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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Tandem expression of *Ganoderma sinense* sesquiterpene synthase and IDI promotes the production of gleenol in *E. coli*

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Abstract

Ganoderma sinense, with more than 2000 years of medicinal history, is a fungus of the basidiomycetes that is rich in polysaccharides and terpenoids. However, the biosynthesis of terpenes, especially sesquiterpenes, has been little studied. The functional identification of sesquiterpene synthases from *G. sinense* is of great significance to the study of fungal terpenoid biosynthesis and regulation. Our research group has completed the functional characterization of 21 sesquiterpene synthase genes from *G. sinense*. It was found that gleenol, biosynthesis of which is catalyzed by the sesquiterpene synthase GsSTS26 and GsSTS27, has the functions of killing termites, antihelminth, and plant growth regulation. In the unmodified *E. coli* Rosetta (DE3) strain, the content of gleenol produced by sesquiterpene synthase from *G. sinense* is low, which makes it difficult to meet the demand of industrial production and the market. Therefore, it is of great significance to obtain high-yielding strains by means of synthetic biology. In this study, we constructed eight recombinant strains by using tandem gene expression and promoter engineering, and the content of gleenol was increased by up to 23-fold. In this study, we realized the de novo synthesis of gleenol in *E. coli* and provided a basis for the biosynthesis of terpenoids in basidiomycetes.

Key points

- *Eight recombinant expression systems were constructed by using tandem genes and promoter engineering.*
- *The recombinant strain promoted the efficient production of gleenol in E. coli Rosetta (DE3).*
- *The recombinant strain achieved de novo production of gleenol in E. coli.*

Keywords *G. sinense* · Sesquiterpene synthase · Tandem expression · Promoter engineering

Introduction

Lingzhi, a fungus of the basidiomycetes subphylum Aphyllum, has been used as a medicine for more than 2000 years (Geng et al. 2020). More recently, Lingzhi has been recognized as the best alternative source of natural bioactive ingredients. The food chemistry of Lingzhi reveals many active compounds, including polysaccharides, triterpenoids, nucleotides, amino acids, steroids, polyphenols, minerals, trace elements, and vitamins (Chaturvedi et al. 2018). According to statistics, Lingzhi is now widely cultivated in many countries in Asia, and the annual sales of Lingzhi products have exceeded 2.5 billion US dollars (Zhang et al. 2017a, b). The global food market demand for Lingzhi extract is estimated to reach USD 3.43 billion by 2024. The huge market demand for Lingzhi is due to the abundance of secondary metabolites with therapeutic and nutritional value. Approximately 400 bioactive compounds have been reported in different parts of Lingzhi (fruiting bodies, mycelium, and spores) (Ahmad et al. 2021). These products have potent pharmacological activity, such as antibacterial, antiviral, antitumor, anti-HIV-1, antioxidant, and cholesterol-lowering activity (Abate et al. 2020; Ahmad 2020; Bhat et al. 2019; Kang et al. 2015; Meng et al. 2019; Vallavan et al. 2020;

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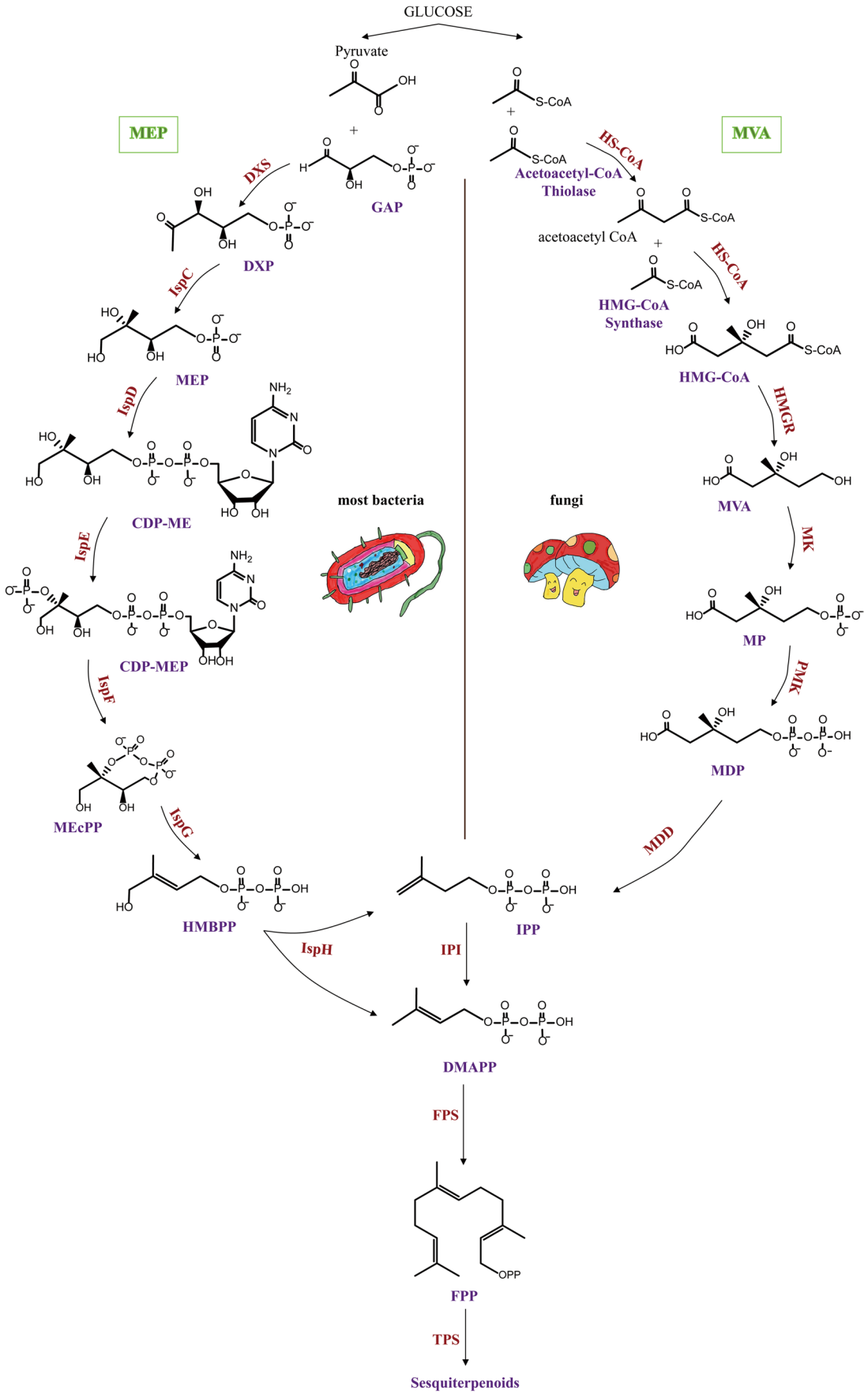

