Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Strategies for terpenoid overproduction and new terpenoid discovery

Guangkai Bian[1], Zixin Deng[1,2] and Tiangang Liu[1,2]

Terpenoids comprise the largest family of natural products and have widespread applications. The overproduction of value-added terpenoids and the efficient mining of new terpenoids represent major challenges in the fields of metabolic engineering and natural product discovery. For terpenoid overproduction in microbial systems, *in vitro* reconstitution strategy guided rational engineering was emphasized, and -omics studies provide further information for targeted engineering. In addition, systematic engineering, including host and pathway engineering for terpenoid overproduction was reviewed. Furthermore, robust precursor supplies with optimized high-production hosts can also be used as an efficient platform to rapidly screen terpene cyclases and accelerate the process of mining new terpenoids.

Addresses
[1] Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education and School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, PR China
[2] Hubei Engineering Laboratory for Synthetic Microbiology, Wuhan Institute of Biotechnology, Wuhan 430075, PR China

Corresponding author: Liu, Tiangang (liutg@whu.edu.cn)

Current Opinion in Biotechnology 2017, **48**:234–241

This review comes from a themed issue on **Pharmaceutical biotechnology**

Edited by **Tiangang Liu** and **Chu-Young Kim**

http://dx.doi.org/10.1016/j.copbio.2017.07.002

Introduction

Terpenoids represent the most diverse group of natural products, and they have been found in almost all life forms. Their structural diversity endows terpenoids with a wide range of applications. Developments in metabolic engineering and synthetic biology provide alternative methods for producing value-added terpenoids in *Escherichia coli*, *Saccharomyces cerevisiae*, and filamentous fungi with high efficiency (Figure 1). As a milestone achievement, Paddon *et al.* reported that after overexpressing key enzymes of the mevalonate (MVA) pathway and the artemisinic acid forming pathway, decreasing the production of squalene, and optimizing the cytochrome P450s and their reductases, a high titer of artemisinic acid could be acquired in engineered *S. cerevisiae* [1]. Ajikumar *et al.* developed a multivariate modular approach and increased the yield of taxadiene about 15 000-fold by balancing the upstream methylerythritol-phosphate (MEP) pathway and downstream terpenoid-forming pathway [2]. To fully utilize mitochondrial acetyl-CoA, Lv *et al.* overexpressed the genes of the MVA pathway in the cytoplasm and mitochondria, increasing the production of isoprene to 2.5 g/L [3•]. All of these approaches have increased the production of terpenoids greatly; however, due to insufficient insight into steady-state kinetic information and an incomplete understanding of the terpenoid biosynthetic pathway, researchers were still hard to build an efficient terpenoid overproduction platform.

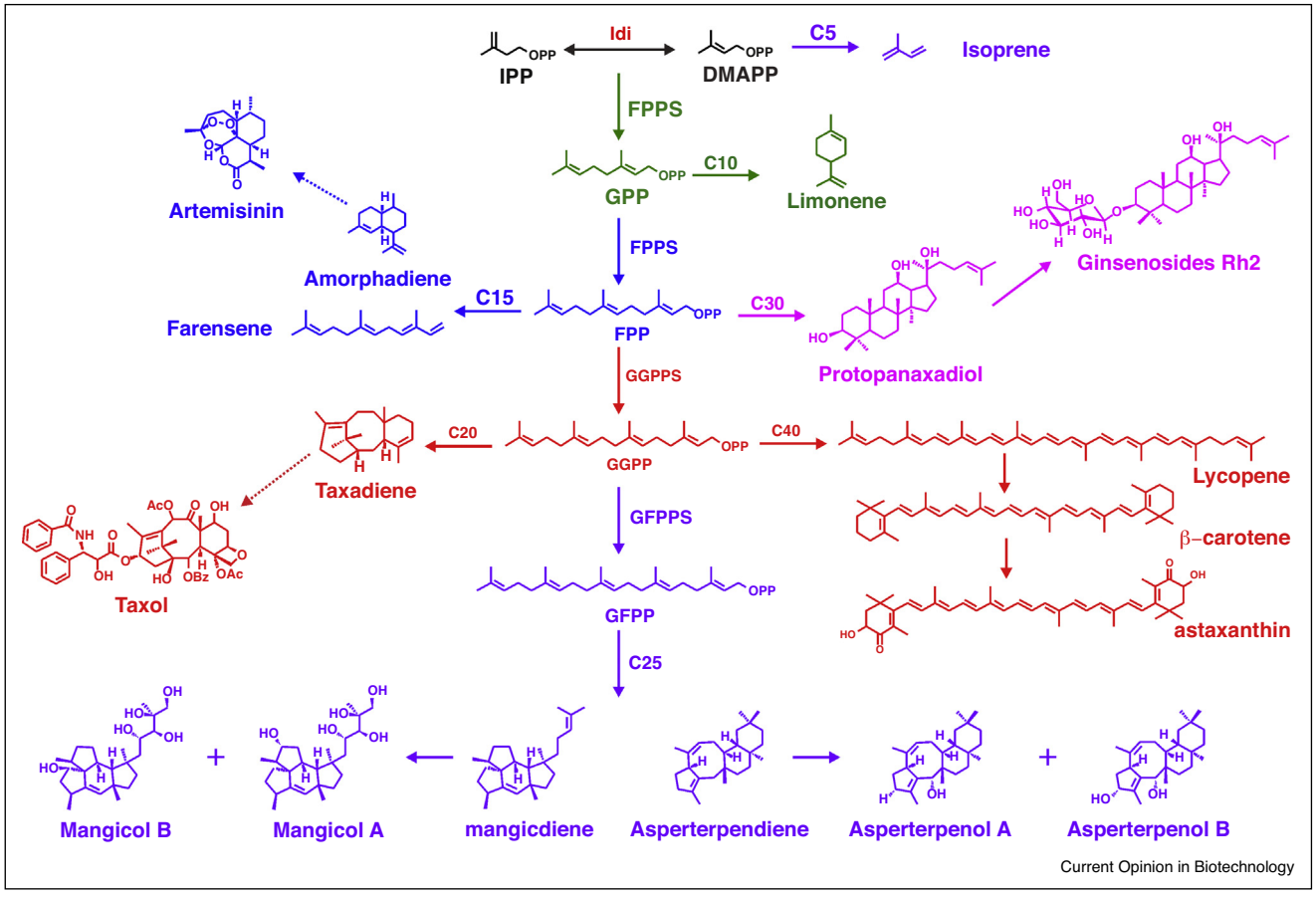
In addition, compared with the 80 000 terpenoids with known structures [4], the characterization of biosynthetic elements (e.g., terpene cyclase and cytochrome P450) involved in the production of value-added terpenoids remains limited. For example, in the production of paclitaxel, the enzyme involved in catalyzing some key steps (e.g., oxetane ring formation and C1, C9, and C2′ hydroxylation) has not yet been identified [5], representing a serious impediment to the engineering of paclitaxel production. In addition to the numerous terpenoids with known structures, it remains unknown how many terpenoids exist in nature that are yet to be discovered. The excavation of novel terpenoids may provide additional candidates for bioactivity screening and other applications. Early strategies, such as the direct isolation and characterization of terpenoids, provided a large number of value-added terpenoids (e.g., paclitaxel, artemisinin, and ginsenoside). However, as the number of characterized terpenoids increases dramatically, the mining of new terpenoids using this strategy becomes more difficult. Therefore, a universal platform that can provide sufficient precursors for the characterization of biosynthetic elements and the efficient mining of novel terpenoids is urgently needed.

