CHAPTER FIFTEEN

Identification of Enzymes Involved in Sesterterpene Biosynthesis in Marine Fungi

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Contents

Abstract

Diverse and bioactive terpene compounds are produced by marine fungi. The biosynthesis of sesterterpene molecules containing a C25 skeleton is catalyzed by chimeric enzymes that carry out both chain elongation and terpene cyclization reactions. These bifunctional characteristics facilitate using different chain length polyisoprenoid

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diphosphates as precursors to yield the geranylfarnesyl diphosphate intermediate, which is then converted to a sesterterpene in one step. In this chapter, we describe the identification of sesterterpene synthase enzymes, together with other related enzymes such as diterpene and farnesyl diphosphate synthases, in a single fungal strain. The processes are based on genome sequencing, in silico analysis of terpene synthase, in vivo gene (cluster) deletion and complementation, and in vitro protein function verification, together with the methods of detecting terpenes using high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS).

1. INTRODUCTION

Sesterterpenes are terpene molecules containing a C25 skeleton, which are rare among terpene compounds (Wang, Yang, Lin, Zhou, & Liu, 2013). Many of them are reported from marine fungi, especially those from mangroves, which include neomangicols A–C and mangicols A–G from the Bahamas mangrove fungus *Fusarium* sp. (Renner, Jensen, & Fenical, 1998; Renner, Jensen, & Fenical, 2000), asperterpenoid A from a south China mangrove endophytic fungus *Aspergillus* sp. (Huang et al., 2013), ophiobolin O produced by *Aspergillus ustus* 094102 from south China mangrove rhizosphere (Yang et al., 2012), and ophiobolins G and K also from marine fungi (Tian, Deng, & Hong, 2017) (Fig. 1).

The biosynthesis of terpenes results from the condensation of five-carbon isoprene building blocks of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). Prenyltransferases (PTs) catalyze chain elongation from these building blocks to yield polyisoprenoid diphosphates such as geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), geranylgeranyl diphosphate (GGPP, C20), geranylfarnesyl diphosphate (GFPP, C25), and hexaprenyl diphosphate (HexPP, C30). Terpene synthases (TS) (or terpene cyclases (TC)) catalyze the cyclization or rearrangement of these polyisoprenoid diphosphates to yield monoterpenes, sesquiterpenes, diterpenes, sesterterpenes and triterpenes, respectively (Christianson, 2007; Quin, Flynn, & Schmidt-Dannert, 2014). Terpene diversity is facilitated by TS (or TC), the catalysis mechanisms of which are very complex (Gao, Honzatko, & Peters, 2012). In the past decade, a new type of bifunctional TC attracted attention of scientists. They possess two catalytically independent domains of PT and TC, and carry out both chain elongation and terpene cyclization reactions. The reported enzymes catalyzed biosynthesis of diterpenes (Minami et al., 2009; Qin et al., 2016; Toyomasu et al., 2007) and sesterterpenes (Chai et al., 2016; Chiba, Minami, Gomi, & Oikawa, 2013; Matsuda, Mitsuhashi, Quan, &

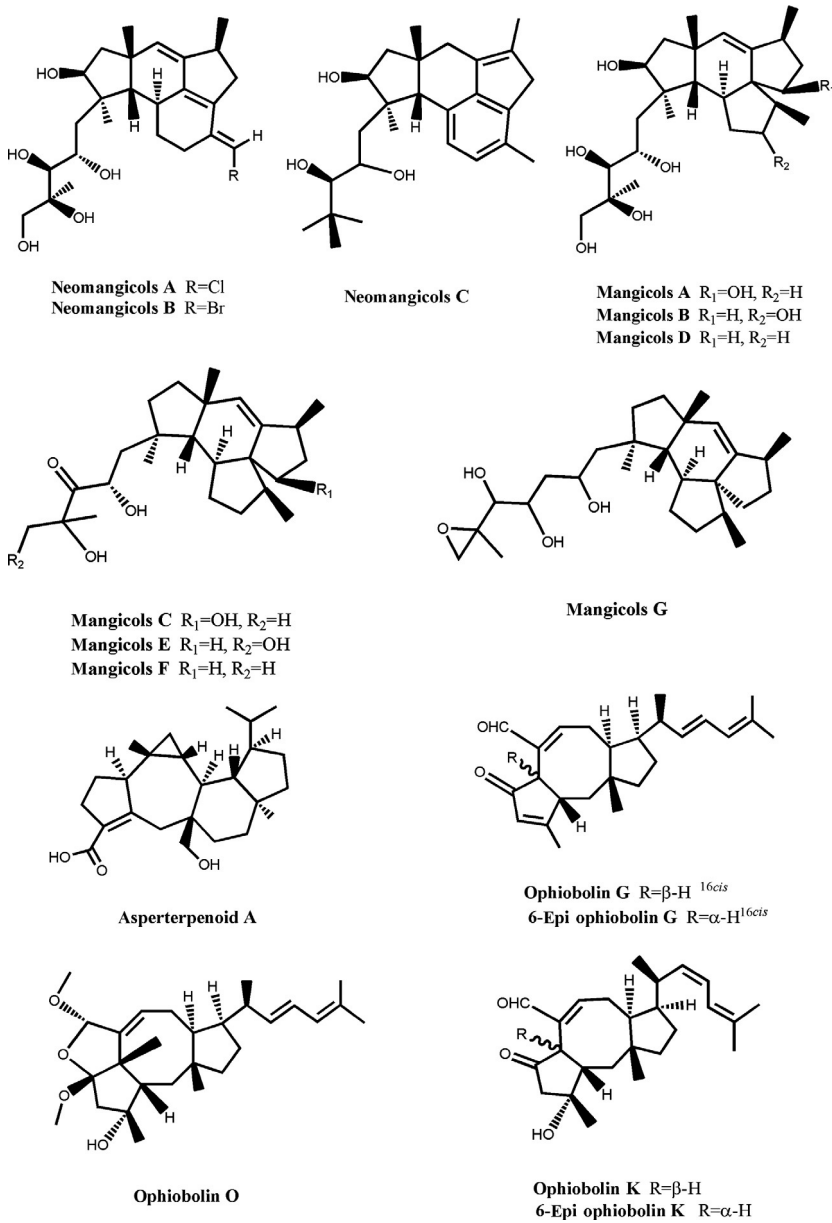

Fig. 1 Sesterterpenes produced by marine fungi.