GLUCOSE
Pyruvate
GAP
MEP
DXS
DXP
IspC
MEP
IspD
CDP-ME
IspE
CDP-MEP
IspF
MEcPP
IspG
HMBPP
IspH
most bacteria
fungi
MVA
Acetoacetyl-CoA Thiolase
HS-CoA
acetoacetyl CoA
HMG-CoA Synthase
HMG-CoA
HMGR
MVA
MK
MP
PMK
MDP
MDD
IPP
IPI
DMAPP
FPS
FPP
OPP
TPS
Sesquiterpenoids

◂**Fig. 1** Sesquiterpenoids are a class of natural products whose biosynthesis occurs through different pathways: MEP (left) and MVA (right). The MVA pathway is found in higher, complex organisms, including fungi. Most bacteria, including *E. coli*, make use of the MEP pathway. Abbreviation: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; IspC(DXR), 1-deoxy-D-xylulose-5-phosphate reductoisomerase or 2-C-methyl-D-erythritol 4-phosphate synthase; IspD, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; IspE, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase; IspF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase; IspH, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPI, isopentenyl-diphosphate delta-isomerase; FPS, farnesyl diphosphatesynthase; HS-COA, Coenzyme A; HMGR, 3-hydroxy-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDD, mevalonate-5-diphosphate decarboxylase; GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate

Wang et al. 2019; Xu et al. 2011). In addition, Lingzhi has been developed due to its promising potential in the treatment of diseases such as diabetes, cancer, convulsions, hyperlipidemia and the immune system (Pan et al. 2013). Lingzhi extract has been transformed into various forms, including tea, powder, and dietary supplements, which are commercially available to treat different ailments (de Mattos-Shipley et al. 2016).

Terpenoids are the largest class of natural products in fungi (Quin et al. 2014). The terpenoids in Basidiomycetes include mainly sesquiterpenes, diterpenes, and triterpenoids, which have great research value and potential. *G. sinense* is a base species of Lingzhi, and terpenoids are the main active components of *G. sinense* (Baby et al. 2015). The functional identification of sesquiterpene synthase from *G. sinense* is of great significance for the biosynthesis and regulation of fungal sesquiterpene. In recent years, scientists have obtained the whole genome sequence of Lingzhi (Li et al. 2013), elucidating the synthetic mechanism of various secondary metabolites of the medicinal fungus. In the early stage of our research group, 21 sesquiterpene synthase genes were cloned from *G. sinense*. Among these genes, *GsSTS26* and *GsSTS27* can produce gleenol in *E. coli* Rosetta (DE3) with the effects of killing termites, repelling insects, and regulating plant growth. The compound has high application value and potential in agriculture. Gleenol is produced at low levels in *E. coli* Rosetta (DE3). Terpenoids are difficult to synthesize chemically due to their complex chemical structures and easily pollute the environment (Xiao and Zhong 2016). In addition, traditional extraction methods to obtain terpenoids from *G. sinense* often result in the loss of active ingredients and waste of resources (Liu et al. 2012). Therefore, the use of genetic engineering technology to obtain high-yielding strains of *G. sinense* sesquiterpene synthase is the focus of research.

There are two main biosynthetic pathways in nature: the mevalonate (MVA) pathway in fungi and the methylerythritol 4-phosphate (MEP) pathway in bacteria (Fig. 1). There are many key enzymes in the two biosynthetic pathways involved in the biosynthesis and regulation of sesquiterpene products. Isopentenyl diphosphate isomerase (IDI) is an important rate-limiting enzyme that exists in both the MVA pathway and the MEP pathway. IDI can catalyze the isomerization reaction of isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and convert IPP and DMAPP to farnesyl diphosphate (FPP) under the catalysis of FPPs. All sesquiterpenes are well known to be derived from one linear precursor, FPP (Quin et al. 2014). FPP is catalyzed by different terpenoid synthases (TPSs) to generate various sesquiterpenoids. At least 121 TPSs are reported to have been catalyzed by FPP (Klapschinski et al. 2016). IDI is a key engineering point to facilitate the conversion of metabolic fluxes to the recombinant MEP pathway (Zhou et al. 2017). In this study, to increase the production of gleenol in *E. coli* Rosetta (DE3), we constructed high-efficiency expression systems of GsSTS26 and GsSTS27 by using IDI and *G. sinense* sesquiterpene synthase tandem expression and promoter engineering and obtained recombinant strains that produced high-yield of gleenol, achieving the de novo synthesis of gleenol in *E. coli*. This study provides experimental data and lays the foundation for the biosynthesis of terpenoids in basidiomycetes.

Materials and methods

Strains, plasmids, and reagents

E. coli strain DH5α (Invitrogen, Waltham, MA, USA) and Rosetta (DE3) (Novagen, Madison, WI, USA) cells were used as the host for gene cloning and protein expression, respectively. The pET32a vector was used to express recombinant protein in Rosetta (DE3) cells. The pET32a-*GsSTS26* (GenBank accession number: MT584777.1) and pET32a-*GsSTS27* (GenBank accession number: OP094045) plasmids were constructed and preserved by our laboratory.

DNA sequences were synthesized by Azenta (Suzhou, China). A capillary column (HP-5 ms 30 m × 250 μm × 0.25 μm) was purchased from Agilent (Santa Clara, CA, USA). Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA). A TIANprep Mini Plasmid Kit was obtained from Tiangen Biotech (Beijing, China). A QIAquick Gel Extraction Kit was purchased from QIAGEN (Hilden, Germany). Pyrobest DNA Polymerase, DNA Ligation Kit Ver. 2.1, and T4

DNA Ligase were purchased from Takara (Beijing, China). SolarGelRed nucleic acid gel stain (10,000×) was purchased from Solarbio (Beijing, China). A Seamless Cloning Kit was purchased from Beyotime Biotechnology (Jiangsu, China).

Phylogenetic analysis and construction of the gene tandem expression system

The phylogenetic tree was built based on full amino acid sequences with the neighbor-joining method by the Molecular Evolutionary Genetics Analysis (MEGA) version 11. The tandem expression gene *IDI* (derived from *Ganoderma lucidum*, named *Gl IDI*, National Center for Biotechnology Information (NCBI) accession number: JX524564.1; derived from *E. coli*, named *E. coli IDI* NCBI accession number: AF119715.1) was fully synthesized by Azenta Company. The method of enzymatic cleavage and ligation was used for the tandem expression of *G. sinense* sesquiterpene synthase and IDI. When designing primers for the gene *GsSTS26* and *GsSTS27* fragments, an *Eco*RI cleavage site was introduced at the 3′ end of the IDI and the 5′ end of sesquiterpene synthase at the same time. The same sticky ends were generated during restriction endonuclease cleavage, and T4 ligase was used to connect the two gene fragments to form a gene tandem fragment. To ensure that the two genes can be coexpressed, the ribosome binding site (RBS, GAAGGA GATATACAT) was added between the two genes (Dao et al. 2022). The target gene was amplified and purified by polymerase chain reaction (PCR). The PCR amplification procedure was as follows: 94 °C for 3 min, 94 °C for 30 s and 30 cycles of 60 °C for 30 s, 72 °C for 90 s, 72 °C for 5 min and 4 °C indefinitely. The purified fragment was digested with restriction endonuclease *Eco*RI, and the tandem gene was ligated with *GsSTS26* and *GsSTS27* by T4 DNA Ligase to form tandem fragments. The *IDI-GsSTS26* tandem fragment and pET32a vector were cut with restriction enzymes *Nco*I and *Hin*dIII, respectively, and then ligated with DNA ligation. The *IDI-GsSTS27* tandem fragment was cleaved with the restriction enzymes *Bam*HI and *Hin*dIII. The tandem fragment was ligated to the pET32a vector using DNA ligation after purification. The ligation product was transformed into DH5α competent cells, and the solid plate with ampicillin resistance was incubated upside down at 37 °C for 16 h in a constant temperature incubator. Single colonies were picked for colony PCR. The plasmids were extracted according to the instructions of the TIANprep Mini Plasmid Kit. The recombinant plasmids were sequenced correctly and stored at − 20 °C.