Herein, according to the above major concerns, we review recent strategies for the engineering of value-added terpenoid production and genome mining of new terpenoids.

Basic biochemistry-directed terpenoid overproduction

The lack of detailed information regarding the steady-state kinetics and the factors that influence metabolic flux toward terpenoid biosynthetic pathways requires

Figure 1

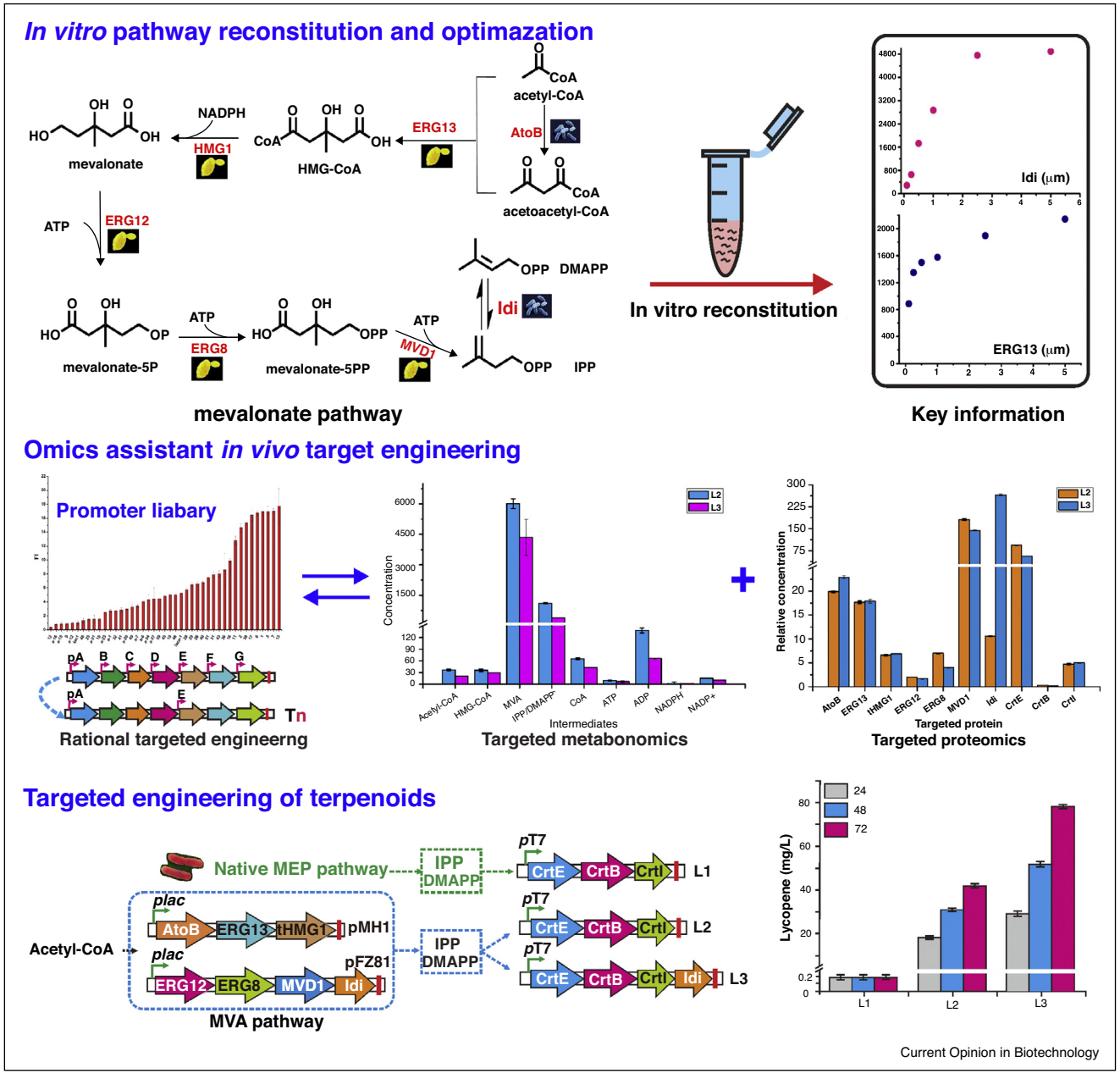


Production of value-added terpenoids using the universal precursors IPP and DMAPP. Abbreviations: C5, C10, C15, C20, C25, C30, and C40 represent hemiterpene, sesquiterpene, diterpene, sesterterpene, triterpene, and tetraterpene, respectively; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; Idi, isopentenyl diphosphate isomerase; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GFPP, geranylfarnesyl diphosphate; FPPS, GGPPS, and GFPPS represent the FPP, GGPP, and GFPP synthases, respectively.

researchers to construct numerous mutants to obtain high-yield strains. For those terpenoids that cause conspicuous phenotypes, such as a color change or change in growth rate (e.g., lycopene), high-throughput screening methods provide an ideal way for screening for high-yield mutants [6•], compensating for the shortcomings of these methods to a certain extent. However, for others (e.g., valencene), such methods would represent time-consuming and labor-consuming processes [7].

In vitro assays (reviewed by Lowry *et al.* [8]) are powerful methods for capturing detailed information regarding enzymatic reaction mechanisms, kinetics, and the identity of natural products. These have been widely used to direct the engineering of value-added natural product production efficiently [9••,10•]. Instead of constructing a series of mutants to evaluate the contributions of enzymes to the yield of terpenoids [11], key information provided by *in vitro* assays reveals directions for the engineering of terpenoid production in microorganisms [9••]. Zhu *et al.