Microbial Cell Factories

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Development of a *Komagataella phaffii* cell factory for sustainable production of (+)-valencene

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Abstract

Background Sesquiterpene (+)-valencene is a characteristic aroma component from sweet orange fruit, which has a variety of biological activities and is widely used in industrial manufacturing of food, beverage and cosmetics industries. However, at present, the content in plant sources is low, and its yield and quality would be influenced by weather and land, which limit the supply of (+)-valencene. The rapid development of synthetic biology has accelerated the construction of microbial cell factories and provided an effective alternative method for the production of natural products.

Results In this study, we first introduced the (+)-valencene synthase into *Komagataella phaffii* by CRISPR/Cas9 system, and successfully constructed a (+)-valencene producer with the initial yield of 2.1 mg/L. Subsequently, the (+)-valencene yield was increased to 8.2 mg/L by fusing farnesyl pyrophosphate synthase with (+)-valencene synthase using the selected ligation linker. High expression of key genes *IDI1*, *tHMG1*, *ERG12* and *ERG19* enhanced metabolic flux of MVA pathway, and the yield of (+)-valencene was further increased by 27%. Besides, *in-situ* deletion of the promoter of *ERG9* increased the yield of (+)-valencene to 48.1 mg/L. Finally, we optimized the copy number of farnesyl pyrophosphate synthase and (+)-valencene synthase fusion protein, and when the copy number reached three, the yield of (+)-valencene achieved 173.6 mg/L in shake flask level, which was 82-fold higher than that of the starting strain CaVAL1.

Conclusions The results obtained here suggest that *K. phaffii* has the potential to efficiently synthesize other terpenoids.

Keywords (+)-Valencene, *Komagataella phaffii*, Cell factory, CRISPR/Cas9, Synthetic biology

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Introduction

Sesquiterpenes are widely found in plants [1]. Modern studies have found that sesquiterpenoids have a wide range of biological activities such as anti-bacterial, anti-inflammatory, immunosuppressive activity, liver protection, and cardiotonic [2, 3]. (+)-Valencene is an important sesquiterpenoid which is widely used as a flavoring and fragrance additive in food, beverages and cosmetics due to its distinctive sweet orange aroma [4, 5]. In addition, (+)-valencene has a wide range of biological properties such as antioxidant, antiallergic, and immunomodulatory [6, 7]. At present, the supply of (+)-valencene mainly relies on extraction from citrus fruits or chemical synthesis, which have the drawbacks of high cost, low yield and environmental friendliness [8, 9].

With the growing demand for natural resources and increasing awareness of environmental issues, there is strong interest in the fermentation of renewable biomass by microorganisms to produce valuable natural products [10–12]. In recent years, with the development of synthetic biology, microbial cell factories have realized the biosynthesis and fermentation of a variety of natural products [13, 14]. *Komagataella phaffii* has been used as a multifunctional cell factory for the production of biomolecules [15]. As a yeast, *K. phaffii* owns many advantages as a robust eukaryotic expression system, such as protein processing, protein folding, and post-translational modifications [16, 17], while it is as easy to manipulate as *Escherichia coli*. Compared with *Saccharomyces cerevisiae*, *K. phaffii* is more convenient for molecular genetic fabrication and has advantages in protein expression [17, 18].

With the successful construction of gene editing technology [19], *K. phaffii*, as a non-traditional yeast, has high hopes for its significant advantages in the biosynthesis of natural products. There have been many natural products that are biosynthesized in *K. phaffii*, including: nepetalactol [20], fatty acid [21], β-elemene [22], lovastatin [23], α-farnesene [24], α-santalene [15], patchoulol [25], vinblastine [26] et al. Cai et al. developed a novel library of artificial transcription devices for *K. phaffii* and realized the fine expression control of multi-enzyme pathways, which provided new ideas and strategies for the biosynthesis of other natural drugs and chemicals with high value complex structures, and facilitated to expand the application potential of *K. phaffii* in the field of synthetic biology and metabolic engineering [27]. Recently, Ye et al. have successfully established an efficient *K. phaffii* cell factory for de novo synthesis of nepetalactol [20]. Zhou et al. achieved high-performance methanol conversion biosynthesis of fatty acids and fatty alcohols by optimizing the central metabolism and methanol utilization pathways in *K. phaffii* [21]. What's even more exciting, Gao et al. achieved de novo synthesis of vinblastine in *K. phaffii* for the first time [28]. The vinblastine pathway is the most complex biosynthetic pathway constructed in non-model strains to date, demonstrating the advantages and great potential of *K. phaffii* as a cell factory for plant natural products. In previous studies, *K. phaffii* has been considered as an ideal host for the production of (+)-valencene, and the yield of (+)-valencene in the shake flask of the starting strain (expressing only valencene synthase form *Callitropsis nootkatensis*) reached 51 mg/L [29]. Gao et al. used a multi-level metabolic control strategy to achieve a high yield of (+)-valencene in *S. cerevisiae*, and finally the yield of (+)-valencene in the shake flasks reached 1.2 g/L through batch fed-feed fermentation [30]. However, the yield still does not meet the industrial applications, thus it needs a higher yield through other synthetic biology methods.