Enhancement of biological elements

The pET32a expression vector contains the T7 promoter, and an exogenous Lac promoter was added to enable the expression of the two genes alone with the T7 promoter. The designed primers were added to the Lac promoter with a length of 78 bp. After amplification, the gene fragment was purified. The PCR procedure was as follows: 94 °C for 3 min, 94 °C for 30 s and 10 cycles from 65 °C for 30 s, 1 °C drop per cycle, 72 °C for 2 min, 94 °C for 30 s and 30 cycles of 55 °C for 30 s, 72 °C for 90 s, 72 °C for 5 min, and 4 °C forever. The recombination system was obtained by the seamless cloning technology of homologous recombination, and the specific operation was in accordance with the Seamless Cloning Kit. The reaction product was transformed into DH5α competent cells, and the plasmid was extracted.

Expression of the recombinant expression system in *E. coli*

The recombinant plasmid was transformed into *E. coli* Rosetta (DE3) competent cells and cultured at 37 °C for 16 h. A single colony was picked and inoculated into Luria–Bertani (LB) liquid medium with ampicillin resistance and cultured at 37 °C and 220 rpm for 16 h. The bacteria cultured overnight were transferred to fresh LB liquid medium at a 5% transfer volume, and the OD_{600} value of the cultured bacteria reached 0.8 at 37 °C. Isopropylthio-β-D-galactoside (IPTG) solution was added to induce protein expression at a final concentration of 0.5 mM, and the bacterial solution was cultured at 18 °C and 220 rpm for 24 h. Thirty milliliters of induced bacterial solution was centrifuged at 4 °C and 5000 rpm for 20 min. The bacterial pellet was washed twice with 1 × phosphate-buffered saline (PBS) buffer. Five milliliters of PBS was added to suspend the bacterial pellet, which was then disrupted ultrasonically to obtain soluble protein. Ultrasonication conditions were as follows: ice bath, power 30% (total power 950 W), on for 3 s, off for 2 s, and lasting for 10 min. A total of 300 μL of crude protein sample was centrifuged at 13,400 × *g* for 1 min and boiled for 15 min to denature and inactivate the protein.

Headspace solid phase microextraction–gas chromatography/mass spectrometry analysis

Headspace injection was performed automatically by MPS-2XT from GERSTEL (Linthicum Heights, MD, USA). Volatile components were extracted using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (50/30 μm; Supelco, Bellefonte, PA, USA). The specific procedure was as follows: incubate at 50 °C for 20 min, extract for 15 min, decompose at 250 °C for 5 min, and age the fiber for 3 min before extraction and after desorption to eliminate cross-contamination between different samples.

The GC/MS system (Agilent 7890B-7000D) was equipped with a capillary column (HP-5 ms 30 m × 250 μm × 0.25 μm) with splitless injection (solid-phase microextraction (SPME)

desorption) at an injection temperature of 250 °C. The helium flow rate was set to 1 mL/min. The heating program is as follows: hold at 60 °C for 2 min, then raise to 250 °C at a rate of 6 °C/min, and hold at 250 °C for 3 min; the total running time is 36.67 min. The solvent was allowed to delay for 2 min before acquiring mass spectral data. Mass spectrometer conditions are as follows: electron ionization source, ion source temperature 230 °C, mass selective detector (MSD) operation at 70 eV, and scanning range of volatile compounds 30–500 m/*z*.

Statistical analysis of the relative content of gleenol in recombinant strains

All recombinant expression plasmids were transformed into *E. coli* Rosetta (DE3) recombinant strains and incubated for 24 h at 18 °C and 220 rpm to produce secondary metabolites. Thirty milliliters of bacterial solution was centrifuged at 5000 rpm for 20 min at 4 °C. Eight milliliters of centrifuged supernatant was transferred to a 20-mL SPME Flask (GERSTEL), and 1 μL of β-caryophyllene dilution was added to each vial as an internal standard (10,000-fold dilution with methanol) (Yang and Nie 2016). This sample was the HS-SPME-GC/MS sample of *E. coli* in vivo culture. The volatile organic compounds (VOCs) were then identified using computer searches on the National Institute of Standards and Technology (NIST) Mass Spectral Library Search Chromatogram (Babiy et al. 2021; He et al. 2021).

Results

Bioinformatics analysis of GsSTS26 and GsSTS27

As a traditional Chinese medicinal fungus, *G. sinense* has completed genome sequencing and annotation work (Zhu et al. 2015), which provides a research basis for the analysis of *G. sinense* sesquiterpene synthase. Our research group has completed the cloning and characterization of 21 sesquiterpene synthase genes of *G. sinense* (unpublished data). The sesquiterpene synthase GsSTS26 was found to be able to produce the sesquiterpene gleenol with good biological activity. Gleenol has termiticidal, anthelmintic, and plant growth–regulating effects. In addition, GsSTS27, which is the closest relative to GsSTS26, also has the same function. The phylogenetic tree of the sesquiterpene synthase of *G. sinense* was drawn using the Molecular Evolutionary Genetics Analysis (MEGA) version 11. Analysis of

