Identification of (-)-bornyl diphosphate synthase from Blumea balsamifera and its application for (-)-borneol biosynthesis in Saccharomyces cerevisiae

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A B S T R A C T

Borneol is a precious monoterpenoid with two chiral structures, (-)-borneol and (+)-borneol. Bornyl diphosphate synthase is the key enzyme in the borneol biosynthesis pathway. Many (+)-borneol diphosphate synthases have been reported, but no (-)-borneol diphosphate synthases have been identified. Blumea balsamifera leaves are rich in borneol, almost all of which is (-)-borneol. In this study, we identified a high-efficiency (-)-diphosphate synthase (BbTPS3) from B. balsamifera that converts geranyl diphosphate (GPP) to (-)-bornyl diphosphate, which is then converted to (-)-borneol after dephosphorylation in vitro. BbTPS3 exhibited a $k_{\text{cat}}$ value of 4.93 ± 1.38 μM for GPP, and the corresponding $k_{\text{cat}}$ value was 1.49 s⁻¹. Multiple strategies were applied to obtain a high-yielding (-)-borneol producing yeast strain. A codon-optimized BbTPS3 protein was introduced into the GPP high-yield strain MD, and the resulting MD-B1 strain produced 1.24 mg L⁻¹ (-)-borneol. After truncating the N-terminus of BbTPS3 and adding a Kozak sequence, the (-)-borneol yield was further improved by 4-fold to 4.87 mg L⁻¹. Moreover, the (-)-borneol yield was improved by expressing the fusion protein module of ERG20PROM27W, YRSQ1-t14-BbTPS3K2, resulting in a final yield of 12.68 mg L⁻¹ in shake flasks and 148.59 mg L⁻¹ in a 5-L bioreactor. This work is the first reported attempt to produce (-)-borneol by microbial fermentation.

1. Introduction

(-)-Borneol, a bicyclic monoterpene alcohol that is present in the essential oils of Blumea balsamifera (L.) DC., is widely used in the perfumery, cosmetics, and pharmaceutical industries [1,2]. It has been used medicinally for centuries in China for the treatment of stroke, and it is also used as a messenger drug to facilitate the transport of multiple drugs to specific sites by increasing blood-brain barrier permeability [3-5]. In addition, (-)-borneol and its derivatives exhibit a wide range of medicinal properties, such as anti-inflammatory, antimicrobial, and antiviral activities [6,7]. (-)-Borneol is mainly obtained from the fresh leaves of the plant B. balsamifera through distillation. However, the current production levels are not sustainable and cannot meet the increasing global market demand. As a result, synthetic borneol, which contains a few toxic compounds (e.g., isoborneol), is used widely as an alternative [8].

Recently, the use of emerging synthetic biology platforms has proved to be a promising alternative for monoterpenoid production; sustainable and reliable means of production can be established via engineering the biosynthetic pathways in microbes. Successful examples include the production of geraniol, limonene, linalool, (-)-borneol, citronellol, and nerol. Efforts to improve the efficiency of monoterpene production mainly focus on: (1) improving the geranyl diphosphate (GPP) pool by overexpressing the upstream pathway genes; (2) selecting for monoterpene synthases with higher activity and the same catalytic function; (3) improving the expression level or catalytic activities of monoterpene synthases by truncating the N-terminal transit peptide; and (4) constructing genetic fusions to bring enzymes together for the catalysis of a

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cascade of reactions \[9-13\]. It is highly possible that high-titer production of \((-\)-borneol) could be achieved by integrating these strategies to maximize flux toward target products.

\((-\)-Borneol) is mainly derived from GPP, which is synthesized from two common \(C_3\) building blocks, isopentenyl diphosphate and dimethylallyl diphosphate, via either the cytoplasmic mevalonate (MVA) pathway or the plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway \[14\]. However, the specific monoterpen synthase responsible for cyclization of GPP to form \((-\)-borneol) diphosphate remains enigmatic, although many \((\,-\,-\)-borneol) diphosphate synthases have been reported in plants (e.g., \textit{Cinnamomum burmannii}, \textit{Salvia officinalis}, \textit{Anomum villosum}, \textit{Lippia dulcis}, and \textit{Lavandula angustifolia}) \[10,15-18\]. Thus, no work has reported the \textit{de novo} synthesis of \((-\)-borneol) in microbes to date.

