic acid in 24 hrs without hindering cell growth of *E. coli* cultures, more efficient hydroxylase comparing to the mutated cytochrome P450 CYP199A2.

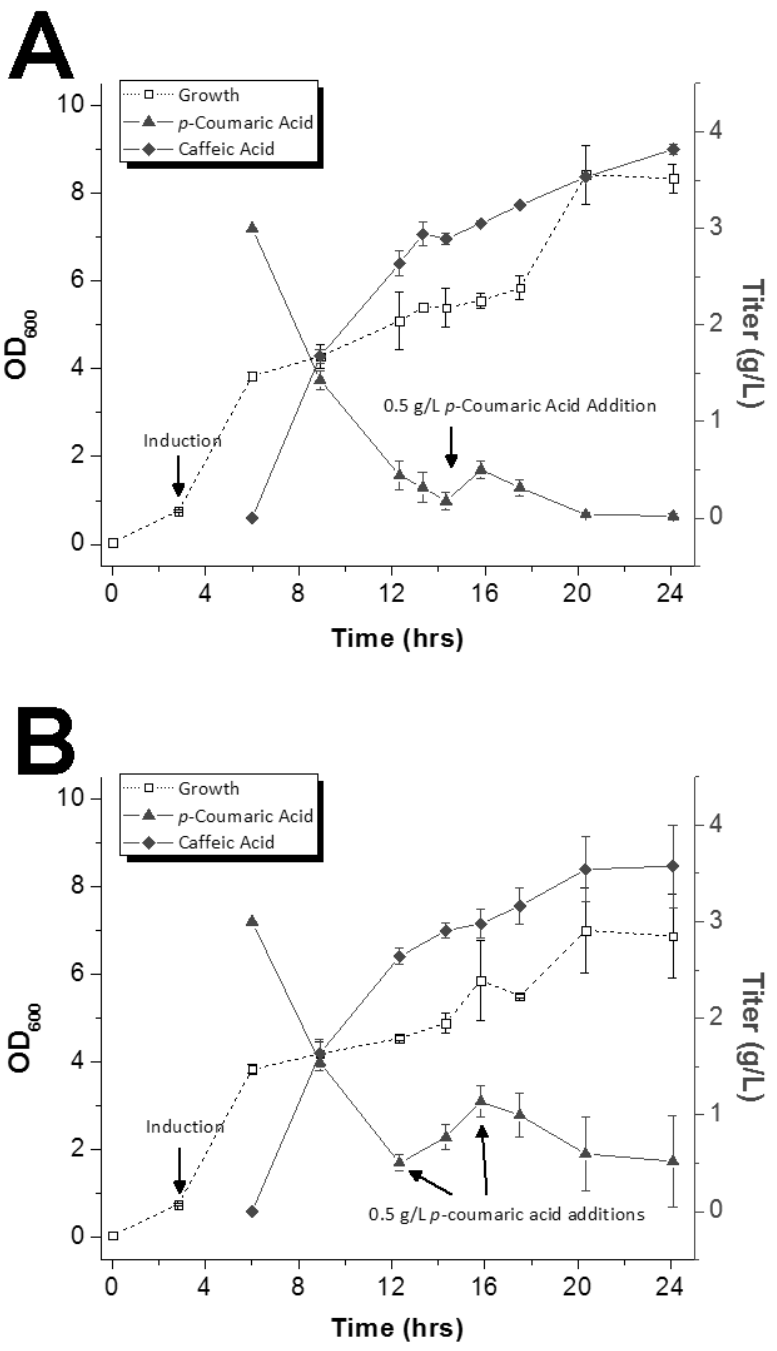

Figure 2. Whole-cell conversion of *p*-coumaric acid to caffeic acid by 4HPA3H. 3 g/L *p*-coumaric acid was fed 3 hrs after induction. Additional *p*-coumaric acid (0.5 g/L per time) was fed into the cultures once (A) and twice (B) into the cultures as shown by arrows. Three independent experiments were conducted to generate the data.

We examined the 4HPA3H from *T. thermophilus* HB8 because it has been characterized and its crystal structure is available for further structural engineering despite its relatively limited catalytic efficiency in this study (Soulimane et al., 2010, Kim et al., 2008, Kim et al., 2007).