* reported the *in vitro* reconstitution-based targeted metabolic engineering approach (Figure 2) for rationally and accurately engineering terpenoid production in microorganisms with high efficiency. In this system, eight enzymes from the MVA pathway in *E. coli* and *S. cerevisiae* and farnesene synthase from the apple were reconstructed *in vitro*. Previously unavailable key information that significantly influences the production of terpenoids, such as the concentration of isopentenyl diphosphate (IPP) isomerase (Idi) and the optimum ratios among components for terpenoid overproduction, can be acquired, providing a clear direction for the *in vivo* engineering of terpenoid production. Then, the relative expression levels of enzymes and the accumulation of intermediates and co-factors provided by proteomics and metabolomics analyses enable the identification of the rate-limiting steps for researchers to further engineer terpenoid production and increase performance step by

Figure 2



In vitro reconstitution-based targeted engineering of terpenoids. *In vitro* reconstitution of the mevalonate pathway provides key information for terpenoid overproduction, enabling researchers to select appropriate promoters for engineering terpenoid production. Targeted-omics data can help increase the production of terpenoids step by step, making it possible for researchers to overproduce terpenoids with a high yield by simply constructing a limited number of mutants.

step [9••]. By using the same high efficiency IPP and DMAPP (dimethylallyl diphosphate) platforms, the overproduction of lycopene in *E. coli* was realized by overexpressing the lycopene-forming enzymes CrtE, CrtB, and CrtI and was further enhanced after the integration of another copy of Idi. As a result, the titer of lycopene was increased to 1.23 g/L (34.3 mg/gDCW) in a 100-L fermenter [12]. For astaxanthin overproduction, the same farnesene precursor-providing platform, together with the lycopene-forming pathway and the optimal CrtY, CrtZ, and CrtW were selected and integrated, achieving the highest yield of 8.64 mg/gDCW in *E. coli* using only one-step construction [13]. In addition to the engineering of terpenoid overproduction in *E. coli*, this strategy can also be used for engineering the production of terpenoids (e.g., taxadiene) in filamentous fungi

[14], demonstrating the high efficiency and extensive applicability of the targeted engineering strategy.