Abe, 2015; Mitsuhashi, Rinkel, Okada, Abe, & Dickschat, 2017; Ye et al., 2015).

In filamentous fungi, genes coding for the enzymes that catalyze secondary metabolites (SM) synthesis, together with those coding for specific

regulatory functions and resistance proteins, are usually contiguously aligned in the genome (Hoffmeister & Keller, 2007; Keller, Turner, & Bennett, 2005; Quin et al., 2014). Biosynthetic gene clusters responsible for the production of terpenes can be rapidly identified by genome sequencing and bioinformatics analysis (Quin et al., 2014). Using the program BLASTP or Profile Hidden Markov models, conserved protein sequences, which are a characteristic set of universally conserved functional domains typical of all terpene synthases, can be used to mine and identify terpene synthases. The two universally conserved domains are the aspartate-rich DDXX(D/E) motif and the NSE/DTE triad, (N/D)DXX(S/T)XX(K/R)(D/E) (Cane & Ikeda, 2012). The true biochemical function of a presumed terpene synthase can then be affirmed by experimentation. The approaches are (i) in vivo gene (gene cluster) knockout and complementation (Chai et al., 2016; Gao et al., 2011); (ii) incubation of the expressed recombinant protein with acyclic, allylic diphosphate substrates and identification of the resultant terpene hydrocarbon or alcohol (Chai et al., 2016); and (iii) in vivo expression in heterologous hosts, i.e., *Escherichia coli* (Minami et al., 2009), or other fungi such as *Aspergillus oryzae* (Chiba et al., 2013; Qin et al., 2016; Ye et al., 2015), that can support the production of terpene metabolites.

C25 sesterterpene synthases were discovered only in recent years. Ophiobolin F synthase (AcOS) was found by accident during genome mining for diterpene synthase from *Aspergillus clavatus* (Chiba et al., 2013). Four other sesterterpene synthases were reported thereafter by genome mining, including NfSS for sesterfisherol biosynthesis from *Neosartorya fischeri* (Ye et al., 2015), EvSS for stellatic acid biosynthesis from *Emericella variecolor* (Matsuda et al., 2015), PbSS of sesterbrasiliatriene synthase from *Penicillium brasilianum*, and PvPS of preasperterpenoid A synthase from *Penicillium verruculosum* synthase (Mitsuhashi et al., 2017).

A. ustus 094102 was found to produce sesterterpene ophiobolin and three other kinds of terpenoids, including the sesquiterpenoids drimane, isochromane, and sterols and dipeptides (Lu et al., 2009; Yang et al., 2012). The biosynthesis of sesterterpene ophiobolins was found involving five gene clusters related to C15, C20, C25, and C30 terpenoid biosynthesis (Chai et al., 2016). In this chapter, we describe how to identify sesterterpene biosynthesis enzymes, together with other enzymes such as diterpene and FPP synthases. The processes are based on our former work (Chai et al., 2016) with updated information on genome sequencing, in silico analysis of terpene synthesis, in vivo gene (cluster) deletion and complementation, and in vitro protein function verification, together with the methods of how to detect terpenes using HPLC and GC-MS methods.

2. GENOME SEQUENCING AND BIOINFORMATICS ANALYSIS OF TERPENE-RELATED GENES IN *A. USTUS* 094102

Nowadays whole-genome sequence is becoming more and more important to investigate the biosynthesis pathways of SM (Cane & Ikeda, 2012; Chiba et al., 2013; Matsuda et al., 2015; Ye et al., 2015). After preparing high quality genomic DNA (gDNA) and submitting for sequencing, complete genome information can be readily obtained by gene annotation and assembly.

2.1 Preparation of gDNA

The methods of fungal gDNA extraction include the cetyltrimethylammonium bromide (CTAB) method (Lipp et al., 1999; Zhang, Yang, Castlebury, & Cerniglia, 1996), sodium dodecyl sulfate (SDS) (Goldenberger, Perschil, Ritzler, & Altwegg, 1995), alkaline guanidine thiocyanate boiling (Sandhu, Kline, Stockman, & Roberts, 1995), Triton X-100 lysis (Sinzger, Knapp, Schmidt, Kahl, & Jahn, 1999), microwave (Orsini & Romano-Spica, 2001), and commercial kit methods (Wang, Teng, Tian, Guan, & Wang, 2012).

The gDNA of *A. ustus* 094102 was extracted via the improved CTAB method. The resulting gDNA (Fig. 2) for whole-genome sequencing was submitted to the Beijing Genomics Institution (BGI) in Shenzhen, China.

2.1.1 Buffers and Reagents

- Sodium chloride–EDTA–Tris (SET) buffer: 75 m*M* NaCl, 25 m*M* EDTA (pH 8.0), 20 m*M* Tris–HCl (pH 7.5).
- Liquid nitrogen.