Phenylalanine overproducer to tyrosine overproducer

Upon understanding 4HPA3H's capability as a potent hydroxylase in our artificial pathway, we aimed at elevating metabolic afflux into *p*-coumaric acid via tyrosine. To enhance tyrosine biosynthesis, we selected *E. coli* ATCC31884, a well developed phenylalanine overproducer, and engineered the strain to a tyrosine overproducer. A similar approach was reported using TetRA circle method to delete *pheLA* genes (Olson et al., 2007). In our work, we first examined aromatic amino acid biosynthetic pathway in *E. coli* where prephenate is the branch point towards either phenylalanine or tyrosine (Fig. 3). Also, *pheLA* genes direct prephenate to phenylalanine and prephenate dehydrogenase encoded by *tyrA* C-terminus is subject to feedback inhibition regulation by tyrosine (Lütke-Eversloh and Stephanopoulos, 2005). We decided to disrupt *pheLA* and *tyrA* genes and express a feedback inhibition resistant mutant of *tyrA* ($tyrA^{fbr}$) to improve tyrosine production. Accordingly, Strains QH-4, QH-5 and ATCC31884's genomic variances are shown in Fig. 4. Strain QH-4 showed the removal of kanamycin-resistant gene comparing to strain QH-5 which is 318 bps shorter at *pheLA-tyrA* locus than *E. coli* strain ATCC31884 because of kanamycin-resistant gene's substitution of the *pheLA-tyrA* fragment. In shake flask study using 2 g/L glucose as carbon source in MOPS minimal media, QH-4 cultures supplemented with 10 mg/L phenylalanine and 10 mg/L tyrosine were able to recover growth, while no supplement of tyrosine or phenylalanine led to less than 0.01 of OD600 values after 24 hrs in shake flasks. Both PCR results and shake flasks study confirmed the disruption of the *pheLA* and *tyrA* fragment.

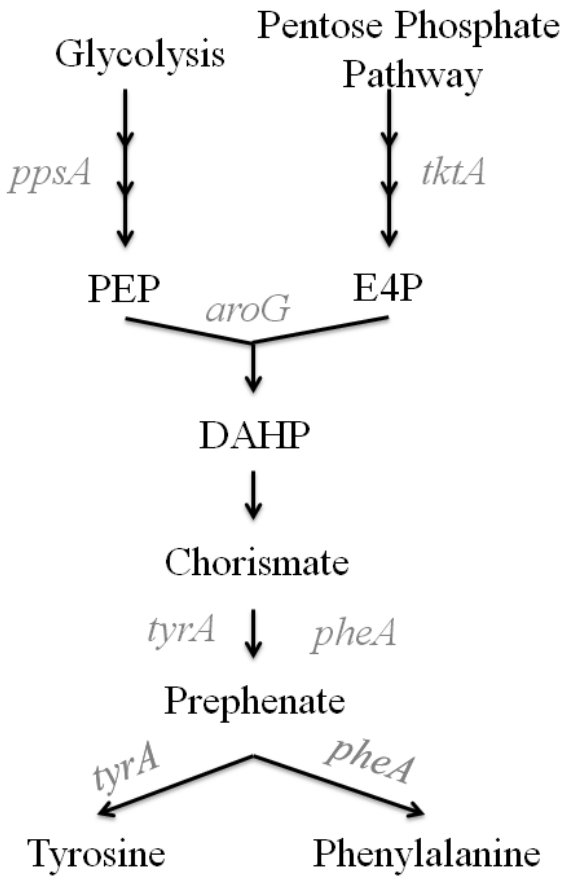

Figure 3. Aromatic amino acid biosynthesis in *E. coli*. Important intermediates in the pathway: DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate. Genes (grey italic letters) encode enzymes catalyzing corresponding reaction steps: *tktA* encodes transketolase; *ppsA* encodes phosphoenolpyruvate synthetase; *aroG* encodes 2-dehydro-3-deoxyphosphoheptonate aldolase; *tyrA* and *pheA* encode chorismate mutase and prephenate dehydrogenase.

