Research Article

Production of taxadiene by engineering of mevalonate pathway in \textit{Escherichia coli} and endophytic fungus \textit{Alternaria alternata} TPF6

Guangkai Bian$^1$,
Yujie Yuan$^1$,
Hui Tao$^1$,
Xiaofei Shi$^1$,
Xiaofang Zhong$^1$,
Yichao Han$^2$,
Shuai Fu$^2$,
Chengxiang Fang$^3$,
Zixin Deng$^{1,4}$,
Tiangang Liu$^{1,4,*}$

$^1$Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education and School of Pharmaceutical Sciences, Wuhan University Wuhan 430071, P. R. China

$^2$J1 Biotech Co., Ltd., Wuhan 430075, P. R. China

$^3$China Center for Type Culture Collection (CCTCC), College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China

$^4$Hubei Engineering Laboratory for Synthetic Microbiology, Wuhan Institute of Biotechnology, Wuhan 430075, P. R. China

Submitted: 23-Nov-2016
Revised: 13-Feb-2017
Accepted: 16-Feb-2017
Correspondence: Prof. Tiangang Liu, Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education and Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, P. R. China.

E-mail: liutg@whu.edu.cn

Keywords: Taxadiene, Mevalonate pathway, Alternaria alternata TPF6, Agrobacterium tumefaciens

Abbreviations: MVA, Mevalonate; TS, taxadiene synthase; Idi, isopentenyl diphosphate isomerase; tHMG1, truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase; ATMT, Agrobacterium tumefaciens-mediated transformation; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate

Abstract

Taxol (paclitaxel) is a diterpenoid compound with significant and extensive applications in the treatment of cancer. The production of Taxol and relevant intermediates by engineered microbes is an attractive alternative to the semichemical synthesis of Taxol. In this study, based on a previously developed platform, we first established taxadiene production in mutant E. coli T2 and T4 by engineering of the mevalonate (MVA) pathway. We then developed an Agrobacterium tumefaciens-mediated transformation (ATMT) method and verified the strength of heterologous promoters in Alternaria alternata TPF6. We next transformed the taxadiene-producing platform into A. alternata TPF6, and the MVA pathway was engineered, with introduction of the plant taxadiene-forming gene. Notably, by co-overexpression of isopentenyl diphosphate isomerase (Idi), a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase (tHMG1), and taxadiene synthase (TS), we could detect 61.9 ± 6.3 μg/L taxadiene in the engineered strain GB127. This is the first demonstration of taxadiene production in filamentous fungi, and the approach presented in this study provides a new method for microbial production of Taxol. The well-established ATMT method and the known promoter strengths facilitated further engineering of taxaenes in this fungus.
1 Introduction

Taxol (paclitaxel) is a diterpenoid compound with significant and extensive applications in treatment of cancer [1]. Taxol was first isolated from the bark of the yew tree by Wani and colleagues [2]. However, because of high demand and limited supply, the price of Taxol has remained high. Moreover, production of Taxol is still dependent on the plant, thus limiting the amount of available drug.

As one of the most important precursors in the biosynthesis of Taxol, taxadiene contains a C20 backbone derived from the condensation and cyclization of the universal C5 unit isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Currently, there are two pathways that produce IPP and DMAPP (Figure 1): the mevalonate (MVA) pathway, which uses acetyl-CoA as a precursor, HMG-CoA reductase (HMGR) for production of mevalonate, and isopentenyl diphosphate isomerase (Idi) for isomerization of IPP and DMAPP [3-5]; and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, which uses pyruvate and glyceraldehyde-3-phosphate as precursors and 1-deoxyxylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspD), 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (IspF), and Idi as key enzymes in the biosynthesis of IPP and DMAPP [6, 7].