In this study, we identified a high-specificity \((-\)-borneol) diphosphate synthase, \textit{BbTPS3}, from \textit{B. balsamifera}, which is the first monoterpen synthase reported to catalyze the cyclization of GPP forming \((-\)-borneol) diphosphate, and then dephosphorylated to \((-\)-borneol) by phosphatases. Among the products obtained using GPP as a substrate in an \textit{in vitro} assay, \((-\)-borneol) accounted for 95.30% of the total products. Thus, no work has reported the \textit{de novo} synthesis of \((-\)-borneol) in microbes to date.

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were extracted with an equal volume of ethyl acetate for 1 h, and USA) to obtain the (-)-borneol producing strains (Table 1).

For (-)-borneol production, the yeast codon-optimized BbTPS3 as well as three truncated variants of BbTPS3 (at positions T14, I18 and N127) were cloned into the pESC-Leu vector (Agilent Technologies, USA) according to the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China), yielding the plasmids pESC-LEU::t14-BbTPS3, pESC-LEU::t14-BbTPS3K, and pESC-LEU::t14-BbTPS3K2. This study

Table 1

| Strains or Vectors | Description | Source |
|--------------------|-------------|--------|
| MATa, URA3-52, TRP1-289, LEU2-3112, HIS31, MAL2-8C, SUC2 | This study |
| MD | This study |
| CEN.PK2-1D | This study |
| pESC-LEU::t14-BbTPS3K | This study |
| pESC-LEU::t14-BbTPS3K2 | This study |

2.7. Shake flask fermentation

For (-)-borneol production, the positive strains were picked into flasks (50 mL) containing 10 mL of synthetic drop-out medium without leucine and uracil (SD-Leu-Ura) at 30 °C and 200 rpm for 48 h. Next, the cells were collected and诱导 by GAL promoters in 10 mL of YPL (1% yeast extract, 2% peptone, and 2% galactose) medium at 30 °C and 200 rpm for 48 h. The fermentation products were extracted with an equal volume of ethyl acetate for 1 h, and centrifuged at 13,000×g for 10 min to separate the upper organic phase for analyzing by GC-MS (described previously [21]). The calibration curves for content determination are shown in Supplemental Fig. S1. All assays were performed in triplicate.

2.8. Fed-batch fermentation for (-)-borneol production

We use strain MD-B12 for fed-batch fermentation in a 5-L bioreactor (Shanghai Bailun Biotechnology Co., Ltd., China) to produce larger amounts of (-)-borneol. Firstly, a single clone was seeded into a 50 mL flask containing 10 mL of SD-Leu-Ura medium with 2.0% glucose and grown at 30 °C and 200 rpm for 48 h. The resulting exponential culture was diluted to an initial OD of 0.3 in 200 mL of fresh SD-Leu-Ura medium and cultivated for another 12 h until the OD reached approximately 5.0. Centrifuge the seed culture at 3000×g, resuspend it and...
with 50 mL of fresh SD-Leu-Ura medium, and inoculate into the bioreactor containing 1.9 L of SD-Leu-Ura medium with 2.0% glucose at an initial OD$_{600}$ of 0.3. The temperature was 30 $^\circ$C and the pH was maintained at 5.0 by automatically adding 40% (v/v) NH$_3$⋅H$_2$O. Air flow was set at 1vvm and the dissolved oxygen (DO) concentration was controlled above 40% saturation by agitation cascade (300–500 rpm).

Concentrated 20× optimized YP medium (OYP, 20% yeast extract, 40% peptone, 16% KH$_2$PO$_4$, and 12% MgSO$_4$) and 50% galactose was added into bioreactor after 24 h. An extraction phase comprising n-dodecane was added to 20% (v/v) of the medium volume after induced 6 h to start the two-phase extractive fermentation. 50% galactose was fed periodically into the fermentation to keep the galactose concentration under 1.0 g L$^{-1}$. Additionally, 20×YP mixture (5% yeast extract, 15% peptone) was fed periodically to provide adequate nutrition for cell growth. At least independent duplicate samples were collected to determine the cell density, the (-)-borneol titer and GPP titer.