In order to further increase the yield of (+)-valencene in *K. phaffii*, we adopted a classical synthetic biology strategy and CRISPR/Cas9 system to engineer *K. phaffii*. In this study, we used a systematic synthetic biology strategy to produce (+)-valencene in *K. phaffii* (Fig. 1), and the final yield at shake flask level was 173.6 mg/L. These strategies include fusion expression, mevalonate pathway (MVA) pathway enhancement, down-regulation of metabolic flux in competitive pathway, and multiple copies of key expression cassette. This work further suggests that *K. phaffii* cell factories have great potential in natural product biosynthesis.

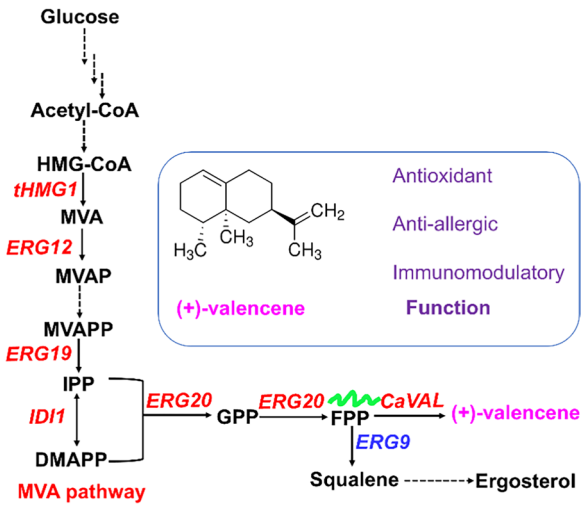

Fig. 1 Schematic diagram of (+)-valencene biosynthesis in *K. phaffii*. *HMG-CoA* 3-Hydroxy-3-methylglutaryl-CoA, *tHMG1* truncated HMG-CoA reductase, *ERG12* mevalonate kinase, *ERG19* mevalonate pyrophosphate decarboxylase, *IPP* isopentenyl pyrophosphate, *IDI1* isopentenyl diphosphate isomerase 1, *DMAPP* dimethylallyl pyrophosphate, *ERG20* farnesyl diphosphate synthase, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate

Materials and methods

Strains and culture condition

E. coli DH5α was used for plasmid design and *K. phaffii* GS115 was used as the host strain for the biosynthesis of (+)-valencene. The plasmid HZP-gRNA (PARS1, Zeo, *SERp*-IntX-*sgRNA*) was used for gRNA expression. IntX-TEF1 (single gene expression vectors, where X represented different insertion sites) and IntX-TEF1-GAP (Dual gene expression vectors, where X represented different insertion sites) were used for the construction and expression of target genes.

Plasmid construction

The relevant plasmids and primers used in this study are listed in Supplementary Table S1 and Table S2, respectively. The (+)-valencene synthase gene *CaVAL* from *Callitropsis nootkatensis* was synthesized by Nanjing GenScript Biotechnology Co., Ltd., and the codon of gene *CaVAL* was optimized and its sequence information was in Supplementary Table S3. Key genes in the mevalonate pathway we used, including truncated HMG-CoA reductase (*tHMG1*), mevalonate kinase (*ERG12*), isopentenyl diphosphate isomerase1 (*IDI1*), mevalonate pyrophosphate decarboxylase (*ERG19*), Farnesyl diphosphate synthase (*ERG20*), were amplified from the yeast genome and subsequently cloned into donor plasmids using a seamless cloning method to obtain the corresponding expression plasmids. The sgRNA plasmid was designed by Benchling CRISPR tool (https://benchling.com/crispr/).