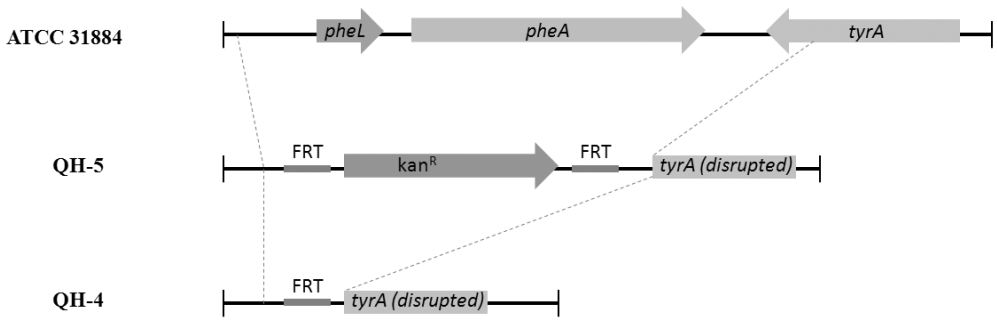

Figure 4. Profiles of the chromosomal region near *pheLA* and *tyrA* genes for ATCC31884 and its derived knockout strains. Sequences of primes phe_F and phe_R are shown in Table 1. Chorismate mutase and prephenate dehydrogenase domains are represented in different colors in *tyrA*. kan^R in QH-5 is kanamycin resistant gene.

After disruption of endogenous biosynthesis of tyrosine and phenylalanine in QH-4, it was not able to survive minimal salt media. To achieve tyrosine over-production, we employed a previously constructed tyrosine over-producing plasmid expressing $tyrA^{fbr}$, *ppsA, tktA* and $aroG^{fbr}$ (Fig. 3), namely pCS-TPTA. On one hand, expression of the feedback inhibition resistant mutant of *tyrA* ($tyrA^{fbr}$) can recover tyrosine biosynthesis in the knockout strain. On the other hand, the enzymes encoded by *ppsA*, *tktA* and $aroG^{fbr}$ can help boost the availability of precursors and finally promote the production of tyrosine. To test its capability of tyrosine production, pCS-TPTA was co-transformed into QH-4 together with the blank plasmid pZE12-luc, resulting in strain QH-28. We tested tyrosine production with QH-28 to assess tyrosine availability when co-expressing pCS-TPTA with caffeic acid biosynthetic pathway on the pZE-luc vector. As we expected, QH-28 could not survive minimal salt media without phenylalanine supplement. And we found that the concentration of phenylalanine had a great impact on

tyrosine production. As shown in Fig. 5, we compared 10 mg/L, 20 mg/L, and 30 mg/L phenylalanine supplement to the QH-28 cultures by taking samples at 24, 48 and 72 hrs for HPLC analysis. Bacterial cultures were induced at re-inoculation to initiate tyrosine biosynthesis for cell growth in the modified M9 minimal media. Sample results from the cultures with 20 mg/L supplement performed better than those with 10 mg/L or 30 mg/L supplement. The highest production of tyrosine (718.53 ± 148.76 mg/L) from QH-28 was observed at 48 hrs from 20 mg/L phenylalanine supplement, while 637.55 ± 195.32mg/L and 351 ± 149.54mg/L tyrosine was detected from 10 mg/L and 30 mg/L phenylalanine supplement, respectively. Therefore, we determined that 20 mg/L of phenylalanine was optimal for tyrosine over-production in the modified M9 minimal media.