3. Results

3.1. Identification of the chirality of borneol in Blumea balsamifera and Cinnamomum burmanni leaves

The chirality and content of borneol in fresh leaves of B. balsamifera and C. burmannii were determined by GC-MS with a chiral column. After extracting the characteristic ion chromatograms of m/z 95, authentic standards of (-)-borneol (peak 1) and (+)-borneol (peak 2) were detected at retention times of 29.71 min and 30.02 min, respectively (Fig. 1A). Both peak 1 and peak 2 were detected in the leaves of B. balsamifera and C. burmannii. Peak 1 was significantly higher than peak 2 in B. balsamifera; in contrast, peak 2 was higher than peak 1 in C. burmannii. Next, we determined the contents of (+)- and (-)-borneol in plant leaves using a standard curve. Almost all of the borneol in B. balsamifera was (-)-borneol (4.80 ± 1.38 g kg$^{-1}$), accounting for...
99.40% of the total borneol in leaves. (+)-Borneol accounted for 94.95% of total borneol in *C. burmannii* leaves (3.79 ± 0.75 g kg⁻¹).

### 3.2. Transcriptome-based discovery of BbTPS3 in *Blumea balsamifera*

In our previous study, we identified a highly specific (+)-bornyl diphosphate synthase, CbTPS1, in *C. burmannii*, whose main product in *vitro* was (+)-borneol [10]. The fresh leaves of *B. balsamifera* containing large amounts of (+)-borneol were used to produce a transcriptome library, and then TPS candidates were screened by performing homology-based searches of the transcriptome. TRINITY_DN19922_c0_g2_i1 had the highest identity with the reported TPS homology-based searches of the transcriptome. TRINITY_DN19922_c0_g2_i1 had the highest identity with the reported TPS candidate gene from *B. balsamifera* cDNA, and the sequence was annotated as BbTPS3. BbTPS3 has an open reading frame of 1671 bp and encodes a 556-residue enzyme with a molecular mass of 64.56 kDa. Plant terpene synthases are divided into seven different subfamilies, named TPS-a to TPS-g, according to amino acid sequence similarity [25]. BbTPS3 belongs to the TPS-b group, which mainly contains angiosperm monoterpene synthases. All members of the TPS-b clade, including BbTPS3, contain the highly conserved aspartate-rich motifs DDXXD and NSE/DTE, which take part in substrate binding and metal-dependent ionization, and the RRX₆W motif, which is responsible for monoterpenoid cyclization [26]. Comparison of amino acid sequences showed that BbTPS3 shared the highest identities with SBS (38.37%) from *S. officinalis* [15] (Fig. 2), followed by LaBPPS (37.21%) from *L. angustifolia* [16], LdBPPS (36.33%) from *L. dulcis* [16], CtTPS1 (36.16%) from *C. burmannii* [10], and AvBPPS from *A. villosum* (34.33%) [18].

### 3.3. Functional characterization of BbTPS3

*In vitro* activity was analyzed using GPP as a substrate. The purified BbTPS3 protein generated borneol as its main product and other minor monoterpenoids (Fig. S4). No product formation was found in the absence of alkaline phosphatase, calf intestinal (CIAP) or when the empty vector was transformed into E. coli (DE3) cells. The chirality of borneol was investigated using a chiral column (Fig. 3A). One peak (peak 1) was the same as that of the authentic standard (+)-borneol. When the authentic standard (+)-borneol was added to the reaction product, the peak remained unchanged, but when the authentic standard (+)-borneol was added, one more peak (peak 2) was detected. This result confirmed that BbTPS3 catalyzed GPP to (+)-borneol in the presence of CIAP. Next, BbTPS3 was purified using Ni-NTA affinity chromatography (Fig. 3B). Kinetic analysis demonstrated that BbTPS3 had a *Kₗₐₜ* value of 4.93 ± 1.38 μM for GPP, and the corresponding *kₗₛ* value was 1.49 s⁻¹ (Fig. 3C).