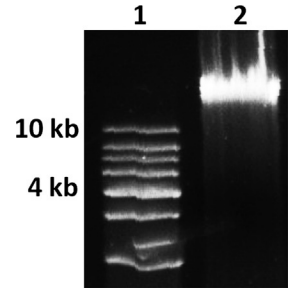

Fig. 2 The genomic DNA of *A. ustus* 094102 with 1.0% agarose gel electrophoresis. Lane 1: 1 kb plus DNA ladder; lane 2: genomic DNA extracted with CTAB method.

- 2% CTAB: 1 *M* Tris–HCl (pH 8.0) 50 mL; 0.5 *M* EDTA (pH 8.0) 20 mL; NaCl 40.908 g; CTAB 10 g; add ddH_2O to 500 mL, store in brown flask, autoclave, add 10 mL beta-mercaptoethanol (β-ME) before use.
- Phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1), isopropanol, 70% ethyl alcohol.
- 1 kb DNA ladder (Fermentas).
- RNase A (Thermo Scientific, 10 mg/mL).
- Tris–EDTA (TE) buffer: 25 m*M* EDTA (pH 8.0); 25 m*M* Tris–HCl (pH 7.5).
- 3 *M* NaAc (pH 5.2): dissolve 40.8 g $CH_3COONa{\cdot}3H_2O$ in 50 mL ddH_2O, adjust pH to 5.2 with acetic acid, add ddH_2O to 100 mL, autoclave.

All materials are used for extraction of gDNA of *A. ustus* 094102.

2.1.2 Procedure

1. Inoculate fresh mycelium in 100 mL fungal II medium (mannitol 20 g; yeast extract 3 g; $MgSO_4{\cdot}7H_2O$ 0.3 g; sodium glutamate 10 g; KH_2PO_4 0.5 g; maltose 20 g; corn steep liquor 1 g; glucose 10 g; add ddH_2O to 1 L, and adjust pH to 6.5.) at 28°C, 220 rpm for 2–3 days.
2. Collect the mycelium at 18,000 × *g* for 15 min, and wash it with sterile ddH_2O, then resuspend with SET buffer (the volume is to cover the mycelium). Sop up the water with a filter paper and store at −40°C.
3. Grind 20 mg mycelium with liquid nitrogen, add 400 μL 2% CTAB, 65°C for 30 min.
4. Add the same volume of phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1), shake well to make the protein fully denatured for 30 min. Centrifuge at 13,000 × *g* for 10 min.
5. Remove the supernatant, add RNase to 20 μg/mL, keep at 37°C for 30 min.
6. Repeat steps 3–5 for 1–2 times.
7. Add two volumes of precooled isopropanol, shake softly, and then separate filamentous DNA out.
8. Remove filamentous DNA, wash twice with 70% ethyl alcohol, and then dry.
9. Add TE buffer to dissolve DNA.
10. Use agarose gel electrophoresis to detect the gDNA with 1% agarose.
11. Store the gDNA at −40°C.

2.1.3 Notes

1. Preheat CTAB to 65°C before use to avoid CTAB precipitation.
2. Sop up the water before grinding and precool the mortar and pestle. Keep cool while grinding to avoid DNase renaturation and grind the mycelium to a powder.
3. Remove the gDNA with cut tips softly to avoid gDNA breakage.
4. Dissolve the gDNA with RNA-free water and add about 0.8 μL 10 mg/mL RNase.

2.2 In Silico Analysis for Terpene Synthesis Genes

Submit *A. ustus* 094102 gDNA for whole-genome sequencing to BGI in Shenzhen, China. Genome assembly can be carried using the short oligonucleotide alignment program denovo 1.05 (Li et al., 2010). The genome size of strain 094102 is 40.09 Mb, assembled as 301 contigs and 174 scaffolds (Table 1).

Software and databases for gene prediction and annotation are generally used as following: gene prediction by defining putative open reading frames (ORFs) with GeneMark-ES 2.3e (Ter–Hovhannisyan, Lomsadze, Chernoff, & Borodovsky, 2008); protein-encoding gene annotation through BLASTP searches against the SwissProt, GO, COG, KEGG, and NR databases. Two bioinformatics tools, SMURF (Khaldi et al., 2010) and antiSMASH (Weber et al., 2015), have been developed based on the common features of SM biosynthetic gene clusters and are most frequently used to predict genes and gene clusters involved in biosynthesis of SM.

Based on bioinformatics analysis, 13,982 protein-coding genes from *A. ustus* 094102 were predicted by BLASTP searches against the SwissProt, GO, COG, KEGG, and NR databases. Eleven terpene gene clusters were identified using antiSMASH analysis. Further manually scanning key words of "terpene synthesis" or "terpene cyclase," using the typical conserved protein sequences aspartate-rich DDXX (D/E) motif and the NSE/DTE triad, (N/D)DXX(S/T)XX(K/R)(D/E), resulted in 27 putative terpene synthesis genes which classified to four groups, including sesquiterpene, diterpene, triterpene synthases, and a "function unknown" group of hypothetical proteins (Table 2).

2.2.1 Software and Database

- GeneMark-ES 2.3e for Gene prediction.
- SwissProt (2012-04), GO (release: 1.419), COG (release: 20090331), KEGG (release: 59), and NR (2012-04) databases for protein-encoding genes annotation.