Whole-cell pathway balance and the production of terpenoids

Except an efficient precursor-providing platform, the systematic engineering of terpenoid production should comprehensively optimize the precursor supply, balance the redox reactions, and introduce a dynamic regulating system. The *in vitro* reconstitution strategy has been used to realize terpenoid and fatty acid overproduction by using glucose as substrate, which provides a clear biochemical background and a series of clear targets for optimizing the host and pathways [10•,15]. For the acetyl-CoA supply, Meadows *et al.* developed a fantastic and efficient strategy to rewrite the central carbon metabolism by introducing four non-native enzymes (xPK, PTA, ADA, and NADH-HMGr) to reduce the ATP required for the production of acetyl-CoA, reducing the loss of carbon and ensuring redox balance simultaneously. As a result, the production of farnesene was increased by 25% with a decrease in the consumption of oxygen of 75% [16••]. For redox balance, Zhao *et al.* engineered the tricarboxylic acid (TCA) cycle (*sucAB*, *gltA*, and *sdhABCD*), the pentose phosphate pathway (PPP; *tktA* and *talB*), and the ATP synthesis pathway (*nuo*, *cyd*, *cyo*, and *atp*) to increase the supply of ATP and NADPH. A synergistic effect was observed when the TCA and PPP modules were engineered together, resulting in a 64% increase in the β-carotene yield [17]. In addition, Liu *et al.* engineered the PPP and Entner–Doudoroff (ED) pathway to provide sufficient precursors and NADPH for the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, increasing isopentenol production 1.9-fold [18]. Furthermore, the efficient regulation system is essentially a safeguard for maximizing the production of terpenoids, minimizing the burdens of accumulated metabolic intermediates and competitive metabolic pathways [19,20•]. For dynamic regulation, the dynamic sensor-regulator system (DSRS) was developed by Zhang *et al.* and has been widely used in fatty acid and terpenoid overproduction [21,22•]. Dahl *et al.* used stress-response promoters to regulate farnesyl pyrophosphate production. As a result, the production of amorphadiene in *E. coli* was increased twofold without the need of expensive inducers [22•]. Similarly, Xie *et al.* developed the inducer/repressor-free sequential control strategy (based on the concentration of glucose) to dynamically control the expression of genes related to the biosynthesis of carotenoids and the competitive squalene pathway (ERG9) [20•]. In addition, the accumulation of hydrophobic terpenoids (e.g., nootkatone) intracellularly may result in cell toxicity and restrict the production of terpenoids with high yields and titers. The overexpression of *tolC* together with ABC family or MFS family exporters therefore increases the efflux of terpenoids [23].

The combination of these well-developed strategies makes it possible for researchers to engineer and produce terpenoids efficiently. On the basis of the sequential control strategy, Xie *et al.* reported that after systematic engineering of the lycopene production pathway together with the directed evolution of CrtE and CrtYB and an optimized two-stage feeding process of fed-batch fermentation, the yield of lycopene was increased to 1.61 g/L [6•]. By deleting potential distant genetic targets and overexpressing transcription factors together with the selection of key enzymes and the optimization of the lycopene-forming pathway, the production of lycopene was increased 22-fold for a maximum yield of 55.56 mg/gDCW [24]. By iteratively integrating multi-copy pathway genes and implementing fed-batch fermentation with an optimized medium, Gao *et al.* overproduced β-carotene in *Yarrowia lipolytica* with a titer of 4 g/L [11]. We expect that a targeted engineering-directed high-efficiency terpenoid overproduction platform, together with the optimization of the precursor supply, restoration of redox balance, and dynamic regulation system, would provide a rational way for engineering terpenoid production efficiently.

Genome mining of terpenoids: robust precursor supply and optimized host as a platform

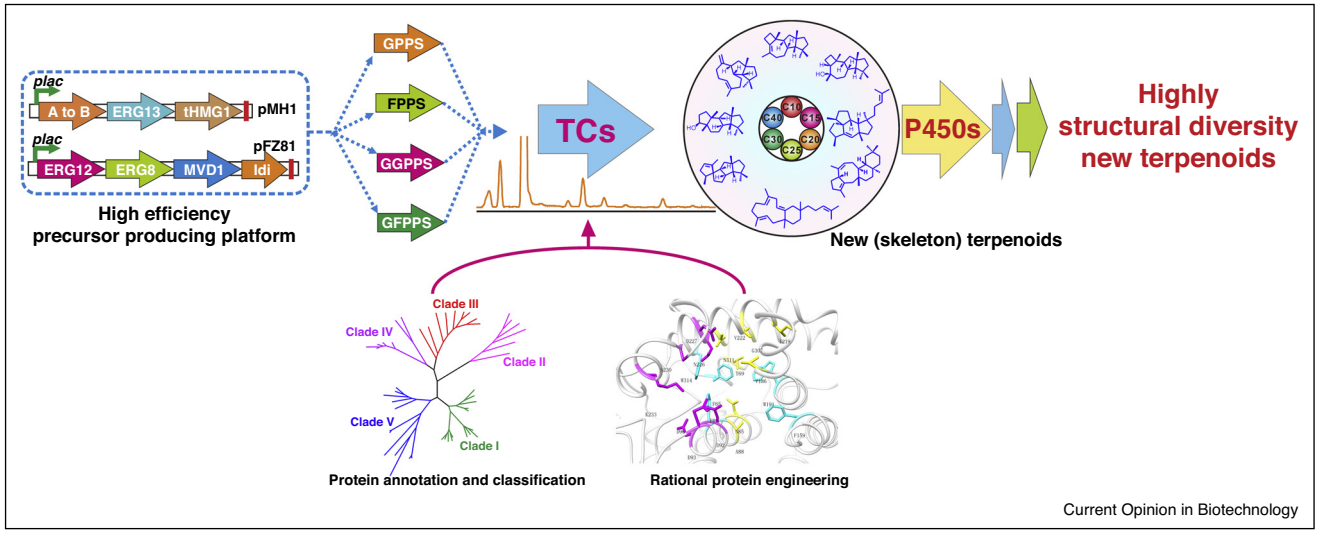
The mining of new terpenoids is the basis for researchers to enrich the structural diversity and screening of new bioactive compounds. The direct fermentation of microorganisms, a traditional strategy for natural product discovery, made great contributions to the process of terpenoid discovery [25,26]. However, for those compounds with very low production levels, it is difficult for researchers to obtain a sufficient amount of compound for structural characterization. In addition, researchers are unable to determine terpenoid biosynthetic information using this strategy. *In vitro* enzymatic reaction-based strategies provide a rapid method for characterizing the functions of enzymes and for mining new terpenoids [27]. However, the very low expression levels of some enzymes [28], the high costs of the substrates, and the complexity of the reactions (e.g., the reaction catalyzed by cytochrome P450) restricts widespread application to some extent.