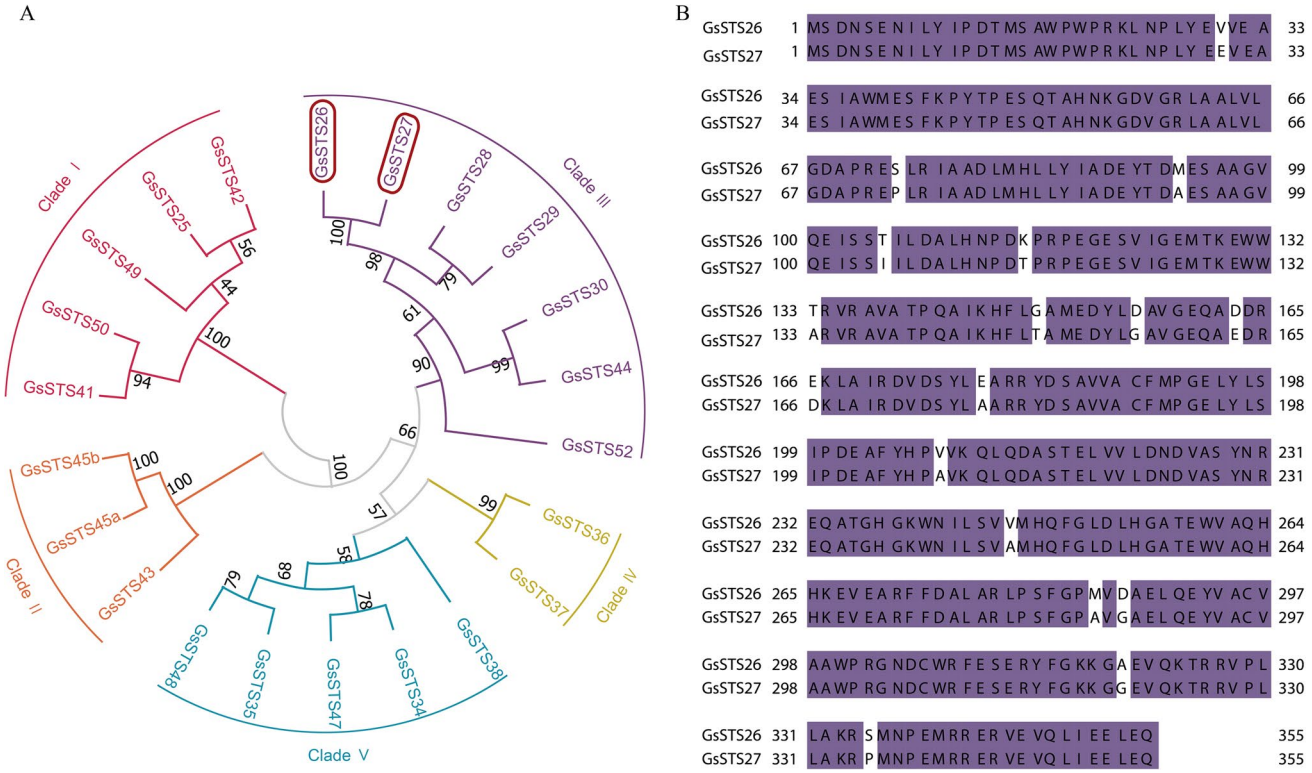
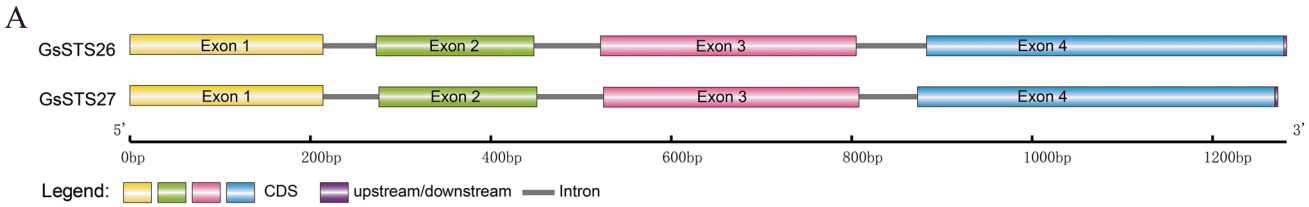
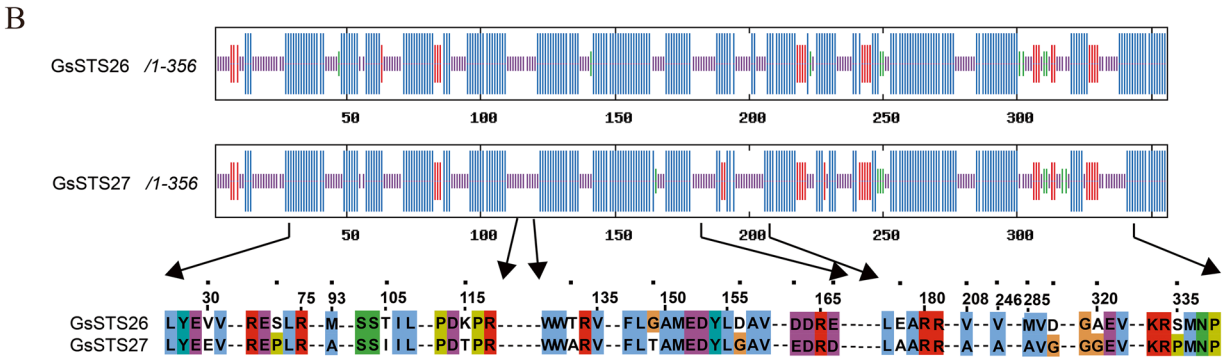
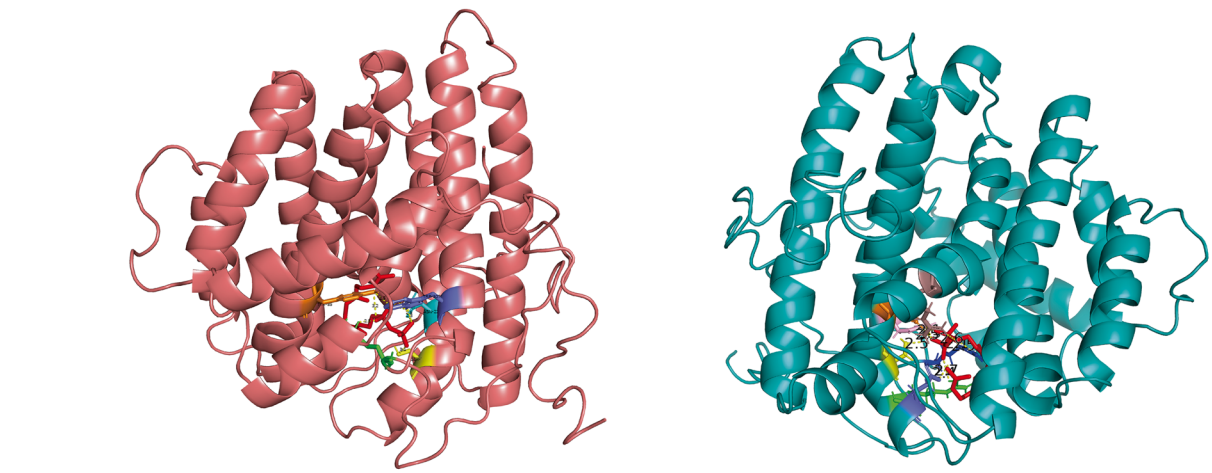
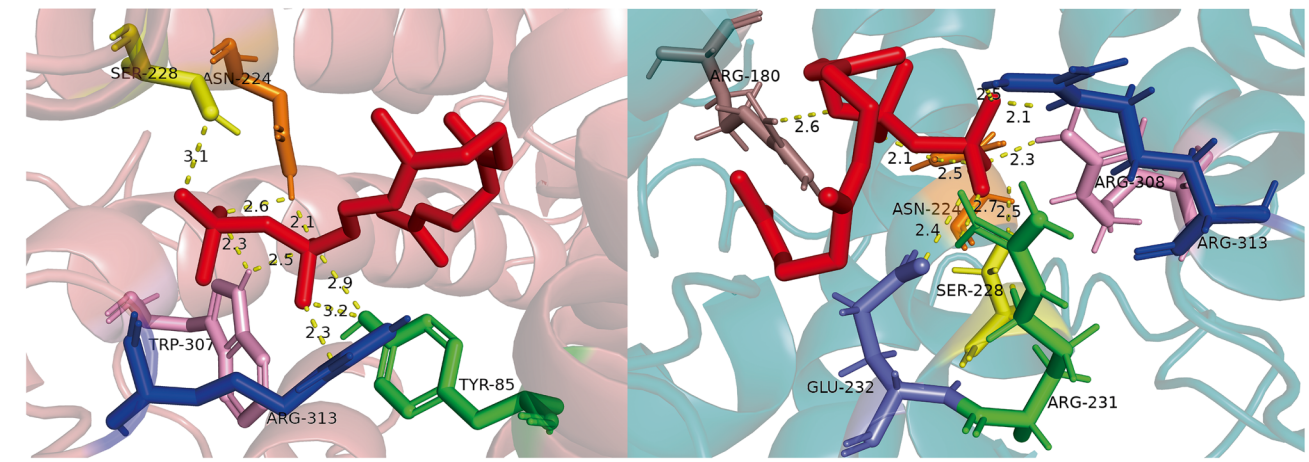