Table 1 *Aspergillus ustus* 094102 Genome Assembly and Main Feature

Statistic	Value
Coverage	>68 ×
Genome assembly size (Mb)	40.09
Number of scaffolds	174
Scaffold N50 (Kb)	1814
Number of contigs	301
Contig N50 (kb)	675
CDS total length (Mb)	20.44 (50.98% of genome)
CDS average size (bp)	1461.62
Predicted protein-coding genes (Number)	13,982
Predicted protein-coding genes, total length (Mb)	23.19
Predicted protein-coding genes, mean length (kb)	1658.33
Exon total length (Mb)	20.44
Exon total number	43,400
Exon average length (bp)	470.88
Intron total length (Mb)	2.75
Intron total number	29,418
Intron average length (bp)	93.50
GC content (%)	51.22

- GenBank.
- MEGA (version 5.0) for amino acid phylogenetic analysis.
- BioEdit 7.0 for multiple sequence alignments.

2.2.2 Procedure

1. Determine putative ORFs with GeneMark-ES 2.3e.
2. Annotate protein-coding genes through BLASTP searches against the databases of SwissProt (2012-04), GO (release: 1.419), COG (release: 20090331), KEGG (release: 59), and NR (2012-04)
3. Search manually terpene synthesis genes using the key words of "terpenoid synthase" or "terpenoid cyclase" from the local genome database. Then check the typical conserved protein sequences

Table 2 BLASTP of the Presumed 27 Terpene Synthases in *A. ustus* 094102 Genome (done in Oct, 2012)

Presumed AUPTS Type	AUPTS ID (Au)	AA No.	Intron No.	RTS	Max Score	Query Coverage (%)	Max Identity (%)
Di AUPTS	6061	343	0	|BAF45925.1| fusicoccadiene synthase [*Phomopsis amygdali*]	207	97	37
	6241	744	7		288	89	29
	7240	762	8		258	94	28
	8003	725	3		517	100	39
	11189	710	5		292	94	30
	13192	708	8		425	98	36
	13606	755	7		307	85	31
	13676	717	9		294	85	30
Unknown AUPTS	606	387	1	|EAA65430.1| hypothetical protein AN0654.2 [*Aspergillus nidulans* FGSC A4]	696	98	86
	7669	362	2	gb|EAA59165.1| hypothetical protein AN8143.2 [*Aspergillus nidulans* FGSC A4]	593	100	83
	11565	692	7	|EAA64518.1| hypothetical protein AN2407.2 [*Aspergillus nidulans* FGSC A4]	759	99	53
	13624	689	7	|EAU32979.1| conserved hypothetical protein [*Aspergillus terreus* NIH2624]	1036	100	72
	329	336	2	|CAK48316.1| unnamed protein product [*Aspergillus niger*]	529	99	74
	3446	456	1	|EAA63201.1| hypothetical protein AN2767.2 [*Aspergillus nidulans* FGSC A4]	832	98	90
	6064	404	3	|CCD54927.1| BcSTC5, similar to sesquiterpene cyclase [*Botryotinia fuckeliana*]	38.1	18	31
	6298	345	1	|EAA59634.1| hypothetical protein AN8012.2 [*Aspergillus nidulans* FGSC A4]	643	99	88

Note: AUPTS: *Aspergillus ustus* presumed terpene synthases; Prefix "Au" for all the ID of the AUPTS.

aspartate-rich DDXX(D/E) motif and the NSE/DTE triad, (N/D)DXX(S/T)XX(K/R)(D/E).
4. Confirm the search result using BLASTX analysis against the GenBank database.
5. Use the BioEdit 7.0 software to perform multiple sequence alignments.
6. Use MEGA (version 5.0) to construct the phylogenetic analysis-based amino acid sequence.

2.2.3 Notes

1. The updated software and database are recommended for further use, as now the above corresponding software and database have been updated to SwissProt (2012-06), GO (release: 1.419), COG (release: 20090331), KEGG (release: 76), NR (20160219), and MEGA (version 7.0)

3. IN VIVO IDENTIFICATION OF GENE (CLUSTERS) INVOLVED IN SESTERTERPENE OPHIOBOLIN BIOSYNTHESIS

Up to now, the tools for in vivo identifying gene function in filamentous fungi include overexpressing (Leger, Joshi, Bidochka, & Roberts, 1996), transposon arrayed gene knockouts (TAGKO) (Hamer et al., 2001), T-DNA tagging (Combier, Melayah, Raffier, Gay, & Marmeisse, 2003), gene trapping (Aichinger et al., 2003), transposon tagging (Firon, Villalba, Beffa, & D'Enfert, 2003), RNA interference (RNAi) (Nakayashiki, 2005), and yeast one/two hybrid (Duyvesteijn et al., 2005). Gene editing, including knockout and complementation experiments, is one of the most direct and effective ways to study gene function. It is based on the principle of DNA homologous recombination to attenuate or terminate the expression of related genes and determine the gene function according to the change of product yield. In addition to random integration, gene targeting based on homologous recombination, RNAi technologies, and emerging gene-editing technologies such as Zinc-finger nucleases (Wang et al., 2013), transcription activator-like effector nucleases (TALENs) (Ménoret et al., 2014), and clustered regulatory interspaced short palindromic repeat/associated systems (CRISPR/Cas) technology can accomplish gene knockout or gene replacement in vivo (Liu, Chen, Jiang, Zhou, & Zou, 2015; Nielsen et al., 2017; Wang et al., 2017).

Generally, the in vivo gene deletion and complementation for fungi is based on the following procedures: protoplast preparation, gene-targeting

cassette construction, transformation, and transformants identification. In this section, we describe the in vivo gene (cluster) deletion and complementation of terpene synthases in the marine *Aspergillus* strain 094102.

3.1 Preparation of Fungal Protoplast

The principle of protoplast preparation may be found in the manual described in *Fungal Biology* (Berg & Maruthachalam, 2015), in which the fungal growth phase and conditions, the type of enzyme combinations, the osmotic stabilizer, the incubation time and temperature for cell wall lysing are included.