Improvements in genome sequencing technologies and the exploitation of bioinformatics tools, such as antiSMASH and SMURF [29,30], have provided a large number of genes with unknown functions that are relevant to the biosynthesis of terpenoids [31,32••]. To take advantage of this large amount of genetic information, genome mining approaches have been developed to identify new terpene cyclases and new terpenoids [13,33]. Using *Aspergillus oryzae* as a heterologous host coupled with prenyltransferase domain swapping, Qin *et al.* characterized the function of the chimeric terpene

cyclase EvSS and discovered terpenoids with new skeletons, variediene and cericerene [34]. Likewise, the silent terpene cyclase NfSS was characterized in *A. oryzae* and the sesterterpenes sesterfisherol and sesterfisheric and their derivatives with novel, complicated skeleton were characterized [35•]. However, owing to the lack of an efficient precursor-providing platform, it remains challenging for researchers to acquire enough products from compounds with extremely low expression levels.

A growing amount of bioinformatics data has shown that a large number of genes related to the biosynthesis of terpenoids have not yet been characterized [36–38]. Terpene cyclase catalyzes the synthesis of the terpenoid core framework, which is the basis of the structural diversity of terpenoids. Thus, the excavation of new terpene cyclases and new terpene skeletons is a decisive step in enhancing the structural diversity of terpenoids. For example, the terpene cyclases FgMS and FgGS, which belong to a family of enzymes that are potentially with promiscuous feature, are reported to produce a series of new sesterterpene and diterpene [32••]. To fully display this promiscuous feature, Bian *et al.* established the combinatorial biosynthesis platform (Figure 3), composed of an optimized MVA pathway, a prenyltransferase (FPPS, GGPPS, and GFPPS) module, and a promiscuous terpene cyclase module [32••]. With a sufficient supply of targeted precursors, mutants for sesquiterpene, diterpene and sesterterpene production were constructed. As a result, the potential of the chimeric FgMS was fully displayed, with 35 terpenoids produced, including five terpenoids with characterized structures and two new sesterterpenes. Similarly, based on the combinatorial biosynthesis platform, the biosynthetic potential of the promiscuous terpene cyclase FgGS was exhibited, and five new diterpenes were characterized with three new skeletons [32••]. To further enhance product diversity, rational site-directed mutagenesis was carried out. On the basis of the theory that exchanging non-aromatic hydrophobic residues and aromatic hydrophobic residues in the binding pocket can significantly influence the overall pocket volume, 13 potentially non-catalytic sites within the binding pocket were engineered. With the help of an efficient precursor-providing platform, a series of potentially new sesterpenoids were produced by FgMS-F65L and FgMS-F159G, with one new sesterterpene confirmed [32••]. This represents the first time that researchers have produced a novel sesterterpene through enzymatic engineering. The integration of combinatorial biosynthesis and rational site-directed mutagenesis strategies with the genome mining of terpenoids provides a promising method for accelerating the characterization of terpene cyclases and new terpenoids. Furthermore, by using well-engineered precursor-providing platforms, various sources of class II (which produce bicyclic diphosphate intermediates for class I terpene synthase) and class I diterpene synthase-based combinatorial biosynthesis can also enable researchers discover new terpenoids with high efficiency [39,40]. It is possible for researchers to apply the combinatorial biosynthesis strategy to other platforms, such as *S. cerevisiae*, *Streptomyces*, and filamentous fungi, to produce terpenoids and other products [41–44].

Figure 3



Accelerated mining and characterization of new terpene cyclases and skeletons via combinatorial biosynthesis. Terpene cyclases (TCs) are predicted and classified by bioinformatics and phylogenetic analysis. Then, targeted substrates are provided via a high-efficiency precursor-providing platform, allowing researchers to discover new terpene cyclases and skeletons efficiently. The novel skeletons, combined with the promiscuous cytochrome P450 (P450s) and other post-modification enzymes, enables researchers to further expand the diversity of new terpenoids.

Conclusion and future perspectives

In vitro reconstitution provides a better understanding of the rate-limiting steps and working mechanisms of the MVA pathway. Key information provided by this system (e.g., the concentration of Idi and optimum component ratios) provides clear directions for engineering research. As briefly reviewed in this paper, in order to engineer terpenoid pathways for commercial production, the platform and the pathway should systematically and selectively integrate the advantages of various strategies, such as optimizing pathways using the targeted engineering approach, balancing the flux of acetyl-CoA and redox reactions, dynamically controlling the fermentation process and competitive metabolic pathways [9••,16••,17]. Additionally, emerging tools such as CRISPR-Cas9 and CRISPRi, as well as protein engineering combined with high-throughput screening, should promote advances in terpenoid overproduction [45–48]. With the help of efficient terpenoid overproduction platforms, it is possible for researchers to discover new terpene cyclases and skeletons. Moreover, these platforms can be extended by combining terpene cyclase and downstream enzymes of the terpenoid biosynthetic pathway (Figure 3), such as cytochrome P450 and other post-modification enzymes, to further enlarge the diversity and accelerate the mining of new terpenoids [49•,50], providing an elegant blueprint for improving the excavation of new terpenoids. After the active or target compounds are selected, overproduction can be achieved by increasing the selectivity and catalytic capability of enzymes using the direct evolution and semi-rational design methods [6•,51–53]. The large number of uncharacterized enzymes relevant to the biosynthesis of terpenoids and the existence of promiscuous enzymes provide the potential for broad applications of the results of genome mining for terpenoids.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 31500072), Hubei Science and Technology Project (2013BKB019) and the Young Talents Program of National High-level Personnel of Special Support Program (The "Ten Thousand Talent Program").