Fig. 2 Phylogenetic tree of sesquiterpene synthases from *G. sinense* and alignment of the protein sequence between GsSTS26 and GsSTS27. **A** The 21 sesquiterpene synthase phylogenetic trees obtained by cloning were drawn, and cluster analysis showed that GsSTS26 and GsSTS27 belonged to the same clade. **B** The protein sequence alignment of GsSTS26 and GsSTS27 showed that the two exhibited ≥ 75% identity


A
GsSTS26
Exon 1
Exon 2
Exon 3
Exon 4
GsSTS27
Exon 1
Exon 2
Exon 3
Exon 4
5'
3'
0bp
200bp
400bp
600bp
800bp
1000bp
1200bp
Legend:
CDS
upstream/downstream
Intron


B
GsSTS26 /1-356
GsSTS27 /1-356
GsSTS26 LYEVV--RESLR--M--SSTIL--PDKPR-----VWTRV--FLGAMEDYLDAV--DDRE-----LEARR--V--V--MVD--GAEV--KRSMNP
GsSTS27 LYEEV--REPLR--A--SSIIL--PDTPR-----VWARV--FLTAMEDYLGAV--EDRD-----LAARR--A--A--AVG--GGEV--KRPMNP


C


D
SER-228
ASN-224
TRP-307
ARG-313
TYR-85
ARG-180
ASN-224
SER-228
ARG-308
ARG-313
GLU-232
ARG-231

◂**Fig. 3** Analysis of gene structure and protein tertiary structure prediction. **A** Gene structure of *GsSTS26* and *GsSTS27*. **B** Secondary structure prediction and sequence alignment of *GsSTS26* and *GsSTS27*. **C** *GsSTS26* (left) and *GsSTS27* (right) bind the ligand FPP. **D** Binding sites of *GsSTS26* (left) and *GsSTS27* (right) to the ligand FPP

the phylogenetic tree showed that GsSTS27 and GsSTS26 exist in the same branch of the phylogenetic tree (Fig. 2A). The protein sequence alignment analysis showed that the two genes showed ≥ 75% identity, possibly having the same activity (Fig. 2B). Therefore, in this experiment, the *G. sinense* sesquiterpene synthases GsSTS26 and GsSTS27 were selected for further research.

GsSTS26 and *GsSTS27* gene structures were drawn online using GSDS 2.0 (Fig. 3A). The gene structures of *GsSTS26* and *GsSTS27* are very similar; they contain the same number of exons and introns, and both open reading frames are 1071 bp, encoding 356 amino acids. The secondary structures of *GsSTS26* and *GsSTS27* were predicted online by SOPMA and PSIPRED, and their structures were analyzed (Fig. 3B). The α-helix structure accounted for the largest proportion in the secondary structure of *GsSTS26*, with a total of 209 amino acid residues, accounting for 58.71%. Twenty-four amino acid residues are β-sheet structures, accounting for 6.74%. A total of 114 amino acid residues are random coil structures, accounting for 32.02%. Nine amino acid residues are β-turn structures, accounting for 2.53%. In the secondary structure of GsSTS27, 196 amino acid residues are α-helix structures, accounting for 55.06%. Twenty-eight amino acid residues are β-sheet structures, accounting for 7.87%. A total of 124 amino acid residues are random coil structures, accounting for 34.83%. Eight amino acid residues are β-turn structures, accounting for 2.25% (Table S1).

The tertiary structure was predicted online using I-TASSER and phyer2, the obtained protein was further evaluated online using SAVES, and the evaluation parameters were calculated (Table S2).

The protein model was optimized using Chiron. Molecular docking was performed with AutoDock Vina software, and the proteins and their complexes were visualized and analyzed with PyMOL software. Finally, the binding sites of the complex ligand FPP to GsSTS26 and GsSTS27 were predicted (Fig. 3C, D). See Supplementary Table S3 for websites used for bioinformatics analysis.

Construction of recombinant plasmids

In this experiment, the *IDI* gene cloned from *G. lucidum* and the *IDI* gene cloned from *E. coli* were selected as tandem genes. Gene tandem expression vectors were constructed with the sesquiterpene synthases GsSTS26 and GsSTS27 of *G. sinense* (Fig. 4). The recombinant plasmid was transformed into *E. coli* Rosetta (DE3) to obtain a recombinant strain. The strains and plasmids used in this study are shown in Table 1. The plasmid was correctly sequenced by GENEWIZ, indicating that the recombinant plasmid was successfully constructed.

To further enhance the expression of the sesquiterpene synthase gene of *G. sinense*, we added the Lac promoter to the above recombinant plasmid so that the T7 promoter and Lac promoter could activate the expression of *IDI* and *GsSTS26* and *GsSTS27*, respectively (Fig. 4). The length of the Lac promoter is 78 bp. The promoter sequence was directly added to the *GsSTS26* and *GsSTS27* forward primers, and a homologous sequence was added simultaneously. Gene fragments were amplified using a touchdown PCR program. The recombinant plasmid was cleaved with the restriction enzymes *BamHI* and *NcoI* to produce two bands of the expected size. The plasmid was correctly sequenced by GENEWIZ. The experimental results showed that the recombinant plasmid was successfully constructed.