### 3.4. Improving the (+)-borneol yield by generating tailored truncations

BbTPS3 was codon-optimized and introduced into the GPP high-yield strain MD, which harbors an optimized exogenous MVA pathway and was developed in our previous work [10]. (+)-Borneol was produced with a yield of 1.24 mg L⁻¹ in the resulting strain, MD-B1, and no product was found when the codon-optimized BbTPS3 was overexpressed in yeast strain CEN.PK2-1D (Fig. 4A). Plant TPS enzymes, including BbTPS3, have an N-terminal plastidic transit peptide that targets the protein to the plastid stroma and stromules where it is proteolyzed [27–29]. To increase the (+)-borneol titer, we overexpressed different N-terminal truncations of BbTPS3 overexpressed in yeast. Based on the ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) predictions, BbTPS3 was truncated at three different positions (T14, I18, and T38) in the N-terminus; these truncated proteins were named t14-BbTPS3, t18-BbTPS3, and t38-BbTPS3, respectively. As illustrated in Fig. 4B, three (+)-borneol-producing strains were constructed and the titer of (+)-borneol in each strain was determined by GC-MS. Expression of the
truncated t14-BbTPS3 and t18-BbTPS3 proteins led to an increase in (-)-borneol production. Strain MD-B3 expressing t14-BbTPS3 produced 2.13 mg L\(^{-1}\) of (-)-borneol, which was 1.72-fold higher than that amount produced by the strain MD-B1 (expressing full-length BbTPS3), and the strain MD-B5 expressing t18-BbTPS3 produced 1.12-fold higher levels of (-)-borneol (1.39 mg L\(^{-1}\)) than the MD-B1 (Fig. 4C). However, no (-)-borneol was detected in strain MD-B7 expressing t38-BbTPS3 (data not shown).

3.5. Improving the (-)-borneol yield by adding a Kozak sequence

Highly expressed genes in S. cerevisiae seem to prefer a 5'UTR rich in adenine and poor in guanine, particularly in the Kozak sequence that occupies roughly the first six nucleotides upstream of the START codon [22,24]. Thus, the Kozak sequence is used to improve gene expression here. The Kozak sequence “GCCACC” was added in front of the start codon (ATG) of the codon-optimized full-length BbTPS3 proteins to improve (-)-borneol production. The modified proteins were named BbTPS3K, t14-BbTPS3K, t18-BbTPS3K, and t38-BbTPS3K. However, when we introduced the modified proteins into the strain MD, there were no further improvements in (-)-borneol yield. As shown in Fig. 4C, the yields of strains expressing BbTPS3K, t14-BbTPS3K, t18-BbTPS3K, and t38-BbTPS3K were 0.87 mg L\(^{-1}\) (strain MD-B2), 1.85 mg L\(^{-1}\) (strain MD-B4), 0.47 mg L\(^{-1}\) (strain MD-B6), and 0.00 mg L\(^{-1}\) (strain MD-B8, data not shown), respectively. In yeast, “AAAAA” is the specific Kozak sequence. Therefore, “AAAAA” was used in place of “GCCACC” in front of the ATG of t14-BbTPS3, which had the highest (-)-borneol yield, generating the modified protein t14-BbTPS3K2. The introduction of t14-BbTPS3K2 into strain MD (strain MD-B9) significantly improved (-)-borneol production to 4.87 mg L\(^{-1}\) (Fig. 4C).

3.6. Improving the (-)-borneol yield by constructing fusion proteins

Fusion proteins are generated by fusing two or more coding sequences separated by a linker region; this method has been the most widely used to enhance the catalytic activities of enzymes [30]. GPP, which is the precursor for various yeast essential metabolites, such as squalene and sterol, is encoded by the gene ERG20 [31–33]. Therefore, we fused ERG20\(^{F96W-N127W}\) with t14-BbTPS3K2 to improve (-)-borneol production. Previous studies indicated that the length of the flexible linker and the order of the proteins determine the amount of folding interference and whether the two fusion proteins fold correctly [34,35]. In this study, we examined five linkers of different lengths, “GGGS”, “GSG”, “YRSQI”, “VIPFIS”, and “WRFSKLPQ". We constructed five fusion proteins (ERG20\(^{F96W-N127W}\)-GGGS-t14-BbTPS3K2, 

**Fig. 5.** The (-)-borneol production of strains expressing the fusion proteins. The data are averages of 3 biological replicates with error bars representing standard deviations.

**Fig. 6.** Production of (-)-borneol in fed-batch fermentation using the engineered strain MD-B12 in a 5-L bioreactor. The data are averages of 3 biological replicates with error bars representing standard deviations.
The in plants, BbTPS3 is the first reported (-)-bornyl diphosphate synthase. With GPP to produce (-)- borneol, then identified a high-efficiency BbTPS3 from B. balsamifera. Cated that (-)-borneol is the predominant form of borneol in B. balsamifera. Effects. The synthesis of (+)-borneol. Only trace amounts GPP were detected at the end of the fermentation. The titer of (-)-borneol was significantly positively correlated with the biomass of the strains increased rapidly before 60 h, and growth gradually increased until a maximum OD$_{600}$ of 140.70 was reached at 312 h. The titer of (-)-borneol was significantly positively correlated with biomass. (-)-Borneol continued to accumulate until 240 h, reaching a titer of 148.59 mg L$^{-1}$. Thereafter the yield declined, but the biomass remained stable. By contrast, GPP accumulated rapidly with increasing biomass during the first 48 h, but it decreased with the accumulation of (-)-borneol. Only trace amounts GPP were detected at the end of the fermentation.