By using a multivariate-modular strategy, the native MEP pathway of E. coli was engineered, and the production of taxadiene was increased approximately 15,000-fold [7]. Additionally, a high titer of oxygenated taxanes was produced by optimizing both cytochrome P450 and reductase partner interactions and by altering N-terminal modifications of these enzymes [8]. However, the presence of high titers of byproducts catalyzed by CYP725A4 significantly restricted the production of taxadien-5a-ol and the resulting taxanes [9]. To resolve this problem, many studies have been carried out to illustrate the specific mechanisms involved [10, 11]. However, there are seven additional steps catalyzed by P450 in the biosynthesis of Taxol, which hinders the further study of Taxol production in E. coli.
To resolve the problems encountered in the production of oxygenated taxanes in *E. coli*, it is necessary to develop alternative Taxol production systems. Compared with *E. coli*, yeast and filamentous fungi are good choices for the overexpression of cytochrome P450 [8, 12]. Zhou developed a coculture strategy by cocultivating the engineered taxadiene-producing *E. coli* and subsequent oxygenated taxane-producing yeast and increased the production of these products [13]. Compared with yeast and bacteria, filamentous fungus are also a good platform for us to produce terpenoids owing to the advantage of high levels of final biomass and products [14]. Recent developments in metabolic engineering and synthetic biology, coupled with improvements in genetic operation methods and gene regulation studies, have allowed for the activation or overproduction of some complicated compounds in fungi. For example, the heterologous expression of aflatem and pleuromutilin biosynthesis gene clusters in *Aspergillus oryzae* can lead to accumulation of 54 mg/Kg aflatem and 84 mg/L pleuromutilin, respectively [12, 15]. However, in yeast and fungi, despite the addition of IPP and DMAPP by the MVA pathway, the production of taxadiene in engineered yeast is relatively low [4].

Biosynthesis of terpenoids by the MVA pathway has been successfully conducted in *E. coli* for the production of lycopene, farnesene, and astaxanthin in our laboratory [5, 16, 17]. This provides an ideal background for us to carry out metabolic engineering experiments to produce terpenoids via the MVA pathway. The MVA pathway has also been used in yeast for the production of farnesene and artemisinic acid with high titers [18, 19], which implies that engineering of the MVA pathway may allow us to produce terpenoids in yeast and filamentous fungi. Moreover, production of terpenoids in yeast and filamentous fungi through the MVA pathway may also be facilitated by the advantage of cytochrome P450 expression in these organisms.

Based on a previously developed platform, we quickly replaced the farnesene-producing module with taxadiene synthase (TS) and geranylgeranyl diphosphate synthase (GGPPS) genes to produce taxadiene in *E. coli*. We proved that the MVA pathway in *E. coli* could produce taxadiene. Then, we transformed this MVA pathway into filamentous fungi to produce taxadiene. Firstly, we
developed an *Agrobacterium tumefaciens* mediated transformation (ATMT) method [20], and verified the strength of heterologous promoters in *A. alternata* TPF6, which was reported as Taxol-producing endophytic fungus [21]. Next, based on this well-established platform, we engineered the MVA pathway to overcome the obstacles encountered during the first committed step of taxadiene production in fungi and realized the stable production of taxadiene in *A. alternata* TPF6. The results of this study provide a foundation for an alternative method for Taxol production in microorganisms.

2 Materials and methods

2.1 Materials

Phusion High-Fidelity polymerase, T4 DNA ligase, and other enzymes used in plasmid construction were from New England Biolabs (NEB; Ipswich, MA, USA). The RNeasy Plant Mini kit and RNase-Free DNase Set were from Qiagen (Valencia, CA, USA). The PrimeScript Reagent kit and SYBR Premix Ex Taq II were from TaKaRa (Kyoto, Japan). The CFX96 Touch Real-Time system was from Bio-Rad (Hercules, CA, USA). The fluorescence microscopy was from Olympus (Olympus IX71; Melville, NY, USA). The taxadiene standard was kindly provided by Professor Phil S. Baran.