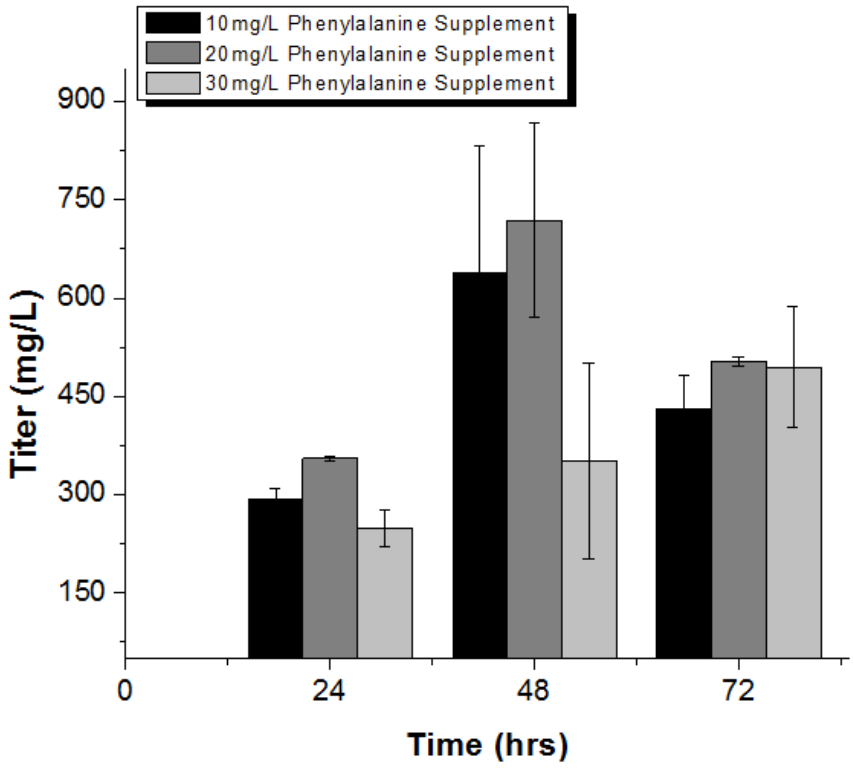

Figure 5. Effect of phenylalanine supplement concentrations on tyrosine production. Three independent experiments were conducted to generate the data.

p-Coumaric acid production by RgTAL

We used the established growth condition after determining optimal phenylalanine supplement, for the production of *p*-coumaric acid, the direct precursor for caffeic acid. We have previously screened the TALs from both *Rhodobacter sphaeroids* and *Rhodobacter ceptulatus* and successfully produced *p*-coumaric acid from tyrosine and caffeic acid from L-dopa (Lin and Yan, 2012), while RgTAL from *Rhodotorula glutinis* was reported to be the most active TAL among all the identified ones (Santos et al., 2011). In this work, we cloned the codon-optimized RgTAL gene into pZE12-luc generating pZE-RgTAL which was then co-transformed into strain QH-4 together with pCS-TPTA, resulting in QH-27. We then tested *p*-coumaric acid production of strain QH-27. In 72 hrs, *p*-coumaric acid reached 294.44 ±22.69 mg/L in modified M9 minimal salt media with 20 mg/L phenylalanine supplement and cell density had been prominently low for all *p*-coumaric acid producing cultures throughout 72 hrs. In the presence of 20 mg/L phenylalanine supplement, the OD_{600} values measured at 24, 48 and 72hrs were around 0.58, 1.12 and 1.15, respectively (Fig. 6). 30 mg/L phenylalanine supplement, on the other hand, enabled 321.32 ±19.67 mg/L *p*-coumaric acid production in 72 hrs. Under both levels of supplement, a trace amount of tyrosine (＜3 mg/L) was detected in cultures. Considering the titer increase of *p*-coumaric acid from the additional 10 mg/L phenylalanine was not significant, we decided to proceed with 20 mg/L phenylalanine supplement indicated by the optimal tyrosine production for caffeic acid production study.

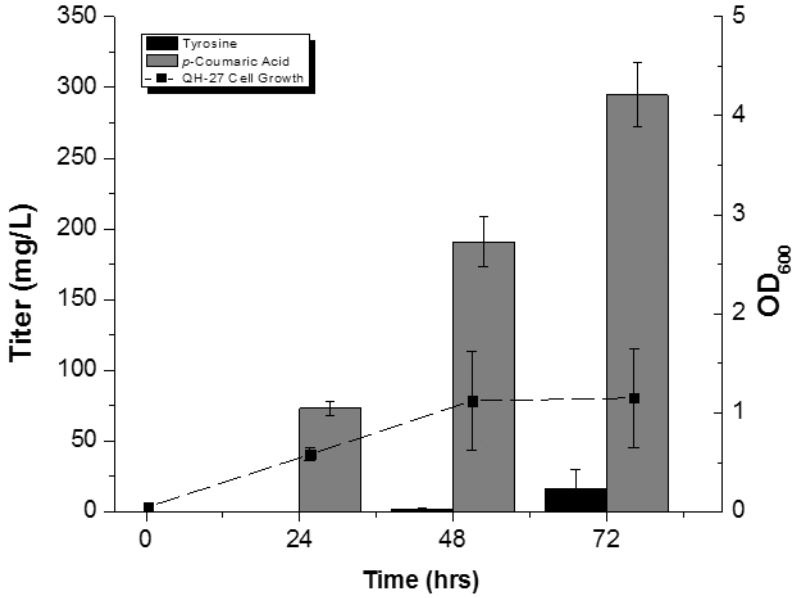

Figure 6. Cell growth and *p*-coumaric acid production in the presence of 20 mg/L phenylalanine supplement. Three independent experiments were conducted to generate the data.

