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Engineering a bacterial platform for total biosynthesis of caffeic acid derived phenethyl esters and amides

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ABSTRACT

Caffeic acid has been widely recognized as a versatile pharmacophore for synthesis of new chemical entities, among which caffeic acid derived phenethyl esters and amides are the most extensively-investigated bioactive compounds with potential therapeutical applications. However, the natural biosynthetic routes for caffeic acid derived phenethyl esters or amides remain enigmatic, limiting their bio-based production. Herein, product-directed design of biosynthetic schemes allowed the development of thermodynamically favorable pathways for these compounds *via* acyltransferase (ATF) mediated trans-esterification. Production based screening identified a microbial O-ATF from *Saccharomyces cerevisiae* and a plant N-ATF from *Capsicum annuum* capable of forming caffeic acid derived esters and amides, respectively. Subsequent combinatorial incorporation of caffeic acid with various aromatic alcohol or amine biosynthetic pathways permitted the *de novo* bacterial production of a panel of caffeic acid derived phenethyl esters or amides in *Escherichia coli* for the first time. Particularly, host strain engineering *via* systematic knocking out endogenous caffeoyl-CoA degrading thioesterase and pathway optimization *via* titrating co-substrates enabled production enhancement of five caffeic acid derived phenethyl esters and amides, with titers ranging from 9.2 to 369.1 mg/L. This platform expanded the capabilities of bacterial production of high-value natural aromatic esters and amides from renewable carbon source *via* tailoring non-natural biosynthetic pathways.

1. Introduction

Nature is the largest arsenal for biologically active compounds and motivates cost-effective manufacturing of valuable natural products. The ever-increasing research and discoveries have brought us a deeper understanding of the biosynthetic machineries of numerous natural products, especially those from plants, on both genetic and enzymatic levels. Instead of direct extraction from plant tissues with low yields or chemical synthesis with high costs and low specificity, knowledge-based microbial production is a sustainable and cost-effective biomanufacturing alternative with high specificity and less complexity. Especially, genetic amenability and the Generally Regarded As Safe (GRAS) status make the well-characterized microbes like *Saccharomyces cerevisiae* and *Escherichia coli* robust industrial microbial platforms for production of non-cognate natural products from simple carbon sources (Xu et al., 2013a). The last decades have witnessed tremendous advances in achieving microbial-based production of plant-derived chemicals with pharmacological activities including polyphenols, alkaloids and terpenoids (Mora-Pale et al., 2013; Wang et al., 2016). In most cases, the entire plant biosynthetic pathways can be directly recruited into microbial workhorses and repurposed to synthesize desired phytochemicals (Brown et al., 2015; Galanie et al., 2015; Nakagawa et al., 2016). However, the limited availability of natural pathways or functional enzymes sometimes presents a significant challenge for biosynthesis through metabolic engineering. In such situations, portions of natural pathways or totally artificial pathways have to be created by capitalizing and assembling enzymes from different organisms or pathways (Lin et al., 2013a; Lin and Yan, 2012; Medema et al., 2012; Ni et al., 2015; Yim et al., 2011). These alternative non-natural pathways might afford production of natural compounds *via* tailoring similar biochemical pathways or integrating promiscuous enzymes, by compensating the absence of natural pathways.

Caffeic acid (CA) is a plant-specific secondary metabolite with potent antioxidant properties (Gülçin, 2006; Lin and Yan, 2012). The CA