Strain construction

We first constructed the plasmid pGAP-Cas9 and transferred it into *K. phaffii* to obtain the host GS115-Gas9 (CaVAL-0) [31]. Different recombinant strains were obtained by integrating (+)-valencene synthase gene *CaVAL* and its biosynthesis-related genes into the *K. phaffii* chromosome by CRISPR/Cas9 method. Subsequently, the gene expression cassette containing the homology arm and the corresponding gRNA plasmid were co-transformed into the Cas9-expressing strain (CaVAL-0). The transformation of *K. phaffii* was carried out using an electroporation method [31]. In this study, all constructed recombinant strains are listed in Table 1 and the corresponding integration sites are listed in Supplementary Table S4.

Flask-scale fermentation of recombinant strains

A single colony strain of the recombinant strain was inoculated into a tube containing 5 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose), incubated for 24 h at 30 °C and 220 rpm, and then transferred to 50 mL of YPD at 1% inoculum, incubated in 250 mL shake flasks for 72 h. After 24 h of incubation, 10% n-dodecane was added into the shake flasks.

Table 1 Strains used in this study

Strain	Genotype	Source
CaVAL0	*K. phaffii* GS115-HIS4::Cas9	This study
CaVAL1	CaVAL0-Int1::TEF1p-AaCPS-AOX1t	This study
CaVAL2	CaVAL0-Int1::TEF1p-AaCPS-AOX1t-GAPp-ERG20-0547t	This study
CaVAL3	CaVAL0-Int1::TEF1p-ERG20-GGS-AaCPS-AOX1t	This study
CaVAL4	CaVAL0-Int1::TEF1p-ERG20-GGGGS-AaCPS-AOX1t	This study
CaVAL5	CaVAL0-Int1::TEF1p-ERG20-(GGGGS)2-AaCPS-AOX1t	This study
CaVAL6	CaVAL0-Int1::TEF1p-ERG20-(PT)4P-AaCPS-AOX1t	This study
CaVAL7	CaVAL0-Int1::TEF1p-ERG20-(PA)5-AaCPS-AOX1t	This study
CaVAL8	CaVAL6-Int2::TEF1p-IDI1-AOX1t	This study
CaVAL9	CaVAL6-Int2::TEF1p-IDI1-AOX1t-GPAp-tHMG1-CYCt1	This study
CaVAL10	CaVAL9-Int3::TEF1p-ERG12-AOX1t	This study
CaVAL11	CaVAL9-Int3::TEF1p-ERG12-AOX1t-GPAp-ERG19-CYCt1	This study
CaVAL12	CaVAL11-ΔERGp (– 50 bp)-L	This study
CaVAL13	CaVAL11-ΔERGp (– 100 bp)-L	This study
CaVAL14	CaVAL11-ΔERGp (– 100 bp)-R	This study
CaVAL15	CaVAL13-Int4::TEF1p-ERG20-(PT)4P- CaVAL-AOX1t	This study
CaVAL16	CaVAL15-Int5::TEF1p-ERG20-(PT)4P- CaVAL-AOX1t	This study
CaVAL17	CaVAL16-Int6::TEF1p-ERG20-(PT)4P- CaVAL-AOX1t	This study

Analytical methods of (+)-valencene

GC–MS was used for the detection of (+)-valencene. The instrument used was a gas chromatograph (GC-TQ8050 NX; SHIMADZU) equipped with a flame ionization detector and an SH-I-SiI column (30 m × 0.25 mm × 0.25 μm). The inlet temperature was maintained at 240 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The heating program was set as follows: the initial temperature was 60 °C, holding for 10 min, and then ramp to 200 °C at 10 °C/min, holding for 6 min. The source temperature and interface temperature were maintained at 250 °C.

Results

Construction of *K. phaffii* cell factory for producing (+)-valencene

The biosynthetic pathway of (+)-valencene has been resolved. *Callitropsis nootkatensis*-derived (+)-valencene synthase was reported to be highly efficient in the biosynthesis of (+)-valencene [8, 32]. We used CRISPR/Cas9 system to transform a expression cassette containing (+)-valencene synthase (CaVAL) into the genome of *K. phaffii* to obtain the starting strain CaVAL1. With GC–MS detection, (+)-valencene was detected in *K. phaffii*

CaVAL1 (Fig. 2). The yield of (+)-valencene in the engineered strain CaVAL1 was 2.1 mg/L.