Effect of expression strategy on caffeic acid biosynthesis

In our previous study, we have observed intermediates accumulation upon production of caffeic acid, and therefore proposed tuning the expression of the biosynthetic pathway (Lin and Yan, 2012). After testing biosynthetic capability of RgTAL and 4HPA3H, we constructed three plasmids (pZE-TH1, pZE-TH2, and pZE-TH3) with different genetic arrangements using *Rgtal* and *hpaBC* genes as described in the section of Materials and Method. We further developed three strains QH-22, QH-23, and QH-24 by co-expressing pCS-TPTA with pZE-TH1, pZE-TH2 or pZE-TH3 respectively, as described in Table 1, for shake flask study. We observed the two-promoter construct (pZE-TH2) in QH-23 gave much better results of caffeic acid production than one promoter constructs QH-22 (pZE-TH1) and QH-24 (pZE-TH3). In 72 hrs, 164.97 ±2.47

mg/L caffeic acid was detected from shake flasks while 47.72 ± 2.95 mg/L *p*-coumaric acid remained unconverted in QH-23 cultures. Meanwhile, no accumulation of *p*-coumaric acid was detected from QH-22 or QH-24 cultures, while the concentrations of caffeic acid were only 28.3 ± 7.8 mg/L and 10.80 ± 0.28 mg/L, respectively. We also observed darkened color of *E. coli* cultures from overnight inoculants and 72 hrs shake flasks.

Caffeic acid production optimization by adjusting inoculation timing

Based on our data analysis and phenotype comparison from established caffeic acid production, we decided to further optimize the pathway. As mentioned above, we have observed coloration from the overnight inoculants and the shake flask cultures of strains QH-22, QH-23 and QH-24. According to reported coloration from oxidation of L-dopa to melanin (Santos and Stephanopoulos, 2008, Satoh et al., 2012), we reasoned that the coloration of overnight inoculants were due to the formation of L-dopa in the cultures and indicated over-maturation of the inoculants. To prevent latent influence of L-dopa accumulation and quality of inoculants on caffeic acid production, we explored the relationship between cell density of inoculants and final titers of caffeic acid. For strain QH-22, caffeic acid production stopped 48 hrs after inoculation, with inoculants' OD_{600} being around 1.00, 1.73 and 3.20 (Fig. 7A). For Strain QH-23, caffeic acid production continued after 48hrs. Therefore, we extended our sampling to 72 hrs after inoculation, with the inoculants' OD_{600} values being around 1.00, 1.66, 2.00, 2.45 and 3.16, respectively (Fig. 7B). For Strain QH-24, we used the inoculants of around 1.40, 2.40, and 3.28 for the OD_{600} values. Results shown in Fig. 7 indicate that QH-23 performed best when inoculants were most robust in log phase, while QH-22 was only slightly influenced by inoculants quality and QH-24 was insensitive to inoculants. Among three constructed caffeic acid producers, the highest caffeic acid productions were 766.68 ± 43.12 mg/L in 72 hrs for QH-

23 with the final OD_{600} values measured as around 4.87, while 7.39 ± 3.10mg/L *p*-coumaric acid and 53.82 ± 15.83 mg/L tyrosine were accumulated. For strain QH-22, 36.39 ± 9.53 mg/L caffeic acid was produced with 9.79 ± 3.19 mg/L *p*-coumaric acid and 27.21 ± 10.13 mg/L tyrosine accumulated as intermediates, and OD_{600} values measured were around 4.98. For strain QH-24, only 12.43 ± 4.93 mg/L caffeic acid was detected after 48 hrs, with the OD_{600} values measured as around 6.55. Meanwhile, 1.26 ± 0.36 mg/L *p*-coumaric acid and 27.66 ± 4.75 mg/L tyrosine were left unconverted in the cultures. Among all three strains, highest caffeic acid production achieved 157.43 mg/L/OD from double-promoter construct in strain QH-23. This result is the highest titer of caffeic acid from *de novo* production so far.