2.2 Strains and genes

A list of the strains used in this study is shown in Table 1, and the relevant oligonucleotides are listed in Supplementary Table 1. The sequences of *ggpps, ts, hptII, idi*, and *thmg1* were codon-optimized for *A. alternata* TPF6 and synthesized by GeneScript (sequences are shown in the Supplementary information).
2.3 Construction of plasmids

The backbone of the pETDuet-1 fragment was amplified using the primer pair P1/P2, and the ts and ggpps genes were synthesized according to the description of Ajikumar and amplified by P3/P4 and P5/P6, respectively [7]. The three fragments were assembled by following the Gibson method to generate pFZ131 [22]. The pSC101 origin was amplified from pSC101 by P7/P8, and the pFZ131 fragment was amplified by P9/P10; these two fragments were then assembled by following the “simple cloning” method to generate pXC02 [23].

The trpC promoter was amplified from Aspergillus nidulans, using the primer pair P13/P26 and digested with EcoRI-HindIII. The synthesized hptII was digested with HindIII-Sall. Next, trpCp and hptII were ligated into the EcoRI-XhoI site of pCAMBIA1301 to construct pGB86. The promoters trpCp, gpdAp, alcAp, and oliCp from Aspergillus nidulans and agdAp from Aspergillus oryzae were amplified by P13/P30, P31/P32, P33/P34, P35/P36, and P37/P38, respectively. The promoter glaA from Aspergillus niger was amplified by P39/P40, and gfp was amplified by P27/P28 and P29/P29. The promoters and corresponding gfp fragments were ligated into the EcoRI-BstEII site of pGB86 to generate pGB92, pGB93, pGB94, pGB96, pGB99, and pGB98, respectively (Supplementary Figure 2).

The ts-Aa gene was amplified using the primer pair P11/P12 and ligated into the SacI-BstEII site of pGB94 to generate pGB123. The primer pairs P13/P14 and P15/P16 were used to amplify the trpC promoter and niaD terminator, respectively. The primer pair P17/P18 was used to amplify idi. These three fragments were assembled by overlap extension polymerase chain reaction (SOE-PCR) method to generate trpCp-idi-Aa-niaDt [24]. The cassette alcAp-ts-Aa was amplified by P14/P19. The cassettes trpCp-idi-Aa-niaDt and alcAp-ts-Aa were ligated into the EcoRI-BstEII site of pGB92 to generate pGB125. The primer pairs P20/P21 and P22/P23 were used to amplify the oliC promoter and agdA terminator, respectively. The primer pair P24/P25 was used to amplify thmg1. These three fragments were assembled using the SOE-PCR method to generate oliCp-
thmg1-Aa-agdAt and ligated into the SpeI site of pGB125 to generate pGB127. All of these plasmids are listed in Table 1.

2.4 Taxadiene production in E. coli

E. coli T2 (harboring pMH1, pFZ81, and pXC02), T4 (harboring pMH1, pFZ81, and pFZ131) were cultivated in 500-mL flasks containing 100 mL LB medium at 28°C with 100 mg/L ampicillin (AMP), 50 mg/L kanamycin (KAN), and 34 mg/L chloramphenicol (CM). When the OD<sub>600</sub> reached 0.6–0.8, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures, which were then cultivated for an additional 3 days. Experiments were repeated three times. One-milliliter cultures were collected at set intervals and extracted with an equal volume of hexane. The mixtures were centrifuged, and the organic layer was prepared for detection by gas chromatography-MS (GC-MS). The cell concentration was measured by OD<sub>600</sub> with a coefficient of 0.3 g DCW/OD<sub>600</sub>.

2.5 Transcriptome sequencing of A. alternata TPF6 for MVA pathway analysis

For transcriptome sequencing, A. alternata TPF6 was cultured in PDB medium with three repeats, and cultures were harvested after 3 days of cultivation and used for extraction of total RNA for transcriptome sequencing. Total RNA was extracted from 100 mg mycelium, using an RNeasy Plant Mini kit. An RNase-Free DNase Set was used to eliminate DNA contamination. A total amount of 3 μg RNA per sample was used as input material for RNA sample preparation. Sequencing libraries were created using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer’s protocols. The transcription level of the MVA pathway in A. alternata TPF6 was analyzed to provide basic information for us to carry out the metabolic engineering study in A. alternata TPF6.
2.6 Establish the genetic operating system for *A. alternata* TPF6