Effect of fusion expression on the yield of (+)-valencene

Protein fusion can help to improve the catalytic efficiency of continuous catalytic reactions [33]. We tried to explore the effect of fusion expression on the yield of (+)-valencene by fusing ERG20 and CaVAL with different linkers. Five different linkers (GGS, GGGGS, (GGGGS)2, (PT)4A, (PA)5) were designed for this study (Fig. 3). The experimental results showed that the fusion ERG20-CaVAL with linker ((PT)4A) was detected with the highest yield of (+)-valencene at 8.2 mg/L, which was 4.7 times higher than that of the starting strain CaVAL1 (Fig. 3). In addition, the yield in the fusion-expressing strains (CaVAL3, CaVAL4, CaVAL5, CaVAL6, CaVAL7) of (+)-valencene were all higher than that of the control strain CaVAL2, which expressed both ERG20 and CaVAL

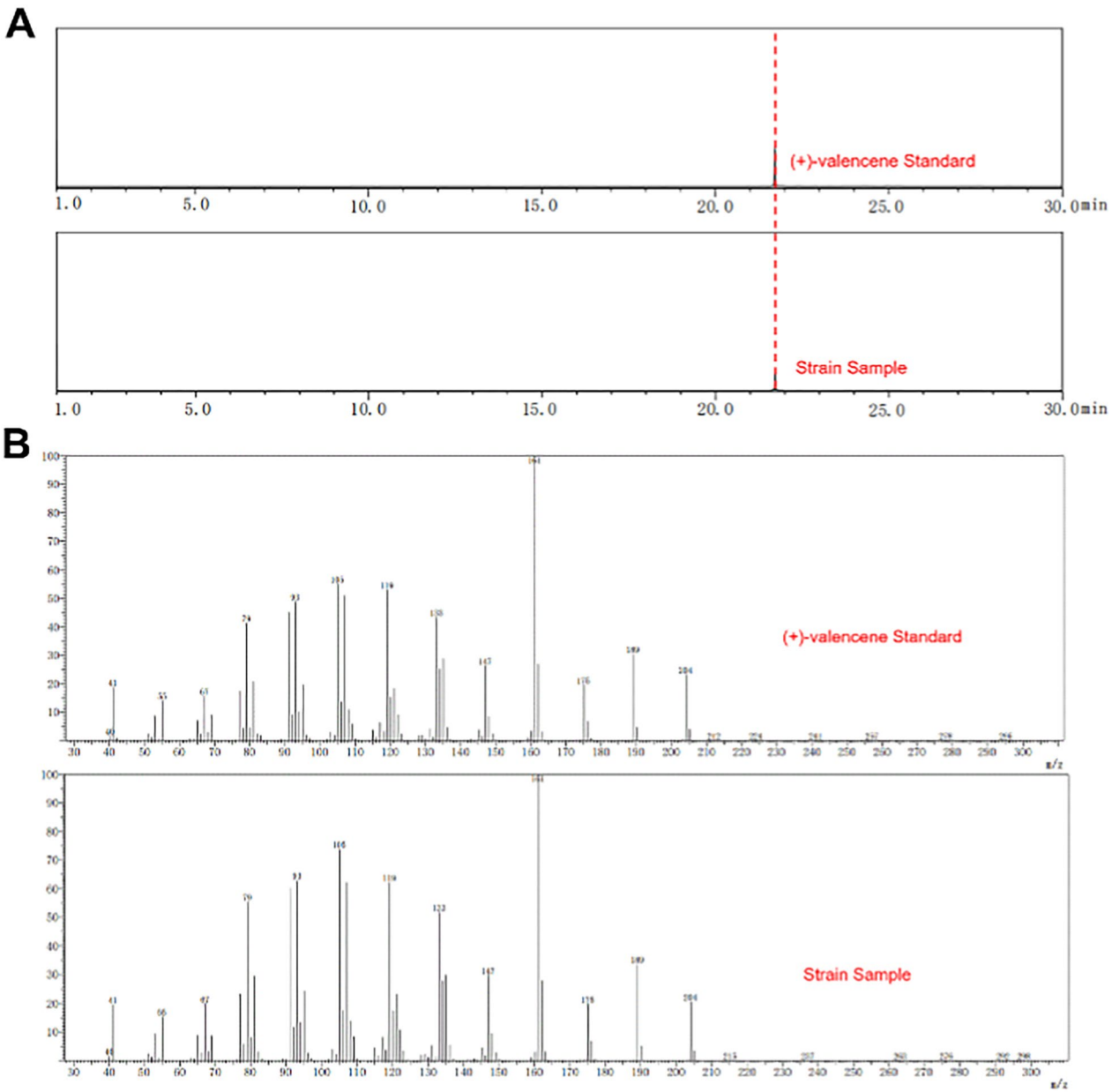

Fig. 2 GC–MS for detection of (+)-valencene standard and fermentation broths of *K. phaffii* expressing CaVAL. **A** Ion chromatography for detection of (+)-valencene standard and fermentation broths of K. phaffii expressing CaVAL. **B** By comparing with the MS fingerprint of the standard, the new compound was determined to be (+)-valencene

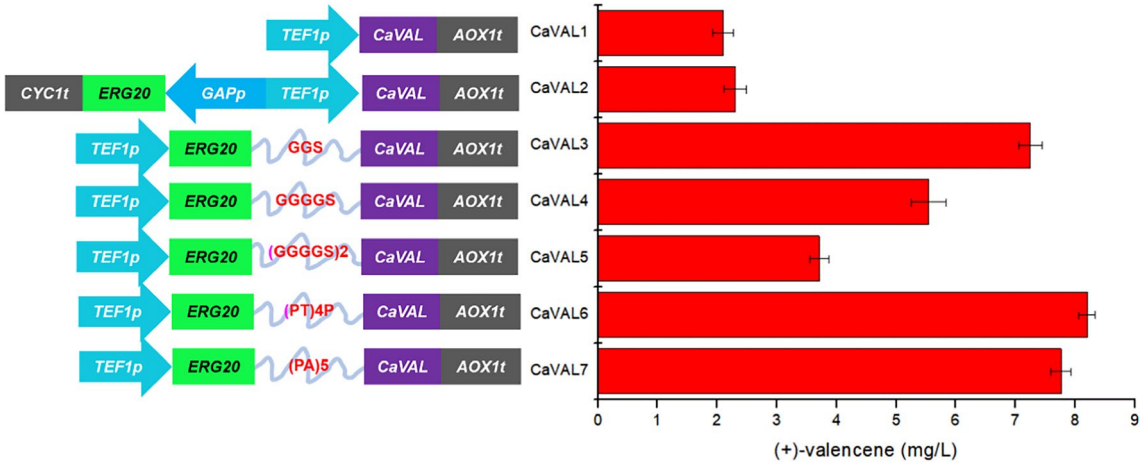

Fig. 3 Effect of fusion expression on the yield of (+)-valencene. Different linkers (GGS, GGGGS, (GGGGS)2 (PT)4P, (PA)5) were constructed to compare their effects on (+)-valencene production. The values of product were the average of three biological replicates