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Paddon CJ, Westfall P, Pitera D, Benjamin K, Fisher K, McPhee D, Leavell M, Tai A, Main A, Eng D: **High-level semi-synthetic production of the potent antimalarial artemisinin**. *Nature* 2013, **496**:528-532.

2. Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G: **Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli***. *Science* 2010, **330**:70-74.

3. • Lv X, Wang F, Zhou P, Ye L, Xie W, Xu H, Yu H: **Dual regulation of cytoplasmic and mitochondrial acetyl-CoA utilization for improved isoprene production in *Saccharomyces cerevisiae***. *Nat Commun* 2016, **7**:12851.

Dual engineering of acetyl-CoA utilization in the cytoplasm and mitochondria results in synergistic effects on isoprene production in *S. cerevisiae*. Coupled with the two-phase culture process and aerobic fed-batch fermentation, the highest titer of isoprene was achieved in engineered eukaryotes.

4. Pemberton TA, Chen M, Harris GG, Chou WK, Duan L, Köksal M, Genshaft AS, Cane DE, Christianson DW: **Exploring the influence of domain architecture on the catalytic function of diterpene synthases**. *Biochemistry* 2017, **56**:2010-2023.

5. Croteau R, Ketchum RE, Long RM, Kaspera R, Wildung MR: **Taxol biosynthesis and molecular genetics**. *Phytochem Rev* 2006, **5**:75-97.

6. • Xie W, Lv X, Ye L, Zhou P, Yu H: **Construction of lycopene-overproducing *Saccharomyces cerevisiae* by combining directed evolution and metabolic engineering**. *Metab Eng* 2015, **30**:69-78.

An unimpeded pathway was set up after increasing the supply of farnesyl diphosphate and strengthening the lycopene-forming pathway. Together with the overexpression of engineered CrtYB11M and the optimization of the two-stage feeding process of fed-batch fermentation, the yield of lycopene was increased to 1.61 g/L with a low yield of the byproduct squalene.

7. Kumaran AP, Lim CG, Li L, Ghosh S, Pirie C, Qualley A: Methods for production of oxygenated terpenes. Google Patents; 2016.

8. Lowry B, Walsh CT, Khosla C: ***In vitro* reconstitution of metabolic pathways: insights into nature's chemical logic**. *Synlett* 2015, **26**:1008-1025.

9. •• Zhu F, Zhong X, Hu M, Lu L, Deng Z, Liu T: ***In vitro* reconstitution of mevalonate pathway and targeted engineering of farnesene overproduction in *Escherichia coli***. *Biotechnol Bioeng* 2014, **111**:1396-1405.

From the *in vitro* reconstituted MVA pathway, the authors obtained an optimal ratio of synthetic components. A farnesene-overproducing *E. coli* strain was designed according to this information and resulted in high productivity and titer.

10. • Korman TP, Opgenorth PH, Bowie JU: **A synthetic biochemistry platform for cell free production of monoterpenes from glucose**. *Nat Commun* 2017, **8**:15526.

Using glucose as a substrate, a biosynthetic pathway for monoterpene production was *in vitro* reconstituted. Twenty-seven enzymes and the supplies of ATP and NAD(P)H were optimized within this system. Unrestricted by cellular toxicity, high titers of limonene, pinene, and sabinene were produced at approximately the theoretical maximum yields.

11. Gao S, Tong Y, Zhu L, Ge M, Zhang Y, Chen D, Jiang Y, Yang S: **Iterative integration of multiple-copy pathway genes in *Yarrowia lipolytica* for heterologous β-carotene production**. *Metab Eng* 2017, **41**:192-201.

12. Zhu F, Lu L, Fu S, Zhong X, Hu M, Deng Z, Liu T: **Targeted engineering and scale up of lycopene overproduction in *Escherichia coli***. *Process Biochem* 2015, **50**:341-346.

13. Ma T, Zhou Y, Li X, Zhu F, Cheng Y, Liu Y, Deng Z, Liu T: **Genome mining of astaxanthin biosynthetic genes from *Sphingomonas* sp. ATCC 55669 for heterologous overproduction in *Escherichia coli***. *Biotechnol J* 2016, **11**:228-237.

14. Bian G, Yuan Y, Tao H, Shi X, Zhong X, Han Y, Fu S, Fang C, Deng Z, Liu T: **Production of taxadiene by engineering of mevalonate pathway in *Escherichia coli* and endophytic fungus *Alternaria alternata* TPF6**. *Biotechnol J* 2017.

15. Liu Z, Zhang Y, Jia X, Hu M, Deng Z, Xu Y, Liu T: ***In vitro* reconstitution and optimization of the entire pathway to convert glucose into fatty acid**. *ACS Synth Biol* 2017, **6**:701-709.

16. •• Meadows AL, Hawkins KM, Tsegaye Y, Antipov E, Kim Y, Raetz L, Dahl RH, Tai A, Mahatdejkul-Meadows T, Xu L: **Rewriting yeast central carbon metabolism for industrial isoprenoid production**. *Nature* 2016, **537**:694-697.