The transformation procedure for *A. alternata* TPF6 was established based on the description of Shao with some modifications [25]. Briefly, *A. alternata* TPF6 was grown on PDA slants for 28 days at 28°C. The spores were washed with sterile distilled water and concentrated for use. Transformants of *A. tumefaciens* EHA105 with the relevant plasmids were incubated overnight at 28°C in LB medium and then diluted to an OD$_{600}$ of 0.15 with induction medium (IM) and cultivated for another 6 h [25]. The spores were diluted with *A. tumefaciens* EHA105 at a concentration of $10^6$ per liter, plated onto sterile cellophane overlaid on the surface of co-cultivation medium (Co-IM) [25], and cultivated at 25°C for 3 days. The cellophane was transferred to a PDA plate with 50 mg/L hygromycin B and 500 mg/L cephalosporin and cultivated at 28°C. Transformants were subcultured three times and verified by PCR amplification.

2.7 Promoter strength verification in *A. alternata* TPF6

Fluorescence microscopy was used to detect the strength of different promoters. Mutants GB92, GB93, and GB96 were cultivated on modified PDA medium plates (glucose was decreased to 1 g/L). GB94 was cultivated on modified PDA medium plates with 12.6 g/L glycerol and 3 g/L threonine. GB98 and GB99 were cultivated on modified PDA medium plates with 20 g/L maltose. The fluorescence intensity was detected after 7 days of cultivation.

*A. alternata* mutants with different promoters were cultivated in the above-mentioned liquid medium for 5 days. For qPCR, total RNA was extracted from 100 mg mycelium using an RNeasy Plant Mini kit. An RNase-Free DNase Set was used to eliminate DNA contamination. cDNA was synthesized using a PrimeScript Reagent kit, and qPCR was performed using a SYBR Premix Ex Taq II kit on a CFX96 Touch Real-Time system. The *gapdh* gene encoding glyceraldehyde-3-phosphate dehydrogenase was selected as an internal control. Relative expression levels of *gfp* were calculated in comparison with the expression of *gapdh* by RT-PCR using primers P41/P42 and P43/P44. Data are presented as averages of three parallel reactions.
2.8 Fermentation of *A. alternata* TPF6 and derivative mutants for the production of taxadiene

Recovered *A. alternata* TPF6 and mutants were inoculated into 500-mL flasks containing 100 mL PGT medium (200 g/L potato, 12.6 g/L glycerol, 1 g/L glucose, and 3 g/L threonine) and incubated at 28°C with shaking at 250 rpm for 14 days. When the fermentation was complete, the cultures were extracted with equal volumes of hexane and ethyl acetate (4:1, v/v) for the detection of taxadiene. Each culture was extracted twice. The organic layer was concentrated using a rotary evaporator, dissolved in hexane, and prepared for GC-MS detection.

2.9 Detection of taxadiene by GC-MS

Taxadiene was detected by GC-MS (Thermo TRACE GC ULTRA combined with a TSQ QUANTUM XLS MS). The samples were injected into a TRACE TR-5MS (30 m × 0.25 mm × 0.25 um). The oven temperature was set at 80°C for 1 min, increased to 220°C at a rate of 10°C/min, and held at 220°C for 15 min. The injector and transfer lines were maintained at 230°C and 240°C, respectively.

3 Results

3.1 Production of taxadiene in *E. coli*

Based on a previous farnesene-producing platform, we quickly replaced the farnesene-producing module with *ts* and *ggpps* to produce taxadiene in *E. coli*. The *ts* and *ggpps* inserted in the low-copy number plasmid pXC02 and the high-copy plasmid pFZ131 were constructed and cotransformed with pMH1 and pFZ81 to generate *E. coli* T2 and *E. coli* T4 (Figure 2A). The produced taxadiene was detected with GC-MS and confirmed by comparing with the standard provided by Professor Phil S. Baran. The titers of taxadiene produced by mutants T2 and T4 at the shaking flask level were 11.3 ± 0.5 mg/L (13.2 mg/gDCW) and 5.0 ± 0.7 mg/L (5.9 mg/gDCW;
Figure 2B), respectively. Taxadiene produced by mutant T2 was purified and further confirmed by $^1$H NMR (Supplementary Figure 3).