separately. This further illustrated that fusion expression can increase the yield of (+)-valencene.

Engineering the mevalonate pathway for (+)-valencene production

Farnesene pyrophosphate (FPP) is a common precursor of sesquiterpenes, and increasing its supply is critical to further improve the yield of downstream products [34]. We introduced the expression cassette containing *IDI1* into recombinant strain CaVAL6 to obtain recombinant strain CaVAL8. Overexpression of isopentenyl diphosphate isomerase gene *IDI1* resulted in a significant increase in (+)-valencene production (Fig. 4). *tHMG1* encodes the truncated 3-hydroxy-3-methylglutaryl-CoA reductase, and is another key enzyme in the MVA pathway which plays an important role in the biosynthetic pathway of (+)-valencene. By designing strain CaVAL8 to overexpress *tHMG1*, named CaVAL9, the yield of (+)-valencene increased to 16.2 mg/L (Fig. 4). In order to further strengthen the metabolic flux of the MAV pathway, expression cassettes containing *ERG12* and *ERG19* were sequentially integrated into the genome of the engineered strain CaVAL9 to obtain the strains CaVAL10 and CaVAL11, respectively (Fig. 4A). The yield of (+)-valencene in the engineered strain CAVAL11 reached 22.8 mg/L, which was 2.7 times of the control strain CAVAL6. This further suggested that enhanced supply of FPP could help to increase the yield of (+)-valencene.