Expression of recombinant protein in the *E. coli* Rosetta (DE3) strain

The pET32a vector carrying the Trx protein can be expressed in tandem with foreign genes (Liu et al. 2020). Using pET32a as a blank control and R101 (pET32a-*GsSTS26*) and R201 (pET32a-*GsSTS27*) as negative controls, the expression of recombinant proteins in *E. coli* Rosetta (DE3) was different. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) electropherogram showed that, among the recombinant proteins of the T7 promoter, the content of the recombinant protein of strain R103 was significantly higher than the content of the recombinant protein of R102. Although *GsSTS26* and *GsSTS27* have high homology and very similar gene structures, the results of tandem expression with *IDI* are different. *Gl IDI* and *E. coli IDI* connected with *GsSTS27* did not increase the target protein content (Supplementary Fig. S1A), possibly due to the weak expression of *IDI-GsSTS27*. To enhance the expression of the target gene, we adopted promoter engineering and added the Lac promoter between the tandem genes. Among the recombinant proteins supplemented with the Lac promoter, although the expression level of the recombinant protein of strain R105 was not as high as the expression level of the recombinant protein of R103, the expression level was also significantly higher than the expression level of the recombinant protein of R101. In contrast, the recombinant protein expression of strain R205 was significantly higher than the recombinant protein expression of R201 (Supplementary Fig. S1B), indicating that the Lac promoter has a certain strengthening effect on the expression of the *G1 IDI-GsSTS27* tandem gene.

Identification of GsSTS26 and GsSTS27 sesquiterpene products

The recombinant plasmids were introduced into *E. coli* Rosetta (DE3) to obtain recombinant strains R101-R105

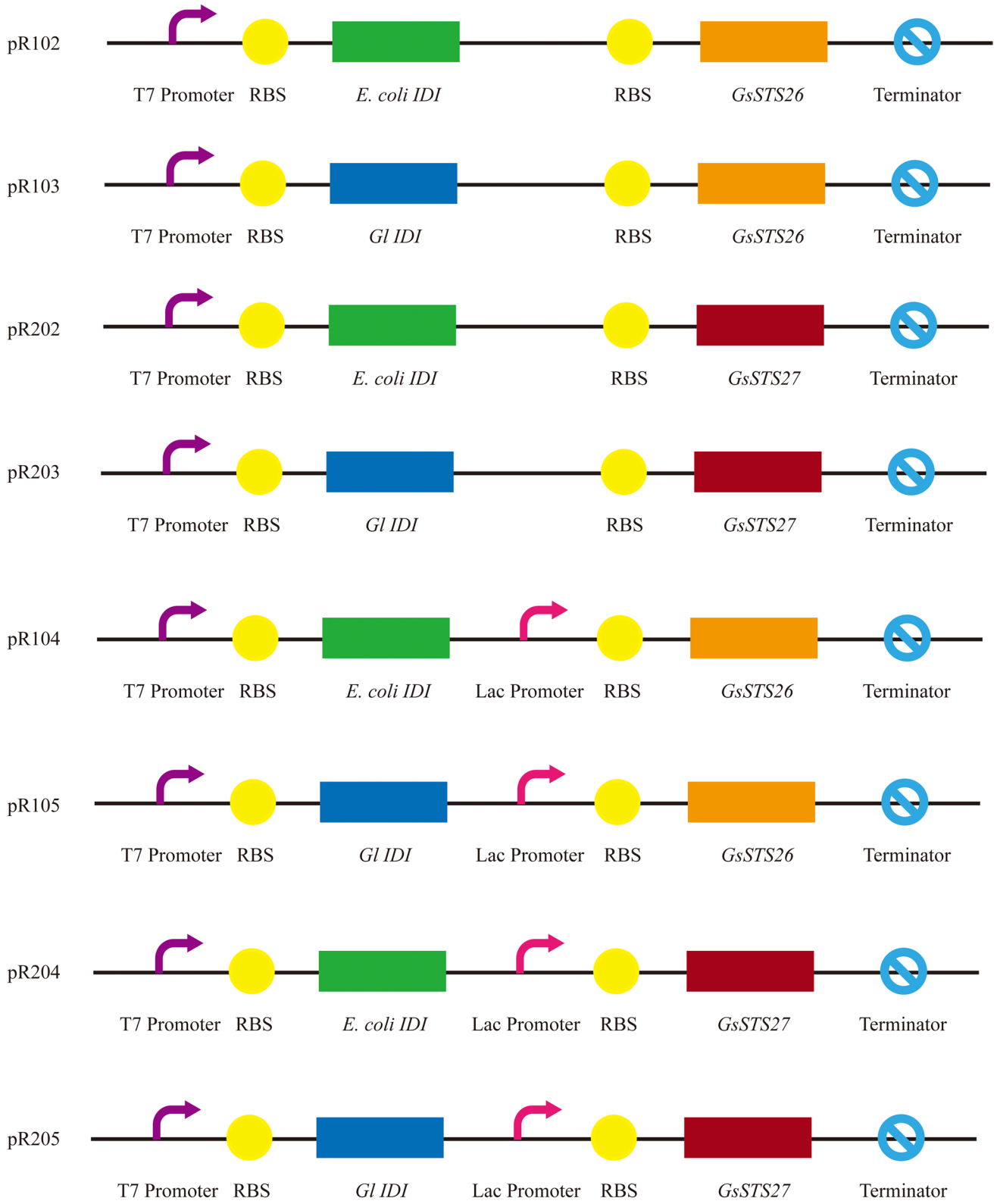


Fig. 4 Schematic diagram of construction of eight recombinant plasmids of *Escherichia coli* producing gleenol

Table 1 Strains and plasmids used in this study

Name	Characteristics	Source
Strains		
Rosetta (DE3)	F^{-}*ompT hsdS*$_B$($r_B^{-}m_B^{-}$) *gal dcm* (DE3) pRARE^2 (Cam^R)	Novagen
DH5α	supE44△*lacU169*(φ80lacZ△M15)	Invitrogen
R101	Rosetta (DE3) *GsSTS26*	Laboratory preservation
R102	R101 with *E. coli IDI*	This study
R103	R102 with *Gl IDI*	This study
R201	Rosetta (DE3) *GsSTS27*	Laboratory preservation
R202	R201 with *E. coli IDI*	This study
R203	R201 with *Gl IDI*	This study
R104	R102 with *Lac*	This study
R105	R103 with *Lac*	This study
R204	R202 with *Lac*	This study
R205	R203 with *Lac*	This study
Plasmids		
pET32a	High-level expression vector fused to Trx•Tag thioredoxin; Amp^R	Laboratory preservation
pR101	pET32a with *GsSTS26*	Laboratory preservation
pR102	pET32a with *GsSTS26* and *E. coli IDI*	This study
pR103	pET32a with *GsSTS26* and *Gl IDI*	This study
pR201	pET32a with *GsSTS27*	Laboratory preservation
pR202	pET32a with *GsSTS27* and *E. coli IDI*	This study
pR203	pET32a with *GsSTS27* and *Gl IDI*	This study
pR104	pET32a with *E. coli IDI* and *Lac-GsSTS26*	This study
pR105	pET32a with *Gl IDI* and *Lac-GsSTS26*	This study
pR204	pET32a with *E. coli IDI* and *Lac-GsSTS27*	This study
pR205	pET32a with *Gl IDI* and *Lac-GsSTS27*	This study

and R201-R205. HS-SPME-GC/MS analysis was performed on the in vivo cultures of *E. coli* of each strain, and C_8–C_{20} was used as a mixed standard compound for GC/MS analysis (Supplementary Fig. S2). Comparing the mass spectrometry data of the volatile products obtained in the experiment with the mass spectrometry data of the standard compounds in National Institute of Standards and Technology (NIST) MS Search 2.3 (Table 2, Supplementary Fig. S3), GsSTS26 and GsSTS27 were found to be able to produce three sesquiterpene products in *E. coli*. The main product is gleenol, and the byproducts are di-epi-1,10-cubenol and Ƭ-muurolol. Compared with the mass spectra of gleenol in the published literature, the types and abundance of ion fragments are consistent (Blay et al. 2005). Using pET32a as a blank control, Origin 2018 was used to draw the GC/MS peak map of the product (Fig. 5). Notably, the two sesquiterpene synthases produce three identical sesquiterpene compounds.