3.2 Transcription level of the MVA pathway in *A. alternata* TPF6

For transcriptome analysis, three samples of *A. alternata* TPF6 were sequenced, and 10612 predicted genes were detected. Transcriptome data provided clear evidence regarding the transcript levels of genes related to the MVA pathway in *A. alternata* TPF6. The results (Supplementary Figure 1) showed that compared with the *in vitro* reconstitution results of the MVA pathway [5], the transcript levels of *erg10*, *erg13*, *erg12*, *erg8*, *mvd1*, and *idi* in *A. alternata* TPF6 were satisfactory for sufficient production of IPP and DMAPP for terpenoid production. In contrast, the transcription level of *hmg1* was relatively low, similar to that in *Saccharomyces cerevisiae*, likely owing to regulation by steroid-based negative feedback [4].

3.3 Development of genetic operating system and evaluation of heterologous promoter strength in *A. alternata* TPF6

Next, we transferred this platform into filamentous fungi. To produce sufficient IPP and DMAPP for the production of taxadiene in *A. alternata* TPF6, we must precisely control these genes by using promoters with different strengths. To evaluate the strength of the heterogeneous promoters in *A. alternata* TPF6, plasmids with *gfp* under the control of different promoters were constructed. Using the above-mentioned *A. tumefaciens* EHA105-mediated transformation method, we obtained strains GB92 (*trpC* promoter), GB93 (*gpdA* promoter), GB94 (*alcA* promoter), GB96 (*oliC* promoter), GB98 (*glaA* promoter), and GB99 (*agdA* promoter) containing GFP under the control of different promoters (Table 1). These promoters can be divided into two categories: the *trpC*, *gpdA*, and *oliC* promoters are constitutive promoters, whereas the *alcA*, *glaA*, and *agdA* promoters are inducible promoters. The promoter *alcA* is repressed by glucose and is strongly...
induced by glycerol plus threonine [26]. The promoters \( glaA \) and \( agdA \) can be induced by maltose [27, 28].

The constructed mutants GB92, GB93, GB94, GB96, GB98, and GB99 were cultivated based on the above-mentioned conditions. Mutant GB94 with promoter \( alcA \) showed the highest fluorescence intensity, whereas the remaining strains showed similar fluorescence intensities (Figure 3A). The strength of the promoters was further quantified by qPCR. In accordance with the fluorescence microscopy results, the qPCR results indicated that the strength of the promoters ranged from \( 4.5 \times 10^3 \) (GB93) to \( 3.4 \times 10^7 \) (GB94), with a span of more than 7,000 times, in the order of \( alcA > agdA > trpC > glaA > oliC > gpdA \) (Figure 3B). After determining the strengths of different promoters, we were able to choose a suitable promoter according to the expression level requirement for different genes in \( A. \ alternata \) TPF6.

3.4 Metabolic engineering of \( A. \ alternata \) TPF6 to promote the production of taxadiene

Our transcriptome data provided a clear background for the MVA pathway in \( A. \ alternata \) TPF6. The qPCR results showed that the promoter strengths in \( A. \ alternata \) TPF6 varied greatly. Based on biochemistry information provided by an in vitro study showing that the best ratio of AtoB:ERG13:tHMG1:ERG12:ERG8:MVD1:Idi for terpenoid production is 1:10:2:5:5:2:5 and prior results regarding the engineering of the MVA pathway [5, 16, 17], we rationally selected and overexpressed tHMG1, Idi, and TS under the control of oliC, trpC and alcA to facilitate the production of taxadiene.