Downgrading the competitive pathway to increase the yield of (+)-valencene

FPP is used as a substrate not only for the reaction to form (+)-valencene, but also for squalene. Therefore, we

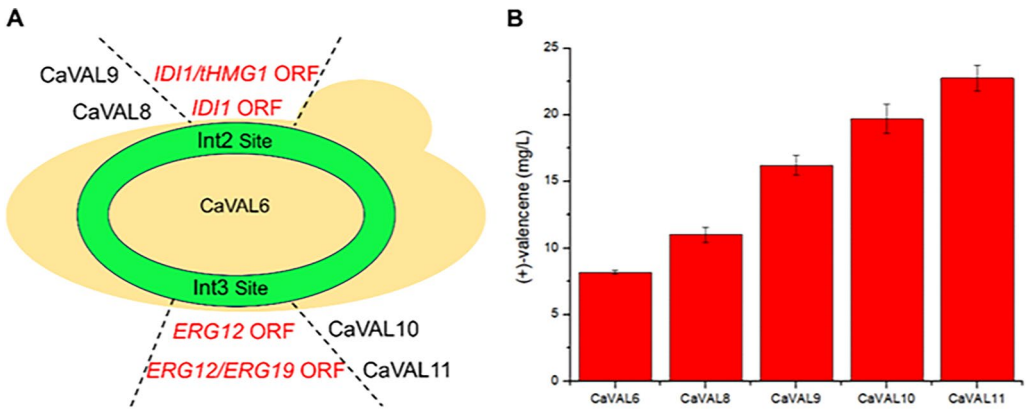

Fig. 4 Engineering the mevalonate pathway for (+)-valencene production. **A** Expression cassettes containing different genes (*IDI1*, *tHMG1*, *ERG12* and *ERG19)* were inserted into specific loci on the genome of *K. phaffii*. **B** (+)-valencene titer of strain CaVAL6 (with integrated IDI1, tHMG1, ERG12, and ERG19)

want to reduce FPP consumption by downgrading the competitive reaction [34]. ERG9 encodes squalene synthase, which can catalyze the conversion of substrate FPP into squalene. Squalene can generate ergosterol through multi-step reactions, and ergosterol is an important component of the cell membrane, so that's why this process cannot be completely blocked. We tried to truncate the promoter P_{ERG9} to regulate the expression of *ERG9*. As shown in the Fig. 5, we made three different designs for the endogenous promoter P_{ERG9}. The results showed that when the promoter P_{ERG9} was truncate by 100 bp (from 5' to 3' ends), the recombinant strain CaVAL13 obtained the highest yield of (+)-valencene (48.2 mg/L), and its yield was increased by 166% compared with the control strain CaVAL11.

The multi-copy strategy further increases the yield of (+)-valencene

In the biosynthesis process, the multi-copy strategy is an effective method to improve the yield of the target product. Based on results mentioned above (Fig. 3), we chose the expression frame ERG20-(PT)4A-CaVAL to perform a multi-copy study. Additional one copy was integrated into the strain CaVAL13 to obtain the engineered strain CaVAL15, and another one copy was integrated into CaVAL15 to obtain the strain CaVAL16. The recombinant strain CaVAL17 was obtained in the same way (Fig. 6). As shown in the Fig. 6, the yield of (+)-valencene was highest when the number of copies of the expression frame ERG20-(PT)4A-CaVAL reached four, and its yield was 173.6 mg/L. This proved again that the multi-copy strategy is an effective way to increase the yield of (+)-valencene.

Discussion

Here, we engineered *K. phaffii* to produce (+)-valencene with CaVAL-encoded (+)-valencene synthase introduction. It is a common application that *K. phaffii* is used for expression of various proteins [17, 18]. In the early stage, we developed an efficient gene editing tool in *K. phaffii* [31], which laid a technical foundation for its application in the biosynthesis of natural products. In this study, we tried to fuse ERG20 and CaVAL to accelerate the catalytic efficiency from the substrate GPP to (+)-valencene. The results showed that this method was very effective. Five different linkers were used to test, and the most effective linker for the yield of (+)-valencene was (PT)4P. In the future, we would try to fuse tHMG1 and ERG12 or ERG19 and IDI1, and these strategies might further increase the yield of (+)-valencene.