HS-SPME-GC/MS analysis of recombinant plasmid *E. coli*in vivo culture

We performed HS-SPME-GC/MS detection on the *E. coli* in vivo culture of the above recombinant strains (see Supplementary Fig. S4 and Supplementary Fig. S5 for the GC/MS analysis of the recombinant expression strains), and the internal standard method was used to calculate the relative content changes of gleenol. HS-SPME-GC/MS data were

Table 2 Constituents of sesquiterpenes volatile compounds of *G. sinense*

Compound	Database RI	Calculate RI	CAS number	References
Gleenol	1586	1593	72,203–99-7	(Nakazaki et al. 2007)
Di-epi-1,10-cubenol	1636	1638	73,365–77-2	(Takao et al. 2012)
Ƭ-Muurolol	1660	1652	19,912–62-0	(Ding et al. 2009)

The calculated RI value and the NIST database RI value that differ by no more than 10 can be identified as the same compound

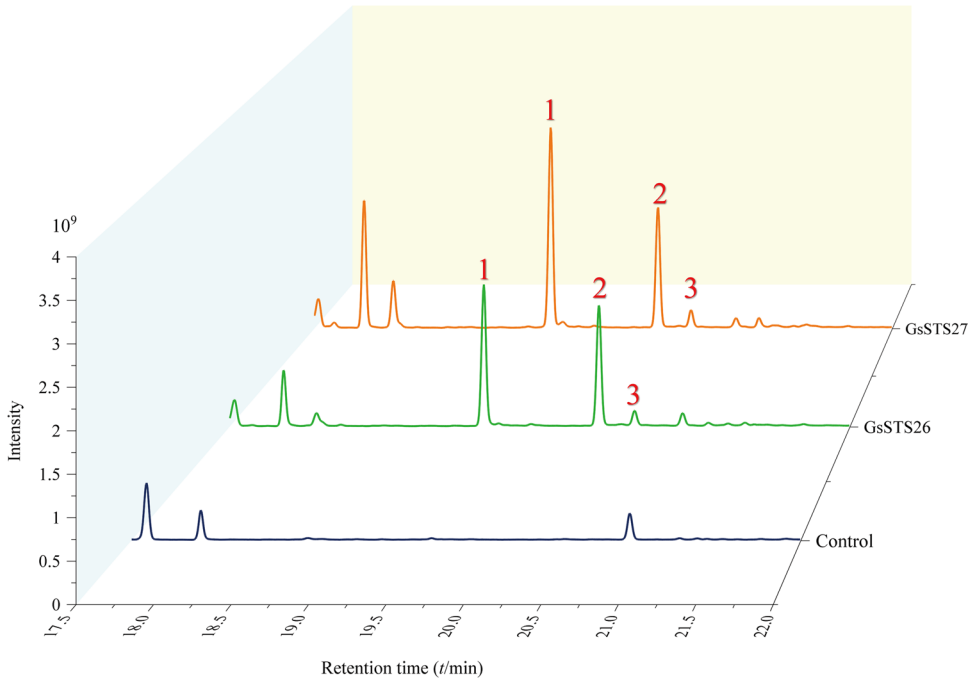


Fig. 5 GC/MS analysis of and GsSTS27 *E. coli* cultures in vivo. Compound 1 is gleenol, compound 2 is di-epi-1,10-cubenol, and compound 3 is Ƭ-muurolol

subjected to one-way analysis of variance (ANOVA). The data results showed that there was a statistically significant difference between the treated samples and the untreated control, with a threshold of $p < 0.01$. In strains R102 and R103, gleenol production concurrently showed a 16-fold and 23-fold increase compared to the control strain, implying that IDI is important for an increase in the pathway flux. However, in strains R202 and R203, gleenol production showed an inhibition compared to the control strain (Fig. 6A, Table 3).

Regarding the strains with the Lac promoter, gleenol production from strains R104 and R105 was 13-fold and 12-fold higher than the gleenol production of the control strain. Notably, strains R104 and R105 with the Lac promoter produced less gleenol than the T7 promoter. This result indicated that the addition of the Lac promoter could not further improve the ability of GsSTS26 to produce gleenol. In contrast, the relative content of gleenol in strains R204 and R205 was increased by approximately 2.7 times and 2.9 times, respectively (Fig. 6B, Table 3), but the production was not as good as the production in R104 and R105. In addition, the relative content of the two byproducts di-epi-1,10-cubenol and Ƭ-muurolol showed the same trend as gleenol. The experimental results showed that the best strain of sesquiterpene synthase in *E. coli* Rosetta (DE3) to produce gleenol was R105, and the best recombinant expression system was pR105 (pET32a-*Gl IDI-GsSTS26*).

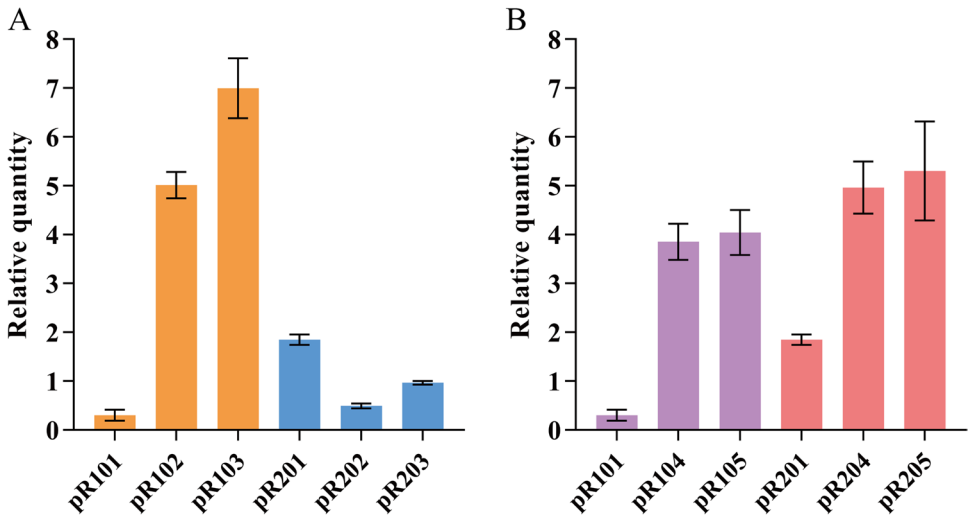


Fig. 6 Relative amounts of gleenol produced by recombinant strains in *E. coli* in vivo cultures. **A** The relative content of recombinant plasmids with the T7 promoter promoting the expression of tandem genes in *E. coli* culture. **B** Relative content of recombinant plasmids with the Lac promoter in *E. coli* cultures