A series of mutants was constructed by overexpressing TS and the key enzymes Idi and tHMG1 of the MVA pathway to facilitate the production of taxadiene (Figure 4A). The mutants GB123 (harboring the \( ts \) gene), GB125 (harboring the \( ts \) gene plus the \( idi \) gene), and GB127 (harboring the \( ts \) gene plus the \( idi \) and \( thmg1 \) genes; Table 1, Figure 4A) were constructed. Due to limited supply of precursor IPP and DMAPP, \( A. \ alternata \) TPF6, GB123, and GB125 were unable to produce sufficient taxadiene for detection by GC-MS. Taxadiene production was significantly increased in
strain GB127 after 14 days of fermentation, reaching a maximum concentration of 61.9 ± 6.3 μg/L (Figure 4B and 4C). This result was reproduced in five parallel experiments, indicating that taxadiene could be constantly overexpressed in this strain. Consequently, strain GB127 was a good platform for the production of taxadiene and subsequent intermediates relevant to the biosynthesis of Taxol, and has the potential for further improvements.

4 Discussion

Filamentous fungi are a good source of biological compounds with clinical and agricultural applications [29]. The observation that endophytic fungi produce Taxol is an attractive concept and may allow us to carry out metabolic engineering studies to produce Taxol and taxanes in filamentous fungi [30]. Taxadiene is the first committed precursor in the biosynthesis of Taxol. In this study, we proved that taxadiene could be produced by the MVA pathway in E. coli. We then transformed this platform into A. alternata TPF6, and taxadiene was detected by engineering of Idi, tHMG1, and TS under the control of different promoters.

A well-established genetic manipulation method is the foundation for engineering of filamentous fungi with increased productivity. A series of genetic manipulation methods, such as the PEG/CaCl₂-protoplast transformation system, electroporation, biolistics, and ATMT, has been successfully developed for different fungi [31, 32]. Among these methods, ATMT has the advantages of various starting materials and single-copy T-DNA integration in filamentous fungi [33]. The ATMT method was successfully established in A. alternata TPF6, which enabled us to carry out engineering studies to produce taxadiene in A. alternata TPF6. Moreover, A. alternata is a ubiquitous plant pathogen that produces host-selective toxins [34, 35]. Consequently, this genetic operation method may facilitate elucidation of the pathogenesis of A. alternata at the genomic level. Moreover, the ATMT method may facilitate the study of genus Alternaria at the genomic engineering level.
Characterization of the strength of promoters is critical for metabolic engineering studies. In *E. coli*, yeast, and *Streptomyces*, precise control of the expression of genes of interest can significantly increase the production of target products [36-38]. However, owing to the complexity of genetic manipulation methods, there is no promoter library available for filamentous fungi. Therefore, it is important to evaluate the strengths of available promoters to meet the needs of metabolic engineering studies. Promoter strength analysis showed that the heterologous promoters of *trpC*, *gpdA*, *alcA*, *oliC*, *glaA*, and *agdA* from different origins functioned well with different strengths in *A. alternata* TPF6. The known strengths of these heterologous promoters allowed us to rationally engineer the taxadiene production pathway in *A. alternata* TPF6 and produce taxadiene as well as other products.

Taking advantage of the high efficiency of the MVA pathway, taxadiene was produced by simply replacing the farnesene-producing module with a taxadiene-producing module. Compared with the titers of taxadiene-producing strains used for the TS engineering study, the titer of *E. coli* T2 was relatively feasible [11]. Production of taxadiene by the MVA pathway in *E. coli* provides an alternative system to produce taxadiene for academia.

Many genes related to post-translational modifications, such as cytochrome P450, can be expressed well in filamentous fungi and yeast [39-41]. Thus, we transformed the taxadiene-producing platform from *E. coli* to filamentous fungi. Based on the information obtained from analysis of promoter strength and the transcriptome for genes involved in the MVA pathway in *A. alternata* TPF6, taxadiene was successfully produced in GB127. This is consistent with earlier efforts demonstrating that simultaneous overexpression of TS, Idi, and tHMG1 could produce taxadiene in *S. cerevisiae* [4]. Moreover, to the best of our knowledge, this is the first demonstration of taxadiene production in filamentous fungi.