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scaffold is abundantly found in biologically active natural compounds like caffeic acid derived flavonoids, esters and amides, thus prompting an upsurge in the development of CA-based therapeutically bioactive compounds (Touaibia et al., 2011). Caffeic acid phenethyl ester (CAPE) is one of the most promising therapeutically bioactive polyphenol components from honey bee propolis, which has been recognized as a potential drug candidate for its potent antioxidant activity (Natarajan et al., 1996; Son and Lewis, 2002). Numerous research studies have demonstrated that CAPE possesses versatile pharmacological properties such as antibacterial, anti-inflammatory, antiproliferative, antiviral, anticarcinogenic, antidiabetic, antileukemic, chemopreventive and neuroprotective effects (Murtaza et al., 2014; Tolba et al., 2013). Recent preclinical studies have shown that CAPE was efficient in inhibiting different kinds of cancers such as colorectal cancer, lung cancer, breast cancer, pancreatic cancer, gastric cancer, melanoma, glioma, cholangiocarcinoma and hepatocellular carcinoma in micromolar concentrations (Chen et al., 2004, 2008; Kuo et al., 2006; Lee et al., 2008; Onori et al., 2009; Wu et al., 2007, 2011; Xiang et al., 2006). CAPE ester analogs including caffeic acid (4-hydroxylphenethyl) ester (CAHPE), caffeic acid (3,4-dihydroxyphenethyl) ester (CADPE) and a series of amide analogs including caffeic acid phenethyl amide (CAPA), caffeoyl tyramine (CATA) and caffeoyl dopamine (CADA) are all potent antioxidants (Guo et al., 2013; Son and Lewis, 2002). Notably, the amide analogs showed improved stability compared to CAPE (Yang et al., 2012).

How caffeic acid derived phenethyl esters and amides are biosynthesized is still unclear in nature. As the prototype, CAPE has been previously chemically or enzymatically synthesized. Chemical synthesis of CAPE *via* acid-catalyzed esterification or base-catalyzed alkylation routinely involves environmentally harmful chemicals and even carcinogenic agents like hexamethylphosphoramide (HMPA) (Son et al., 2001). The most common enzymatic strategy to synthesize CAPE was *via* a lipase-catalyzed esterification involving caffeic acid and 2-phenylethanol, whose efficiency was hindered by non-specificity (Guyot et al., 1997; Wang et al., 2014). In particular, chemical synthesis as well as enzymatic catalysis suffers from a thermodynamic disadvantage, as hydrolysis is favored at ambient temperatures ($\Delta G = -5$ kcal/mol) (Rodriguez et al., 2014). Thus, reconstituting a feasible microbial-based production of CAPE as well as its ester and amide analogs with reliable bio-safety and high specificity will be more attractive, ascribed to its ecological acceptability and economic benefits. Although short and medium-chain esters as well as long-chain fatty esters have been extensively biosynthesized in microbial hosts, aromatic esters and its analog amides like caffeic acid derived ones are rarely produced (Guo et al., 2014; Rodriguez et al., 2014; Steen et al., 2010; Xu et al., 2013b, 2016a; Zhang et al., 2012).

Due to the inaccessibility of natural pathways for synthesis of caffeic acid derived phenethyl esters and amides, we turned to establishing artificial pathways. By examination of the CAPE and CAPA chemical structures, we reasoned that acyltransferase mediated trans-esterification of caffeoyl-CoA with 2-phenylethanol (PE) or 2-phenylethylamine (PA) would facilitate ester or amide formation since the release of free CoA from the acyl groups upon esterification is thermodynamically favored ($\Delta G = -7.5$ kcal/mol) (Rodriguez et al., 2014). In the present work, we took advantage of this CoA-dependent trans-esterification pathway and constituted an *E. coli* platform for production of a collection of caffeic acid derived phenethyl esters and amides from glucose. To construct this platform, we first identified and characterized the acyltransferases that can catalyze the trans-esterification of caffeoyl-CoA with aromatic alcohols or amines. Next, assembly of the whole pathway by combinatorial incorporation of an artificial caffeic acid pathway and various aromatic alcohol or amine biosynthetic pathways enabled successful production of a panel of caffeic acid derived phenethyl esters or amides. Finally, systematic investigation of endogenous (thio)esterases on caffeoyl-CoA degradation enhanced production of both caffeic acid derived esters and amides. Specifically, balancing co-substrates CA and PE with an engineered promoter library enhanced production of CAPE. This work affords the first bacterial production of authentic caffeic acid derived phenethyl esters and amides *via* assembling product-directed non-natural metabolic pathways, which expands the capabilities of bacterial production of high-value aromatic esters and amides.