The authors constructed a genome-scale model of *S. cerevisiae* to calculate the maximum theoretical yields and corresponding flux distributions and then systematically engineered the central carbon metabolism to balance energy and carbon flux, resulting in commercially efficient production of farnesene.

17. Zhao J, Li Q, Sun T, Zhu X, Xu H, Tang J, Zhang X, Ma Y: **Engineering central metabolic modules of *Escherichia coli* for improving beta-carotene production**. *Metab Eng* 2013, **17**:42-50.

18. Liu H, Wang Y, Tang Q, Kong W, Chung W-J, Lu T: **MEP pathway-mediated isopentenol production in metabolically engineered *Escherichia coli***. *Microb Cell Fact* 2014, **13**:135.

19. Tippmann S, Scalcinati G, Siewers V, Nielsen J: **Production of farnesene and santalene by *Saccharomyces cerevisiae* using fed-batch cultivations with RQ-controlled feed**. *Biotechnol Bioeng* 2016, **113**:72-81.

20. • Xie W, Ye L, Lv X, Xu H, Yu H: **Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae***. *Metab Eng* 2015, **28**:8-18.
A sequential control strategy regulated by glucose concentration was used to balance flux to the ergosterol and carotenoid pathways at the FPP branch point.

21. Zhang F, Carothers JM, Keasling JD: **Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids**. *Nat Biotechnol* 2012, **30**:354-359.

22. • Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD: **Engineering dynamic pathway regulation using stress-response promoters**. *Nat Biotechnol* 2013, **31**:1039-1046.
Stress-response promoters were identified and successfully used in regulating farnesyl pyrophosphate production. Finally, in the absence of expensive inducers, the production of amorphadiene in *E. coli* was increased twofold to 1.6 g/L with low acetate accumulation and high cell growth rate.

23. Zhang C, Chen X, Stephanopoulos G, Too HP: **Efflux transporter engineering markedly improves amorphadiene production in *Escherichia coli***. *Biotechnol Bioeng* 2016, **113**:1755-1763.

24. Chen Y, Xiao W, Wang Y, Liu H, Li X, Yuan Y: **Lycopene overproduction in *Saccharomyces cerevisiae* through combining pathway engineering with host engineering**. *Microb Cell Fact* 2016, **15**:113.

25. Katz L, Baltz RH: **Natural product discovery: past, present, and future**. *J Ind Microbiol Biotechnol* 2016, **43**:155-176.

26. He Y, Hu Z, Sun W, Li Q, Li X-N, Zhu H, Huang J, Liu J, Wang J, Xue Y: **Spiroaspertrione A, a bridged spirocyclic meroterpenoid, as a potent potentiator of oxacillin against methicillin-resistant *Staphylococcus aureus* from *Aspergillus* sp. TJ23**. *J Org Chem* 2017, **82**:3125-3131.

27. Meguro A, Tomita T, Nishiyama M, Kuzuyama T: **Identification and characterization of bacterial diterpene cyclases that synthesize the cembrane skeleton**. *ChemBioChem* 2013, **14**:316-321.

28. Görner C, Schrepfer P, Redai V, Wallrapp F, Loll B, Eisenreich W, Haslbeck M, Brück T: **Identification, characterization and molecular adaptation of class I redox systems for the production of hydroxylated diterpenoids**. *Microb Cell Fact* 2016, **15**:86.

29. Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH, Fedorova ND: **SMURF: genomic mapping of fungal secondary metabolite clusters**. *Fungal Genet Biol* 2010, **47**:736-741.

30. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W: **antiSMASH 3.0 — a comprehensive resource for the genome mining of biosynthetic gene clusters**. *Nucleic Acids Res* 2015, **43**:W237-W243.

31. Yamada Y, Kuzuyama T, Komatsu M, Shin-ya K, Omura S, Cane DE, Ikeda H: **Terpene synthases are widely distributed in bacteria**. *Proc Natl Acad Sci U S A* 2015, **112**:857-862.

32. •• Bian G, Han Y, Hou A, Yuan Y, Liu X, Deng Z, Liu T: **Releasing the potential power of terpene synthases by a robust precursor supply platform**. *Metab Eng* 2017, **42**:1-8.
Under the combinatorial biosynthesis strategy, the potential of the promiscuous terpene cyclases FgMS and FgGS were fully displayed. Two new sesterterpenes and five new diterpenes with three new carbon skeletons were structurally characterized from the 12 identified products. Then, rational site-directed mutagenesis was carried out to further enhance product diversity. Six potentially new sesterterpenes were produced by F65L and F159G, and one was confirmed as a new sesterterpene.

33. Matsuda Y, Mitsuhashi T, Quan Z, Abe I: **Molecular basis for stellatic acid biosynthesis: a genome mining approach for discovery of sesterterpene synthases**. *Org Lett* 2015, **17**:4644-4647.

34. Qin B, Matsuda Y, Mori T, Okada M, Quan Z, Mitsuhashi T, Wakimoto T, Abe I: **An unusual chimeric diterpene synthase from *Emericella variecolor* and its functional conversion into a sesterterpene synthase by domain swapping**. *Angew Chem Int Ed* 2015, **54**:1-5.