3.1.1 Equipment

- Microscope (Axio Scope. A1, Zeiss), hemocytometer (10 mm × 10 mm).
- High-speed freezing centrifuge (Centrifuge 5810R, Eppendorf).

3.1.2 Buffers and Reagents

- Glucose minimum medium (GMM): glucose 5 g; 20× nitrate salts 25 mL; trace elements 500 μL; agar 20 g; in 500 mL dH_2O, at pH 6.5, to obtain a large amount of fungal spores.
- Liquid minimal medium (LMM): GMM without agar, for spore germination.
- 20× Nitrate salts: $NaNO_3$ 60 g; KCl 5.2 g; $MgSO_4{\cdot}7H_2O$ 5.2 g; KH_2PO_4 15.2 g; add ddH_2O to 500 mL, for GMM preparation.
- Trace elements: $ZnSO_4{\cdot}7H_2O$ 2.2 g; H_3BO_3 1.1 g; $MnCl_2{\cdot}4H_2O$ 0.5 g; $FeSO_4{\cdot}7H_2O$ 0.16 g; $CoCl{\cdot}5H_2O$ 0.16 g; $CuSO_4{\cdot}5H_2O$ 0.16 g; $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$ 0.11 g; Na_4EDTA 5 g; add ddH_2O to 100 mL, for GMM preparation.
- Mycelium wash: $MgSO_4$ 73.95 g; add ddH_2O to 500 mL, for washing spores.
- Trapping buffer: sorbitol 54.65 g; 1 *M* Tris–Cl (pH 7.0) 50 mL, pH 7.0; add ddH_2O to 500 mL, for protoplast preparation.
- Osmotic medium: $MgSO_4$ (147.9 g) and 2 *M* NaPB (2.5 mL), in 1 L dH_2O; the pH was adjusted to 5.8 with 1 *M* Na_2HPO_4, for protoplast preparation.
- Driselase from *Basidiomycetes* sp., purchased from Sigma.
- Sorbitol/Tris/$CaCl_2$ (STC) buffer: Sorbitol 109.3 g; $CaCl_2$ 0.235 g; 1 *M* Tris–Cl (pH 7.5) 5 mL; add ddH_2O to 500 mL, for protoplast transformation.

3.1.3 Procedure

1. Grow spores of *A. ustus* 094102 on at least five GMM plates for 3 days at 28°C.
2. Collect spores with 10–15 mL ddH_2O containing 0.1% Tween-80.
3. Determine spore concentration with a hemocytometer under microscope.
4. Inoculate 10^9 *A. ustus* 094102 spores into 100 mL sterile LMM and shake at 28°C and 200 rpm for 10–11 h. Check the young germlings under the microscope (Fig. 3A).
5. Harvest the germlings in 50-mL tube at 8000 × *g* and 4°C for 15 min, decant the supernatant, and resuspend the cultures in 50 mL mycelium wash buffer. Centrifuge at 8000 × *g* and 4°C for 15 min and decant the supernatant.
6. Transfer the aggregates of germlings to a sterile 50-mL Erlenmeyer flask containing 10 mL osmotic medium with 1% drislase. Shake at 80 rpm and 28°C for 3.5–4 h (Fig. 3B). Observe the formation of protoplast under the microscope at intervals of 0.5 h.
7. Transfer the protoplast into a new 50-mL tube and carefully add 10 mL trapping buffer to cover the mixture and centrifuge at 3000 × *g* and 4°C for 15 min.
8. Remove the protoplast in the interface with a pipette and transfer to a sterile 15-mL falcon tube. Add at least two volumes of STC buffer to dilute the protoplast. Centrifuge at 5000 × *g* and 4°C for 8 min.
9. Decant the supernatant. Resuspend the protoplast at the concentration of 10^8–10^9 with about 1 mL STC buffer. Count with a hemocytometer under the microscope.

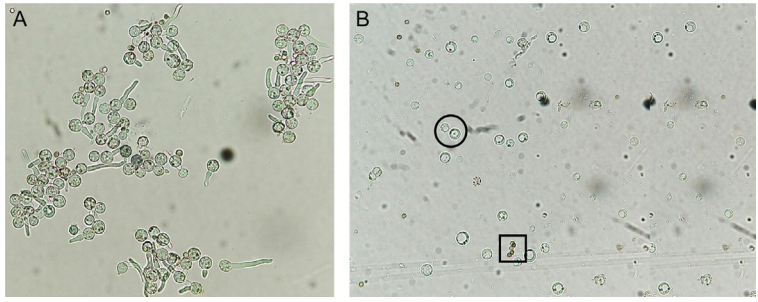

Fig. 3 Protoplast preparation of *A. ustus* 094102. (A) Spore germination in LMM at 28°C and 220 rpm for 11 h. (B) Protoplasts formation in Osmotic medium containing 10 mg mL^{-1} driselase at 28°C and 80 rpm for 4 h. The spores and protoplasts were displayed in the *circle* and *square*, respectively.

3.1.4 Notes

1. Do not adjust the pH of osmotic medium with NaOH, as it will cause precipitation.
2. Do not heat up the osmotic medium in microwave or autoclave, or it will form a precipitate.
3. Time of spore germination must be controlled. If the culture has grown too fast, do ot try to make protoplast.
4. If the protoplast forms rapidly, it will need less than 3–4 h. Protoplasts are thin-walled and about twice as large as spores.
5. Trapping buffer should be kept on the surface of mixture before centrifugation.