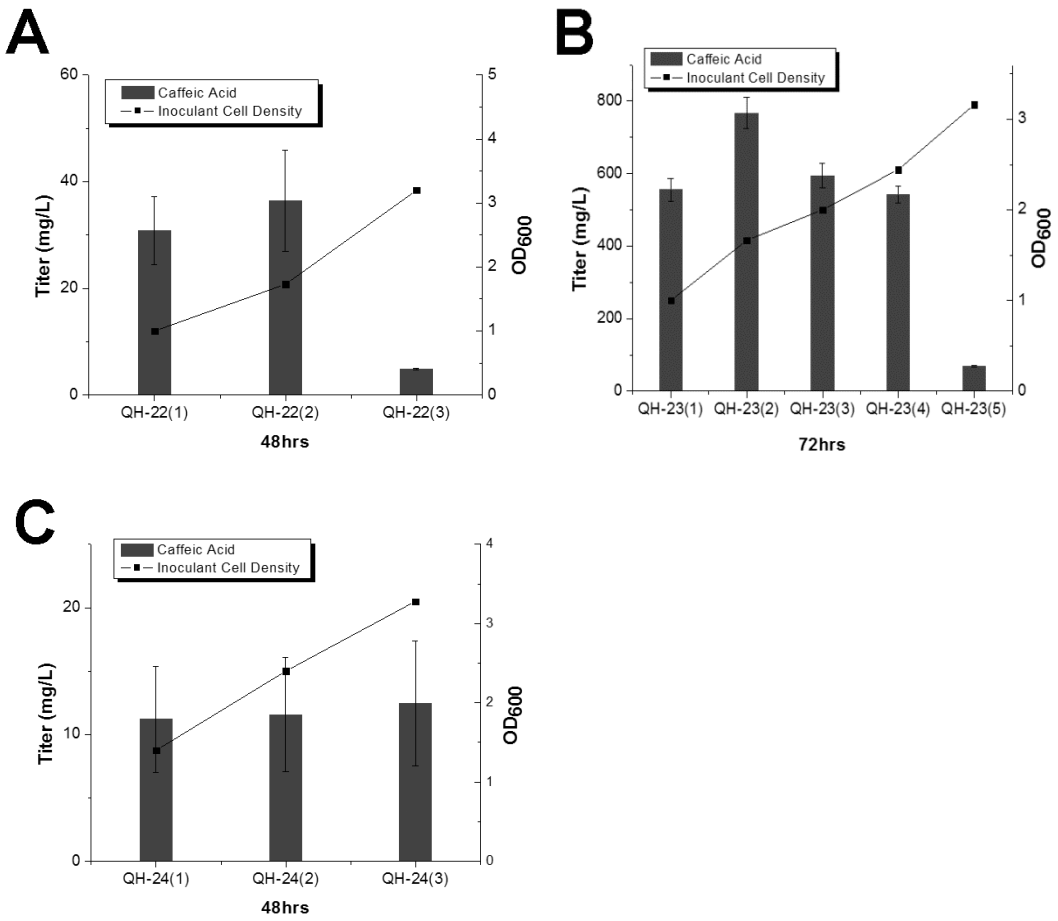

Figure 7. Correlation of inoculation timing with caffeic acid production. A. Strain QH-22; B. Strain QH-23; C. Strain QH-24. Lines and symbols indicate the inoculants' OD_{600} values for re-

inoculation. Columns and error bars indicate caffeic acid titers and standard deviations. Three independent experiments were conducted to generate the data.

Discussion

Caffeic acid and its derivatives have been a frequently reported group of prospective therapeutic compounds in recent years. Renewable biosynthesis strategies of caffeic acid were established by multiple research groups in last year only. While most recently published work regarding caffeic acid production in microbes had aimed at various TALs and *p*-coumaric acid hydroxylase candidates, this work was developed and improved upon our previous publication in which we reported the first established *de novo* production of caffeic acid in genetically modified *E. coli* (Lin and Yan, 2012). In addition to originally proposed pathway, we further engineered two caffeic acid producing strains, QH-23 and QH-32. QH-23 was constructed by following the rationale of step-wise metabolism enhancement. After adjustments for growth and expression of artificial pathway, we achieved 15-fold *de novo* production of caffeic acid over our previous report by engineering a phenylalanine over-producing *E. coli* strain for caffeic acid production. We have also observed the sensitivity of the proposed pathway when adjusting inoculants freshness and thus optimized performances of the full pathway. And with QH-32, we demonstrated 3.82 g/L (21.32 mM) as the highest caffeic acid production so far from feeding of *p*-coumaric acid, using 4HPA3H. Both one-step conversion titer and *de novo* production of caffeic acid are the highest so far among all the microbial production of caffeic acid.