Compared with those in yeast and *E. coli*, the titers of taxadiene produced by *A. alternata* GB127 were still low; there are two main reasons for this result. First, the metabolic pathways of fungi are frequently regulated by global regulatory factors and pathway-specific factors at the
transcript level [42]. For example, inactivation of chromatin-level regulators, such as CclA, hdaA, and LaeA, can activate or inactive the expression of some cryptic secondary metabolite clusters in \textit{A. nidulans} [43-45]. Second, abundant terpenoid biosynthesis gene clusters may compete for the available GGPP with taxadiene. Given that higher production of target products can be acquired by simply transforming heterologous terpenoid-production gene clusters into filamentous fungi [12, 15], it may be possible for us to increase the production of taxadiene by optimizing the product flux and knocking out or downregulating the competitive metabolic pathway to promote the production of taxadiene [46, 47].

In summary, we developed an ATMT method for the genus \textit{Alternaria} for the first time and verified the strength of heterologous promoters in \textit{A. alternata} TPF6. Data regarding the promoter strength and expression levels of genes related to the MVA pathway enabled us to rationally design and overexpress taxadiene in \textit{A. alternata} TPF6. The taxadiene-producing mutant GB127 may facilitate metabolic engineering studies to achieve high production of Taxol in fungi. These efforts may provide an alternative method for Taxol biosynthesis and alleviate the requirement for natural plant sources for the production of this important cancer drug.

**Acknowledgement**

We thank Xiaoying Cao for construction of the plasmid pXC02 and Professor Phil S. Baran for kindly providing the taxadiene standard. This research was supported by grants from the Science and Technology Project of Hubei Province (grant no. 2013ACA011), the 973 Project of National Program on Key Basic Research (grant no. 2012CB721000), and the Young Talents Program of National High-level Personnel of Special Support Program (The “Ten Thousand Talent Program”).

**Conflict of interest**

The authors declare no financial or commercial conflict of interest.
5 References