3.2 Construction of Gene-Targeting Cassette

The gene-targeting cassette contains upstream homology arm (UHA), downstream homology arm (DHA) and a selection marker. In general, homologous recombination in filamentous fungi (Fig. 4A) requires that the length of homology arms be greater than 1 kb (Asch & Kinsey, 1990; Hynes, 1996). The cassette can be constructed using restriction enzyme digestion and ligation, fusion PCR (Szewczyk et al., 20066), double-joint PCR (Yu et al., 2004), *E. coli* RecE/T system (Chaveroche, Ghigo, & D'Enfert, 2000), and TAGKO (Hamer et al., 2001).

Selective markers commonly used in filamentous fungi include auxotrophic complementary genes such as *pyrG* (orotidine-5′-phosphate decarboxylase gene), *ura5* (uracil auxotrophic gene) (Ruiz-Díez, 2002), and *argB* (encoding ornithine carbamoyltransferase) (Jadoun, Shadkchan, & Osherov, 2004). Carbon, nitrogen, sulfur, and other nutrients such as *amdS* (encoding acetamidase) (Hynes, 1994), *niaD* (nitrate reductase gene) (Malardier et al., 1989), *MPI* (mannose-6-phosphate isomerase) (Gui, Li, Liu, Han, & Li, 2014), and the like can be used. Antibiotic resistance genes such as hygromycin, bialaphos, zeocin, chlorimuronethyl (Chang et al., 2010), benomyl, and oligomycin (Wang et al., 2017) are employed.

We describe construction of the gene-targeting cassette here (Fig. 4B), which involves steps of restriction enzyme digestion, ligation, and subcloning. The pMD18T vector was used as a backbone; the *Sh ble* resistance gene is cloned into pMD18T by TA cloning. UHA and DHA of the *Au3446* gene of the strain 094102 are loaded to the two ends of the resistance gene of pMD18T vector through restriction enzyme digestion and ligation.

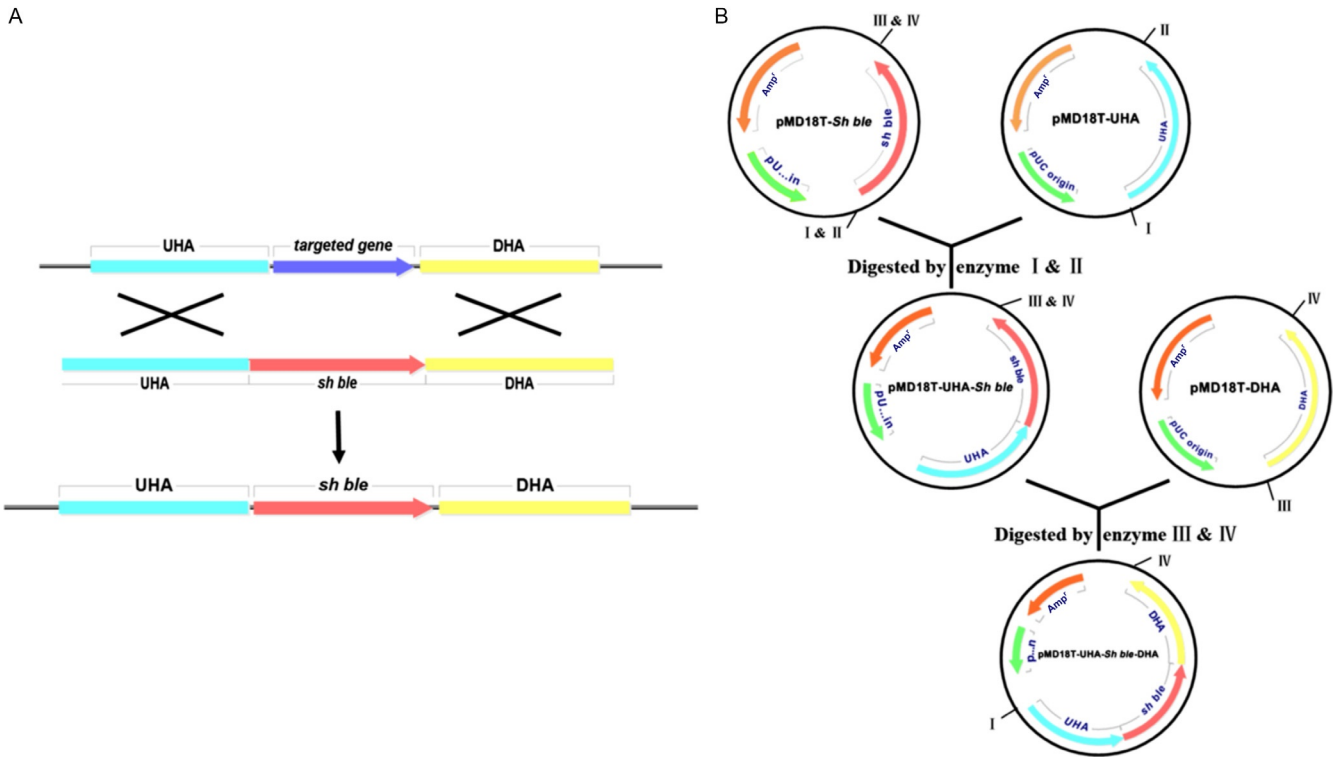

Fig. 4 Gene (cluster) knockout in fungi with UHA-Sh ble-DHA cassette. (A) The schematic diagram of homologous recombination. (B) Construction of the gene-targeting cassette. pMD18T-*Sh ble*, pMD18T-UHA, and pMD18T-DHA were obtained by TA clone. pMD18T-UHA-*Sh ble* and pMD18T-UHA-*Sh ble*-DHA were obtained by restriction enzymes I & II, III & IV digestion, respectively, and ligation of T4 DNA ligase. The gene-targeting cassette was obtained from pMD18T-UHA-*Sh ble*-DHA digested by enzyme I & IV.