During shake flask experiments with L-dopa produced as an intermediate in our pathway, we have observed color change, which is consistent with dark color from L-dopa oxidation from other group's results (Satoh et al., 2012). Adding ascorbic acid into media as antioxidant agent

didn't show significant evidence of production improvement or color-change moderation. During our study, we observed that color change started when caffeic acid production slowed down and stopped. And we were able to alleviate L-dopa oxidation and therefore extend caffeic acid producing time by manipulating inoculants freshness. To avoid L-dopa formation, a hydroxylase with exclusive substrate specificity towards *p*-coumaric acid is desired for further production enhancement.

When constructing the artificial pathway for heterologous production of caffeic acid, we applied different genetic arrangements with two genes and achieved dramatic change of metabolic throughput. Apparently, layout of genes and operons in plasmid has a great impact on gene expression to certain degree. Understanding quantitative relationship of cause and effect will assist more microbial productions of valuable compounds. However, to establish instructional theory of correlations of gene/operon arrangements and enzyme expression, more pertinent experiments need to be performed.

5.3 Isoprenoids

Different from other plant-specific compounds described above, isoprenoids are not derived from aromatic amino acids. Isoprenoids derivatives have been demonstrated for toxicity (Mariita et al., 2011), applied for specific treatments, driving more research interest and effort into overproduce these valuable compounds for economical and therapeutic benefits. Whichever compound studied, the introduction of heterologous mevalonate pathway and genes is the first step. Though microbial enzymes have been replacing plant enzymes that can't function well in microbes, few attempts have succeeded to engineer protein structure of plant cytochrome P450s from native pathways to perform sufficiently in microbes (Chang et al., 2007).

5.3.1 Diterpenes

Like other isoprenoids, taxadiene is derived from a series of conversions from IPP and DMAPP. It serves as the first specific branch point towards synthetic pathway of taxol, a well-known cancer chemotherapy drug. In transgenic *E. coli*, the yield of taxadiene has reached gram per liter level with fermentors through balanced expression of native methylerythritol-phosphate (MEP) pathway towards IPP and heterologous isoprenoids synthetic pathway derived from IPP(Ajikumar et al., 2010b). To further improve yield rate of taxadiene and other isoprenoids, computational methods have been applied to target genes along the integrated pathway for availability of metabolic flux towards products of interest(Boghigian et al., 2012a). The established algorithm shall be utilized for developing over-expression strategies in other isoprenoids production platforms.

To produce taxol in engineered *E. coli*, multistep reactions following taxadiene are being assessed for feasibility. Researchers analyzed protein structures of plant-specific cytochrome P450s and engineered specific membrane-bounding domains to chimeric proteins. (Chang et al., 2007)

Comparisons between B- and K-derived *E. coli* strains are also made by analyzing parameters during biosynthetic process of taxadiene. Yield of final product from K-derived strain manifested a 2.5-fold advantage over B-derived *E. coli*. And K-derived *E. coli* were proved to be more sensitive to growth inhibition introduced by indole feeding. Observations upon pyruvate metabolism in both strains provided clues of huge differences on performances and grounds for choosing proper hosts for further research(Boghigian et al., 2012b).

5.3.2 Carotenoids

Carotenoids in plant cells are generated through multiple mechanisms as pigment and precursors for hormones. And their yields in metabolically engineered *E. coli* have yet to meet up with commercial levels realized by chemical synthetic strategies, while only lycopene, β-carotene and a few other carotenoids have been promised for scale-up bioprocess production. (Bhataya et al., 2009)

Heterologous carotenoids are formed in *E. coli* isoprenoid pathway where pyruvate and glyceraldehydes-3-phosphate serve as two major precursors(Pyung Cheon et al., 2008). Six different carbon sources have been used to test correlations between carotenoids biosynthesis efficiency and carbon source uptake. And glycerol, other than glucose, mannose, maltose, galactose and lactose, performed best towards production of diapolycopene (66.2 μg/g-DCW), diapotorulene (68.1 μg/g-DCW), lycopene (89.1 μg/g-DCW) and β-carotene (64.4 μg/g-DCW) (Jongrae et al., 2010).