The flux of the mevalonate pathway affects the yield of the downstream product. Therefore, we overexpressed the key genes (*IDI1*, *tHMG1*, *ERG12*, *ERG19*) in the mevalonate pathway, and the results showed that high expression of these genes contributed to an increase in (+)-valencene production. Considering NADPH is the driving force for reduction in the biosynthesis of terpenes, we tried to increase the supply of NADPH to increase the production of (+)-valencene. However, the results showed that there was no use for the production of (+)-valencene (data not shown). This may be due to the fact that NADPH is not the key factor restricting the production of (+)-valencene in our work.

Endogenous pathways in microorganisms also compete with the synthesis pathways of target terpenoids. In the synthesis of (+)-valencene, FPP works as the direct precursor, and the competition of FPP in squalene synthesis is unfavorable for the production of our target

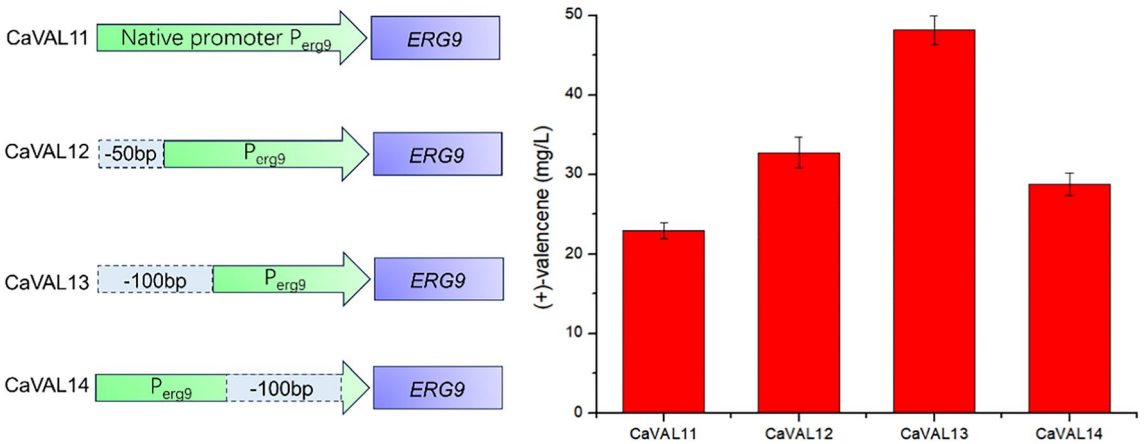

Fig. 5 Downgrading the competitive pathway to increase the yield of (+)-valencene. The ERG9 promoter was truncated to varying degrees, which were 50, 100 bp, from 5 to 3 ends, and 100 bp truncation was also designed, from 3 to 5 ends. The values of product were the average of three biological replicates