3.2.1 Equipment

- PCR instrumentation (C-1000, BIO-RAD)
- Agarose gel electrophoresis equipment (G560E, BIO-RAD)
- Ultramicro spectrophotometer (Nanodrop 2000, Thermo)

3.2.2 Buffers and Reagents

- 2 × ES Taq MasterMix/2 × Pfu MasterMix (CWBio)
- I-5™ 2 × High-Fidelity Master Mix (Molecular Cloning Laboratories)
- Gel/PCR extraction kit/plasmid miniprep kit (50 ×) (OMEGA Bio-tek)
- T Vector pMD18 (Simple) Kit (TaKaRa)
- Restrictive endonuclease/T4 DNA ligase (New England BioLabs)

3.2.3 Procedure

1. Obtain each of *Au3446L′* (1224 bp) and *Au3446R′* (1657 bp) gene (Fig. 5A) using PCR amplified from *A. ustus* 094102 gDNA with primer *Au3446L′-s/a* and *Au3446R′-s/a* (Table 3) and clone these fragments into pMD18-T-Simple vector (Table 4).
2. Amplify the zeocin-resistant gene *Streptoalloteichus hindustanus ble* (*Sh ble*) (1172 bp) (Fig. 5A) that selected as the selection marker from pGPAαA with *sh ble-s′/a′* (Table 4) and cloned into pMD18T vector by TA clone (Table 4).
3. Transform the TA clones to *E. coli* TOP 10 competent cell and incubate at 37°C overnight. Confirm all fragments by DNA sequencing.
4. Digest pMD18-T-Simple-*Au3446L′* and pMD18-T-*Sh ble* with restriction *Eco*RI and *Kpn*I and purify using gel extraction kit, clone *Au3446L′* into linearized pMD18-T-*Sh ble* with T4 DNA ligase to obtain pMD18-T-*Au3446L′-Sh ble* (Table 4).
5. Digest pMD18-T-Simple-*Au3446R′* and pMD18-T-*Au3446L′-Sh ble* with restriction *Pst*I and *Hin*dIII and purify the target DNA fragments. Clone *Au3446R′* into linearized pMD18-T-*Au3446L′-Sh ble* to construct pMD18-T-*Au3446L′-Sh ble-Au3446R′* (Table 4).
6. Digest pMD18-T-*Au3446L′-Sh ble-Au3446R′* with restriction *Eco*RI and *Hin*dIII to obtain linearized *Au3446L′-Sh ble-Au3446R′* target cassette (Fig. 5B).

3.2.4 Notes

1. The size of homology arms is about 1–2 kb.
2. Using the above procedure, gene cassette containing UHA, DHA, and the antibiotic-resistant gene can be used in the deletion of other gene or gene clusters by changing different primers of UHA and DHA.