Among these carotenoids, lycopene has been located from tomatoes, grapes and other fruits. It has been associated with lowering risks of cancer as a potent antioxidant (Johary et al., 2012), and protecting against atherosclerosis(Palozza et al., 2010). By introducing *crtE*, *crtB* and *crtI* of *Pantoea agglomerans* and *Pantoea ananatis* into non-carotenogenic *E. coli*, lycopene in 2YT medium was yielded 27mg/L with *crt* genes from *P. agglomerans* and 12mg/L from crt genes from *P. ananatis* with IPTG induction(Yoon et al., 2007). Systematic genetic modifications on promising knockouts have been tested to improve lycopene yield, and one genotype ΔhnrΔyliE out of 800,000 mutants showed optimal lycopene yield of nearly 15000ppm(Alper and Stephanopoulos, 2008). Genes from non-mevalonate pathway, *dxs* and *idi*, were amplified with *rrnB*, *yjiD* and *ycgW*, together with multiple knock-out *ΔgdhΔaceEΔfdhF*

constructed a lycopene producing *E. coli* strain converting 5g/L glucose into 16000ppm (16mg/g cell) lycopene in M9 minimal medium (Jin and Stephanopoulos, 2007). Deoxyxylulose 5-phosphate synthase (DXS) was over-expressed to provide more precursors. By expressing it under the control of a leaky IPTG-inducible promoter, yield of lycopene was increased 8-fold than without inducer. In addition to it, supplement with mevalonate resulted in 10-fold increase. When incorporated with an engineered *E. coli* BL21 strain harboring an exogenous MVA+ operon, another 2-fold increase to 228mg/L lycopene was obtained(Rodr ́guez-Villal ́n et al., 2008). When lycopene was produced from recombinant *E. coli* carrying its biosynthetic pathway, inocula dependence pattern in its productivity was observed. After adjusting temperature of culture and using low-copy recombinant vector carrying the pathway, final yield of lycopene was increased by 20% to reach 260mg/L after 60hrs (Kim et al., 2009). Influence from copy number of plasmids carrying lycopene biosynthetic pathway was again demonstrated with *crtI* expressed in high-copy plasmid and *crtE* and *crtB* expressed in single copy, tetradehydrolycopene was produced at 253μg/gCDW (Albermann, 2011).

CHAPTER 6

PERSPECTIVES AND FUTURE OPPORTUNITIES

Metabolic Engineering of *E. coli* as host cells for production of AAA derived compounds has been acknowledged as potent strategy for economic manufacturing and environmental impact. However, current progress is still limited in varieties of compounds that can be synthesized and their yields. To foresee the future of development in this field, two directions tackling each obstacle should be clarified, novel synthetic pathway construct and yield enhancement.

Current compound targets following AAA pathway have mapped a remarkably branched web of renewable production of valuable chemicals. A great proportion of this group of compounds has established therapeutic potentials. Therefore, metabolic engineering of microbes to produce more structurally diverse medicinal candidates are needed. So far, artificial pathways are inspired by exogenous enzymes from microbial and plant origins. Screening potent enzymes that are capable of catalyzing certain structure is still a crucial step in building blocks for metabolic engineering. Reported enzymes may still have undetected catalytic ability over novel substrates, directing alternative paths to forming other products.

Meanwhile, as shown in most if not all attempts to express heterologous biosynthetic pathway towards plant secondary metabolites in *E. coli*, realization of conversions towards target compounds through intermediates manifests a step-wise logic. These relocated expression systems generically take advantage of native production of precursors in *E. coli*, which then elongate the assembly line to start from renewable feedstock. Upon adopting optimal enzyme

candidates, engineering additional auxiliary factors can also make differences. Increasing the availability of cofactors and knocking out competitive branches have all been proved to effectively elevate final turnout. Fermentation strategies have also contributed to increasing yield of production both bench-level and industry level.

In addition to current progress and rationale of metabolic engineering for renewable biosynthesis of AAA derived compounds, one should look forward to interdisciplinary applications to reach broader range of product and higher yield for industry manufacture.

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