2. Materials and methods

2.1. Bacterial strains, plasmids and chemicals

Bacterial strains and plasmids used in this study were listed in Supplementary Table 1. *E. coli* XL1-Blue was used for plasmid construction and preservation, and *E. coli* BL21 Star (DE3) was used for protein expression and purification. *E. coli* BW25113 (F′) and its derived knockout strains were used for production. The derived knockout strains were generated from *E. coli* BW25113 (F′) *via* P1 phage transduction method according to standard protocols (Thomason et al., 2007). pZE12-luc (high-copy number), pCS27 (medium-copy number) and pSA74 (low-copy number) were used as backbone plasmids for pathway construction. pETDuet-1 was used for cloning genes to be expressed. Standard chemicals were purchased from Sigma-Aldrich unless otherwise specified. All chemicals and solvents were purchased from Alfa Aesar (Ward Hill, Ma), Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Waltham, MA), J.T. Baker (Center Valley, PA), and Matrix Scientific (Columbia, SC).

2.2. Chemical synthesis of standards

Standard of esters and amides were synthesized to high purity (> 95%) according to standard protocols of carbodiimide couplings. These chemicals included *p*-coumaric acid phenethyl ester, *p*-coumaric acid 4-hydroxyphenethyl ester, caffeic acid phenethyl ester (CAPE), caffeic acid 4-hydroxyphenethyl ester (CAHPE), *p*-coumaroyl phenylethylamine, *p*-coumaroyl tyramine, *p*-coumaroyl dopamine, caffeic acid phenethyl amide (CAPA), caffeoyl tyramine (CATA), and caffeoyl dopamine (CADA). If phenolic groups were present, protection as acetates was carried out prior to coupling, and deacetylations were performed afterward. See the Supplementary note for full details. Caffeic acid (3,4-dihydroxyphenethyl) ester (CADPE) was not synthesized and CADPE quantification was estimated based on the synthesized CAHPE standard because of their structural similarity.

2.3. Plasmid constructions

All genes were amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA polymerase (New England BioLabs). Plasmid constructions and DNA manipulations were performed following the standard molecular cloning protocols (Sambrook et al., 1989). pZE-*TH* is a plasmid previously constructed to express *hpaBC* from *E. coli* BL21 Star (DE3) and the codon-optimized *tal* gene from *R. glutinis* (Huang et al., 2013). pZE-*Eht1-4CL2* was constructed by inserting *Eht1* from *S. cerevisiae* and *4CL2* from *A. thaliana* into the high copy number plasmid pZE12-luc between *Acc65*I and *Xba*I. Similarly, *Atf1*, *Atf2*, *Eeb1* from *S. cerevisiae*, *CAT* from pSA74 plasmid and the codon-optimized *HCT* from *N. tabacum* were constructed into pZE12-luc with *4CL2*. pZE-*THT-4CL2* was obtained by inserting the codon-optimized *THT* from *C. annuum* and *4CL2* into pZE12-luc between *Acc65*I and *Xba*I. pZE-*KA* was constructed by inserting *kivD* from *L. lactis* and *adh6* from *S. cerevisiae* into pZE12-luc in between *Acc65*I and *Xba*I. pCS-*TPTA* containing *tyrA**, *ppsA*, *tktA*, *aroG** was constructed previously (Lin and Yan, 2012). pCS-*TPTA-EC* and pCS-*TPTA-TC* were constructed by amplifying *Eht1-4CL2* and *THT-4CL2* respectively and inserting into pCS-*TPTA* in between *Spe*I and *Sac*I. *LbPDC* from *L. brevis*, *MjTDC* from *M. jannaschii*, *PpDDC* from *P. putida* were firstly inserted into the medium copy number plasmid pCS27 to obtain pCS-*LbTDC*, pCS-*MiTDC*