35. • Ye Y, Minami A, Mandi A, Liu C, Taniguchi T, Kuzuyama T, Monde K, Gomi K, Oikawa H: **Genome mining for sesterterpenes using bifunctional terpene synthases reveals a unified intermediate of di/sesterterpenes**. *J Am Chem Soc* 2015, **137**:11846-11853.
Using a genome mining approach, the bifunctional terpene synthase NfSS was characterized and coupled with cytochrome P450 monooxygenase (NfP450) to produce sesterterpenoids with new skeletons. The cyclization mechanisms were proposed by feeding with isotopically labeled precursors. The initial cyclization mechanisms of diterpene and sesterterpene were classified, and the unified biosynthetic pathway for sesterterpenes in group A was proposed.

36. Nielsen JC, Grijseels S, Prigent S, Ji B, Dainat J, Nielsen KF, Frisvad JC, Workman M, Nielsen J: **Global analysis of biosynthetic gene clusters reveals vast potential of secondary metabolite production in *Penicillium* species**. *Nat Microbiol* 2017, **2**:17044.

37. Li YF, Tsai KJ, Harvey CJ, Li JJ, Ary BE, Berlew EE, Boehman BL, Findley DM, Friant AG, Gardner CA: **Comprehensive curation and analysis of fungal biosynthetic gene clusters of published natural products**. *Fungal Genet Biol* 2016, **89**:18-28.

38. Zerbe P, Hamberger B, Yuen MM, Chiang A, Sandhu HK, Madilao LL, Nguyen A, Hamberger B, Bach SS, Bohlmann J: **Gene discovery of modular diterpene metabolism in nonmodel systems**. *Plant Physiol* 2013, **162**:1073-1091.

39. Andersen-Ranberg J, Kongstad KT, Nielsen MT, Jensen NB, Pateraki I, Bach SS, Hamberger B, Zerbe P, Staerk D, Bohlmann J: **Expanding the landscape of diterpene structural diversity through stereochemically controlled combinatorial biosynthesis**. *Angew Chem Int Ed* 2016, **55**:2142-2146.

40. Jia M, Potter KC, Peters RJ: **Extreme promiscuity of a bacterial and a plant diterpene synthase enables combinatorial biosynthesis**. *Metab Eng* 2016, **37**:24-34.

41. Zhuang X, Chappell J: **Building terpene production platforms in yeast**. *Biotechnol Bioeng* 2015, **112**:1854-1864.

42. Liu Q, Xiao L, Zhou Y, Deng K, Tan G, Han Y, Liu X, Deng Z, Liu T: **Development of *Streptomyces* sp. FR-008 as an emerging chassis**. *Synth Syst Biotechnol* 2016, **1**:207-214.

43. Castillo DA, Kolesnikova MD, Matsuda SP: **An effective strategy for exploring unknown metabolic pathways by genome mining**. *J Am Chem Soc* 2013, **135**:5885-5894.

44. Matsuda Y, Mitsuhashi T, Lee S, Hoshino M, Mori T, Okada M, Zhang H, Hayashi F, Fujita M, Abe I: **Astellifadiene: structure determination by NMR spectroscopy and crystalline sponge method, and elucidation of its biosynthesis**. *Angew Chem Int Ed* 2016, **128**:5879-5882.

45. Jakociunas T, Bonde I, Herrgard M, Harrison SJ, Kristensen M, Pedersen LE, Jensen MK, Keasling JD: **Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae***. *Metab Eng* 2015, **28**:213-222.

46. Li Y, Lin Z, Huang C, Zhang Y, Wang Z, Tang YJ, Chen T, Zhao X: **Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 meditated genome editing**. *Metab Eng* 2015, **31**:13-21.

47. Kim SK, Han GH, Seong W, Kim H, Kim S-W, Lee D-H, Lee S-G: **CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production**. *Metab Eng* 2016, **38**:228-240.

48. Michener JK, Smolke CD: **High-throughput enzyme evolution in *Saccharomyces cerevisiae* using a synthetic RNA switch**. *Metab Eng* 2012, **14**:306-316.

49. • Mafu S, Jia M, Zi J, Morrone D, Wu Y, Xu M, Hillwig ML, Peters RJ: **Probing the promiscuity of ent-kaurene oxidases via combinatorial biosynthesis**. *Proc Natl Acad Sci U S A* 2016, **113**:2526-2531.
Based on the combinatorial biosynthetic approach, the promiscuous nature of the ent-kaurene oxidase AtKO was fully displayed by screening 22 labdane-related diterpenoid substrates in engineered *E. coli*, resulting in a large number of AtKO alcohols and acids.

50. Ignea C, Ioannou E, Georgantea P, Loupassaki S, Trikka FA, Kanellis AK, Makris AM, Roussis V, Kampranis SC: **Reconstructing the chemical diversity of labdane-type diterpene biosynthesis in yeast**. *Metab Eng* 2015, **28**:91-103.

51. Zhuang Y, Yang G, Chen X, Liu Q, Zhang X, Deng Z, Feng Y: **Biosynthesis of plant-derived ginsenoside Rh2 in yeast via repurposing a key promiscuous microbial enzyme**. *Metab Eng* 2017, **42**:25-32.

52. Furubayashi M, Ikezumi M, Takaichi S, Maoka T, Hemmi H, Ogawa T, Saito K, Tobias AV, Umeno D: **A highly selective biosynthetic pathway to non-natural C50 carotenoids assembled from moderately selective enzymes**. *Nat Commun* 2015, **6**:7534.

53. Kolev JN, Zaengle JM, Ravikumar R, Fasan R: **Enhancing the efficiency and regioselectivity of P450 oxidation catalysts by unnatural amino acid mutagenesis**. *ChemBioChem* 2014, **15**:1001-1010.