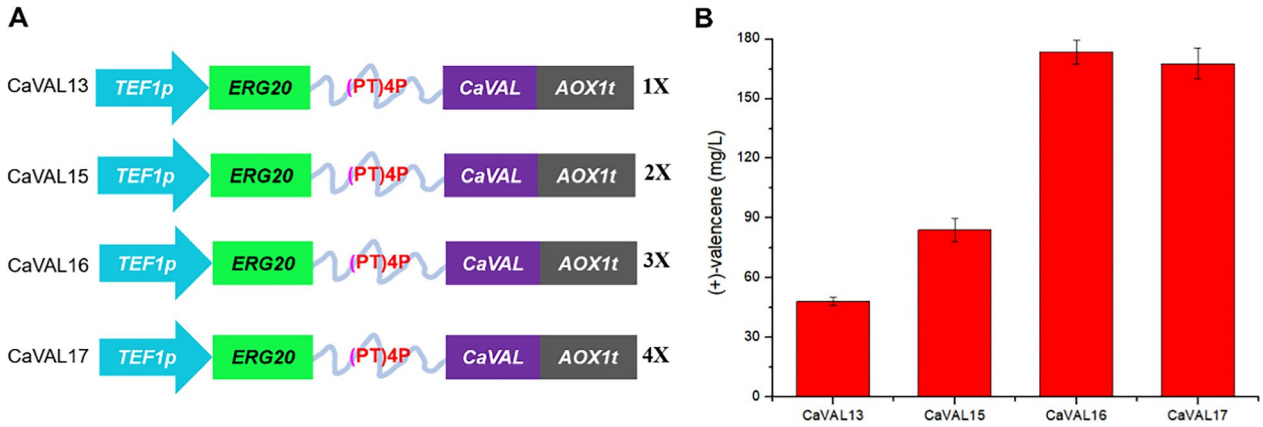

Fig. 6 The multi-copy strategy further increased the yield of (+)-valencene. **A** Expression cassette (ERG20-(PT)4-CaVAL) were inserted into specific loci on the genome of *K. phaffii*. Expression cassette was integrated into the genome of recombinant CaVAL13 at locus 4 (int4) to obtain recombinant CaVAL15. Expression cassette was integrated into the genome of recombinant CaVAL15 at locus 5 (int5) to obtain recombinant CaVAL16. Expression cassette was integrated into the genome of recombinant CaVAL16 at locus 6 (int6) to obtain recombinant CaVAL17. **B** (+)-Valencene titer of different strains. Two multi-copy, three multi-copy, four multi-copy recombinant strains were designed to compare the yield of (+)-valencene, respectively. The values of product were the average of three biological replicates

product. However, ergosterol, a downstream product of squalene, is an important part of membrane structure, and direct knockout of its squalene synthesis gene *ERG9* would impair cell growth. Expression of ERG9 is generally down-regulated by means of promoter replacement. In order to improve the availability of FPP in (+)-valencene biosynthesis, we tried to downregulate the expression of ERG9 using a promoter truncation strategy, which significantly increased the yield of (+)-valencene by 109%. On the other hand, increasing the copy number of genes is an effective strategy to optimize the expression level of exogenous genes for increasing the yield of the target product. The CRISPR/Cas9 tool was used to construct a stable strain CaVAL17 with four copies of ERG20-(PT)4A-CaVAL, and the yield of (+)-valencene was increased to 173.6 mg/L, which was 260% higher than that of the control strain CaVAL13. This work illustrated again that the systematic strategies such as protein fusion, key enzyme overexpression, and multi-copy integration are effective and general methods to improve product yield.

Wriessnegger et al. also used *K. phaffii* to construct a starting strain for the production of (+)-valencene, a minimally engineered *K. phaffii* strain yielded a valence approximately 25-fold higher than the starting value of our manuscript (+)-valencene [29]. There are significant differences in promoters (the inducible strong promoter *AOX1p* used is more active than the constitutive promoter *tef1p* we employed), copy numbers (Wriessnegger et al. screened multiple copy numbers, and we only had one copy), insertion sites (the insertion sites were inconsistent), and media and culture methods, and we believe that these differences may be responsible for the different final results. In addition, the *S. cerevisiae* cell factory was used to achieve efficient biosynthesis of (+)-valencene by Gao et al. [30]. Compared to them, we used *K. phaffii* to produce much lower yields of (+)-valencene. We also use some similar methods to increase the yield of (+)-valencene, but it is clear that Gao et al. have adopted more other strategy to increase the yield of (+)-valencene These include enhanced acetyl-CoA and NADPH supply, knock out of the gene *ROX1* encoding negative transcription factors for terpenoid biosynthesis, and a fed-batch fermentation strategy. These strategies have further increased the yield of (+)-valencene. This is worth learning from.

Conclusions

In this study, we successfully introduced the (+)-valencene synthase expression cassette to *K. phaffii* to obtain the engineered strain CaVAL1 by gene editing system CRISPR/Cas9, and then further increased the yield of (+)-valencene through a variety of synthetic biology strategies. These strategies included fusion expression, MVA pathway enhancement, competitive pathway inhibition, and multi-copy integration. In the end, the yield of (+)-valencene in the recombinant *K. phaffii* strain CaVAL16 reached 173.6 mg/L, which was 82 times higher than that of the starting strain CaVAL1. This work suggests that *K. phaffii* has a great potential for terpenoid biosynthesis as a cell factory.

Abbreviations

FPP	Farnesyl diphosphate
ERG20	Farnesyl diphosphate synthase

IDI1	Isopentenyl diphosphate isomerase 1
tHMG1	Truncated HMG-CoA reductase
ERG12	Mevalonate kinase
ERG19	Mevalonate pyrophosphate decarboxylase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02649-5.

Additional file 1.

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Author contributions
JC, CS and GY conceived the study. JC, CS and GY created its design and drafted the manuscript. JLC, DC, XL, CW, TL and XW performed the experiments. ZW and YJ analyzed data. All authors read and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animal performed by any of the authors.

Consent for publication
All authors have read and approved this manuscript to publish.

Competing interests
The authors declare no competing interests.

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