Table 1 Details of strains and plasmids used in this research.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>E. coli B F dcm ompT hsdSB rB mB g6l</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>T2</td>
<td>E. coli BL21:: pMH1, pFZ81, pXC02</td>
<td>This research</td>
</tr>
<tr>
<td>T4</td>
<td>E. coli BL21:: pMH1, pFZ81, pFZ131</td>
<td>This research</td>
</tr>
<tr>
<td>EHA105</td>
<td>A. tumefaciens CS8 (Dm³) Ti pEHA105 (pTiBo542DT-DNA) (strept⁶) Succinamopine</td>
<td>[20]</td>
</tr>
<tr>
<td>TPF6</td>
<td>Alternaria alternata</td>
<td>[21]</td>
</tr>
<tr>
<td>GB92</td>
<td>TPF6 derivative; [pGB92: trpCp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB93</td>
<td>TPF6 derivative; [pGB93: gpdAp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB94</td>
<td>TPF6 derivative; [pGB94: alcAp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB96</td>
<td>TPF6 derivative; [pGB96: oliCp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB98</td>
<td>TPF6 derivative; [pGB98: glaAp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB99</td>
<td>TPF6 derivative; [pGB99: agdAp-GFP]</td>
<td>This research</td>
</tr>
<tr>
<td>GB123</td>
<td>TPF6 derivative; [pGB123: alcAp-TS]</td>
<td>This research</td>
</tr>
<tr>
<td>GB125</td>
<td>TPF6 derivative; [pGB125: trpCp-Idi; alcAp-TS]</td>
<td>This research</td>
</tr>
<tr>
<td>GB127</td>
<td>TPF6 derivative; [pGB127: trpCp-Idi; oliCp-tHMG1; alcAp-TS]</td>
<td>This research</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Origin of replication</td>
<td>Description</td>
</tr>
<tr>
<td>pMH1</td>
<td>p15A</td>
<td>lacp: AtoB, ERG13, tHMG1</td>
</tr>
<tr>
<td>pFZ81</td>
<td>pBBR1MCS</td>
<td>lacp: ERG12, ERG8, MVD1, Idi</td>
</tr>
<tr>
<td>pFZ131</td>
<td>pBR322</td>
<td>T7p: TS; GGPPS</td>
</tr>
<tr>
<td>pXC02</td>
<td>pSC101</td>
<td>pXC-TG; replace pBR322 origin with pSC101</td>
</tr>
<tr>
<td>pCAMBIA-1301</td>
<td>pBR322</td>
<td></td>
</tr>
<tr>
<td>pGB86</td>
<td>pBR322</td>
<td>pGB86; trpCp-HPTII (codon optimized)</td>
</tr>
<tr>
<td>pGB92</td>
<td>pBR322</td>
<td>pGB86; trpCp-GFP</td>
</tr>
<tr>
<td>pGB93</td>
<td>pBR322</td>
<td>pGB86; gpdAp-GFP</td>
</tr>
<tr>
<td>pGB94</td>
<td>pBR322</td>
<td>pGB86; alcAp-GFP</td>
</tr>
<tr>
<td>pGB96</td>
<td>pBR322</td>
<td>pGB86; oliCp-GFP</td>
</tr>
<tr>
<td>pGB98</td>
<td>pBR322</td>
<td>pGB86; glaAp-GFP</td>
</tr>
<tr>
<td>pGB99</td>
<td>pBR322</td>
<td>pGB86; agdAp-GFP</td>
</tr>
<tr>
<td>pGB123</td>
<td>pBR322</td>
<td>pGB86; alcAp-TS</td>
</tr>
<tr>
<td>pGB125</td>
<td>pBR322</td>
<td>pGB86; trpCp-Idi; alcAp-TS</td>
</tr>
<tr>
<td>pGB127</td>
<td>pBR322</td>
<td>pGB86; trpCp-Idi; oliCp-tHMG1; alcAp-TS</td>
</tr>
</tbody>
</table>
Figure 1. Production of taxadiene by MEP and MVA pathway in *E. coli* and fungi. Abbreviations: D-glyceraldehyde-3-phosphate (G3P), 1-deoxy-D-xylulose-5-phosphate (DXP), 2-C-ethyl-D-erythritol-4-phosphate (MEP), 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME2P), 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP), 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP), (S)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonate (MVA), mevalonate-5-phosphate (MVP), mevalonate diphosphate (MVPP), isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DAPP), geranylgeranyl diphosphate (GGPP). Enzyme abbreviations: 1-deoxyxylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (IspG), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IspH), AAT, acetyl-CoA C-acetyltransferase (ERG10), 3-hydroxy-3-methylglutaryl CoA synthase (ERG13), 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), mevalonate kinase (ERG12), phosphomevalonate kinase (ERG8), mevalonate diphosphate decarboxylase (MVD1), isopentenyl diphosphate isomerase (Idi), geranylgeranyl diphosphate synthase (GGPPS), taxadiene synthase (TS).
Figure 2. Production of taxadiene in *E. coli*. A: Comparison of the effects of different copy numbers of *ggpps* and *ts* on the production of taxadiene in *E. coli*. The fermentation of *E. coli* T2 with pMH1/pFZ81/pXC02 and *E. coli* T4 with pMH1/pFZ81/pFZ131 was carried out, and the production of taxadiene was detected by GC-MS. The mean ± SD for three biological replicates are shown.
Figure 3. Verification of heterologous promoter strength in A. alternata TPF6. A: Validation of promoter strength through the expression of GFP in A. alternata TPF6. The mutants GB92, GB93, GB94, GB96, GB98, and GB99 contained the promoters for trpC, gpdA, alcA, oliC, glaA, and agdA, respectively. The bar represents 10 μm. B: Quantitative evaluation the mRNA levels of gfp genes in the A. alternata mutant under the control of different heterologous promoters, as determined by qPCR. The mean ± SD for three biological replicates are shown.
Figure 4. Plasmid construction and GC-MS detection of taxadiene in cultures of wild-type and mutant *A. alternata* TPF6. A: T-DNA vectors used for *A. tumefaciens* EHA105-mediated transformation of *A. alternata* TPF6. B: GC-MS chromatogram of extracted taxadiene ions (m/z 122). C: GC-MS analysis of taxadiene produced by wild-type and mutant *A. alternata* TPF6 with a retention time of 16.6 min.