Table 3 Relative content of gleenol produced by recombinant strains and relative increase compared to control strain

Strains	Relative content[a]	Relative increase[b]
R101	0.30 (±0.013)	0.00
R102	5.02 (±0.072)	16.62
R103	6.97 (±0.379)	23.19
R104	3.91 (±0.076)	12.95
R105	4.04 (±0.210)	13.41
R201	1.85 (±0.011)	0.00
R202	0.49 (±0.002)	0.26
R203	0.97 (±0.001)	0.52
R204	4.96 (±0.285)	2.68
R205	5.30 (±1.027)	2.86

[a]The relative content of gleenol was measured in triplicate (±variance)

[b]The relative increase is calculated as the amount of gleenol produced in the recombinant strain divided by the amount of gleenol produced in the control strain containing only the gleenol producing

Discussion

Drug discovery of traditional Chinese medicine (TCM) is usually the extraction of active components from natural products with high biological activity and function from TCM libraries, but this strategy cannot fully utilize the huge resources of TCM (Zheng et al. 2020). For example, *G. sinense*, as a traditional medicinal resource with a long history in ancient China, is considered to be a functional food and medicine that can promote human health (Chen et al. 2020). The comprehensive study on the biosynthesis of active components of *G. sinense* provides new insight for the development of new compounds and large-scale industrial production. Isoprenoids are the most functional and structurally diverse natural products from which many drugs and chemicals are derived. The MEP pathway is an efficient way to produce isoprenoids. In this pathway, overexpression of key enzymes can increase the production of terpenoids (Xue et al. 2015). Promoter engineering is one of the common ways to enhance gene expression. When a promoter promotes the expression of two genes, there is often a problem that the expression level of the second gene is lower than the expression level of the first gene next to the promoter. The production of complexes formed by the expression of two genes initiated by independent promoters has been reported to be higher than the production of complexes formed by a single promoter (Kim et al. 2004). In prokaryotic expression vectors, commonly used strong promoters are the T7, Lac, Tac, trp, and IPL promoters. According to the mode of action, these promoters are divided into inducible promoters and constitutive promoters (Ward et al. 1999). A typical inducible promoter is the lactose operon-Lac promoter from *E. coli*, which forms a complex with a repressor protein to induce transcription under the induction of IPTG (Murakami et al. 2021).

The *G. sinense* sesquiterpene synthases GsSTS26 and GsSTS27 can produce three identical sesquiterpene compounds in *E. coli* Rosetta (DE3). Three compounds have been reported to exhibit significant biological activity. The main product, gleenol, plays an important role in killing termites, repelling insects and regulating the growth of plant seeds (Nakazaki et al. 2007). The byproduct di-epi-1,10-cubenol has a strong antitrichophyton effect (Takao et al. 2012), and Ƭ-muurolol also has a good effect on termite inhibition (Ding et al. 2009). The three compounds are of great agricultural value and were all discovered for the first time in Basidiomycetes. In this experiment, eight recombinant expression systems of *G. sinense* sesquiterpene synthase were successfully constructed by using gene tandem expression and promoter engineering (Fig. 4). Experiments show that the gene tandem expression strategy has more advantages than promoter engineering, and the content of gleenol in the recombinant strain pET32a-*G1 IDI-GsSTS26* is increased by approximately 23 times. The relative content of gleenol products in GsSTS27 was significantly higher than the relative content of Gleenol in GsSTS26 by HS-SPME-GC/MS analysis (Fig. 6), possibly because GsSTS27 has more ligand FPP binding sites than GsSTS26. After the tandem expression of IDI and GsSTS27, the content of gleenol decreased, speculating that feedback inhibition may have occurred. Therefore, the development and utilization of more efficient heterologous expression systems (Zhang et al. 2022) and the discovery of efficient microbial chassis strains (Banerjee et al. 2021; Lynch et al. 2022) are the focus of future research. In addition, due to the antibacterial activity of the product, the efflux of the product may be the focus of future research (Zhang and Too 2020). In this experiment, we successfully obtained a recombinant strain of *G. sinense* sesquiterpene synthase in *E. coli* Rosetta (DE3) with high production of gleenol, which solved the problem of low production of gleenol and realized the de novo synthesis of gleenol in *E. coli*. In this experiment, we successfully obtained a recombinant strain of *G. sinense* sesquiterpene synthase expressing gleenol in *E. coli* Rosetta (DE3). The gene tandem expression strategy and promoter engineering increased the content of gleenol by up to 23-fold and realized the de novo synthesis of gleenol in *E. coli*.

Recent genomic data and bioinformatic analyses have shown that fungi possess a large number of biosynthetic gene clusters for biologically active natural products, but more than 90% are silent. Basidiomycetes are highly productive producers of structurally diverse and biologically active natural products (Masuya et al. 2019). However, despite the great potential of Basidiomycota in natural product discovery, it is a largely unexplored area in drug discovery compared to ascomycetes. Since 2010, heterologous expression of fungal metabolites has been recognized as one of the powerful methods to synthesize natural products (Oikawa 2020). *E. coli* and yeast are currently the most widely used heterologous expression systems, and the development and application of more efficient heterologous expression hosts is also an important

task in synthetic biology. Terpenoids are the largest family of natural products in Basidiomycetes, and it has become a core task to obtain strains with high terpenoid production. With the rapid development of genetic engineering, metabolic engineering, and protein engineering, the use of microbial chassis cells to synthesize terpenoids heterologously has achieved good results (Zhang et al. 2017a). Analyzing the mechanism and regulatory network of terpenoid biosynthesis in Basidiomycetes (Gao et al. 2018) and further analyzing their biosynthetic pathways are the main ways to study Basidiomycetes deeply. The synthetic pathway of terpenoids can be transformed or even reconstructed by means of synthetic biology (Ma et al. 2022). Through the optimization of natural and artificial pathways and the application of the gene editing technology clustered regularly interspersed short palindromic repeats (CRISPR) (Liu et al. 2022), precise regulation of gene expression can be achieved at the transcriptional and translational levels, and the ability of microorganisms to synthesize natural products can be effectively improved. Using genetic engineering technology to develop new technologies under the coregulation of multiple strategies can effectively improve the ability of microorganisms to synthesize terpenoids and provide a powerful means for realizing large-scale industrial production. In short, synthetic biology research on Basidiomycetes is still in its infancy, and we need continuous innovation through modern biological technology to make breakthroughs.

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Author contribution WQ and WLZ conceived the study and designed the experiments; WQ mainly completed molecular experiment, and QPY mainly completed GC-MS experiment and analysed the data; ZCS and ZYN conducted the additional experiments; WQ wrote the manuscript; YHS provided the instruments and performed on GC-MSs.

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Data availability statement The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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