4. Discussion

Borneol is a rare and precious natural product that is widely used in the medicine, perfume, and chemical industries because of its unique aroma; therefore, it is referred to as soft gold. Borneol is a dicyclic monoterpeneoid with two chiral structures, (-)-borneol and (+)-borneol. Both have similar properties and have anti-inflammatory and analgesic effects. The synthesis of (+)-borneol has been well studied, but that of (-)-borneol has rarely been investigated. Here, the chirality of borneol in B. balsamifera was characterized for the first time, and the results indicated that (-)-borneol is the predominant form of borneol in B. balsamifera, accounting for 99.40% of the total borneol in leaves. We then identified a high-efficiency BbTPS3 from B. balsamifera that reacts with GPP to produce (-)-borneol in vitro in the presence of CIAP. Although several (+)-bornyl diphosphate synthases have been reported in plants, BbTPS3 is the first reported (-)-bornyl diphosphate synthase. The $K_m$ value of BbTPS3 (4.93 ± 1.38 μM) for GPP is similar to that of CbTPS1 (5.11 μM) and SBS (3.0 μM) but lower than that of other reported monoterpene synthases (13.10–26.12 μM), indicating that BbTPS3 has a higher affinity for GPP than most monoterpene synthases [15,36–38]. Thus, this gene will be useful in efforts to reconstruct the (-)-borneol biosynthetic pathway for heterologous production.

GPP is the direct precursors of monoterpeneoids [8]. We selected the yeast strain MD, which was engineered to produce high levels of GPP [10]. Then, multiple strategies were applied to increase the production of (-)-borneol. The codon-optimized BbTPS3 was first truncated at the N-terminus and a Kozak sequence was placed in front of the ATG. The highest (-)-borneol titer was achieved in strain MD-B9, which expresses the modified protein t14-BbTPS3K2. The titer (4.87 mg L$^{-1}$) was 4-fold higher than that of strain MD-B1. Next, five linkers with different lengths and protein orders were tested. The strain MD-B12 expressing the fusion module ERG20$^{360}$-127W-YRSQI-t14-BbTPS3K2 produced 12.68 mg L$^{-1}$ (-)-borneol. Therefore, a combination of truncation, Kozak sequence addition, and fusion protein construction is an effective strategy for improving (-)-borneol production. This strain produced a titer of (-)-borneol 148.59 mg L$^{-1}$ in a 5-L bioreactor, which is the highest (-)-borneol titer achieved in heterologous production reported so far.

Though more than 100 mg L$^{-1}$ of (-)-borneol was produced in this study, the yield is still well below the level required for industrial applications and that achieved for other terpenoids [8,39,40], such as artemisinc acid (25 g L$^{-1}$) [41]. The efficiency of generating the final product is affected by many factors. Monoterpeneoids, such as linalool and borneol, alter membrane properties or damage the cell wall and are highly toxic to S. cerevisiae, reducing the production efficiency [42,43]. Here, we used two-phase extractive fermentation to reduce toxicity. It was recently reported that transformation of yeast peroxisomes into microfactories for monoterpeneoid production can achieve up to a 125-fold increase over cytosolic production by reducing cytotoxicity [38,44]. Multiple engineering strategies can be applied to optimize the metabolic pathway of host cells and increase the supply of GPP [11,45]. In addition, directed evolution of enzymes and optimization of the fermentation strategy will further enhance production [46,47]. Our work lays a solid foundation for the biosynthesis of natural (-)-borneol and other active pharmaceutical terpenoids.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Rui Ma: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. Ping Su: Conceptualization, Methodology, Investigation, Writing – original draft. Qing Ma: Methodology, Supervision. Juan Guo: Formal analysis. Suiping Chen: Investigation. Bao-long Jin: Data curation. Haiyan Zhang: Resources. Jinfu Tang: Software. Tao Zhou: Funding acquisition. Chenghong Xiao: Funding acquisition. Guanghong Cui: Supervision, Project administration. Luqi Huang: Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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