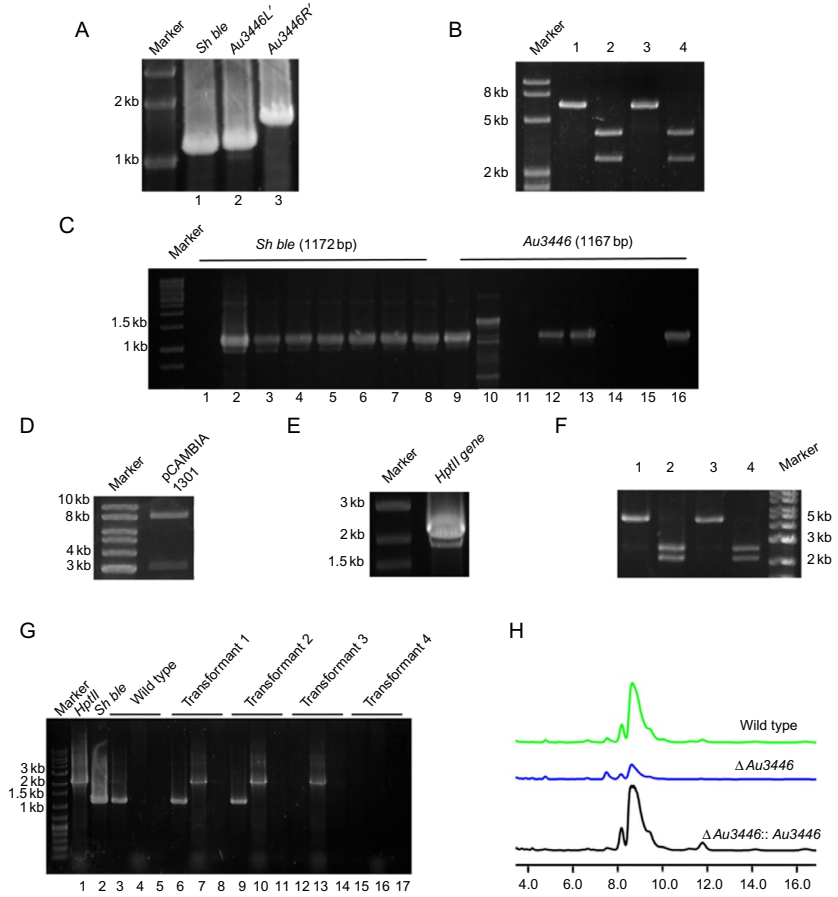

Fig. 5 *Au*3446 gene knockout and complementation in *A. ustus* 094102. (A) *Sh ble*, *Au3446L′*, and *Au3446R′* gene amplification. Lanes 1–3: *Sh ble* (1171 bp), *Au3446L′* (1224 bp), and *Au3446R′* (1657 bp) gene amplified with primers *Sh ble-s/a*, *Au3446L′-s/a*, and *Au3446R′-s/a*, respectively. (B) Digestion verification of pMD18T-*Au3446L′-Sh ble-Au3446R′*. Lanes 1 and 3: pMD18T-*Au3446L′-Sh ble-Au3446R′* digested by *Eco*RI (6720 bp); lanes 2 and 4: pMD18T-*Au3446L′-Sh ble-Au3446R′* digested by *Eco*RI and *Hin*dIII (4085 and 2635 bp). (C) Colony PCR of *A. ustus* 094102::Δ*Au3446*. Lanes 1 and 9: *A. ustus* 094102 genomic DNA amplified with primers *Sh ble-s/a* and *Au3446-s/a*; lanes 2 and 10: pGAPZαA amplified with primers *Sh ble-s/a* and *Au3446-s/a*; lanes 3–8, 11–16: six transformants of *A. ustus* 094102::Δ*Au3446 genomic* DNA amplified with primers *Sh ble-s/a* and *Au3446-s/a*, respectively. Six transformants were picked for PCR identification. Among them, three transformants (in lanes 4/12, 5/13, and 8/16) were positive strains, and ectopic insertion of targeting cassette may have occurred in the others. (D) *hptII* gene amplification. The plasmid pCAMBIA 1301 digested by *Sph*I (8619 and 3230 bp). (E) *hptII* gene amplified from digested pCAMBIA 1301 with primers *hptII-s/a* (2146 bp). (F) Digestion verification of pUC18-*CaMV35S-hptII-CaMV35S polyA*.

3.3 Transformation of Gene-Editing Cassette to Fungal Protoplast

Methods of genetic transformation in filamentous fungi include protoplast-mediated transformation, *Agrobacterium tumefaciens*-mediated transformation, biolistics transformation, electroporation and restriction endonuclease-mediated integration (Wang et al., 2017), and lithium acetate transformation (Olmedo-Monfil, Cortés-Penagos, & Herrera-Estrella, 2004).

After the protoplast preparation, the linearized *Au3446L′-Sh ble-Au3446R′* target cassette obtained in Section 3.2 is transformed into *A. ustus* 094102 protoplast by PEG–$CaCl_2$-mediated protoplast transformation (Jiang, Shen, Liu, & Lu, 2014; Zhong et al., 2012; Zhou, Li, Wang, & Zhuge, 2005).

3.3.1 Equipment

- High-speed freezing centrifuge (Centrifuge 5810R, Eppendorf).

3.3.2 Buffers and Reagents

- PEG solution: PEG 4000 (BDH) 60%; $CaCl_2$ 0.235 g; 1 *M* Tris–Cl (pH 7.5) 5 mL; add ddH_2O to 100 mL, for protoplast transformation.
- Stabilized minimal medium (SMM): GMM with sorbitol 218.6 g/L, for protoplast regeneration and screening of transformants.

3.3.3 Procedure

1. Dilute 10 μg linearized *Au3446L′-Sh ble-Au3446R′* target DNA fragment with STC buffer in a sterile falcon tube and keep the volume at 100 μL.
2. Add 100 μL of protoplast and incubate for 50 min on ice.

CaMV35S-hptII-CaMV35S polyA cassette was cloned into pUC18 vector. Lanes 1 and 3: plasmid digested by *Eco*RI (4840 bp); lanes 2 and 4: plasmid digested by *Eco*RI and *Sph*I (2195 and 2645 bp). (G) Colony PCR of *A. ustus* 094102::Δ*Au3446* complementary strains. Lanes 1, 4, 7, 10, 13, and 16: *hptII* gene amplified with primers *hptII-s/a* (2146 bp); lanes 2, 5, 8, 11, 14, and 17: *Sh ble* gene amplified with primers *Sh ble-s/a* (1172 bp); lanes 3, 6, 9, 12, and 15: *Au3446* gene amplified with primers *Au3446-s/a* (1167 bp). Among these transformants, 1 and 2 were positive mutant strains. (H) HPLC analysis of ophiobolin production in *A. ustus* 094102 wild-type, Δ*Au3446* mutant and the *Au3446Δ::Au3446* complementary strains. The production of ophiobolins decreased in the *ΔAu3446* mutant and recovered almost the same to the wild-type strain in the complementary mutant.

Table 3 Primers Used for *Au*3446 Gene and Gene Clusters Deletion and Complementation of *Au*3446

Type	Full Name	Sequence: 5′–3′ (Restriction Endonuclease)	Product Size (bp)
pUC18	M13-47	CGCCAGGGTTTTCCCAGTCACGAC	156
	RV-M	GAGCGGATAACAATTTCACACAGG	
Selective marker	*Sh ble*-s	CCCACACACCATAGCTTCAAAAT	1172
	Sh ble-a	AGCTTGCAAATTAAAGCCTTCG	
	hptII-s	AAATTGACGCTTAGACAACTTAA	2146
	hptII-a	GCAGCTTGCCAACATGGTG	
Genetic deletion and complementation system (gene *Au_3446*)	*Au_3446*-P1	GTGTGGGTGATCTTGACGGATTC	1245
	Au_3446-P3	CTATGGTGTGTGGGGGATCGGGTGGTATTTGCGTGTTTGG	
	Au_3446-P4	GCAAGCTGGAGACCAACATGCGGCCTAATCGAGATGTGC	1672
	Au_3446-P6	AACGGGTTCCTTCAAAATGG	
	Au_3446-P2	ACATACACTCTCATCCTACGCCTG	3402
	Au_3446-P5	CAGCCCAACTGAACTTTTCTAGC	
	Au_3446L′-s	CGGAATTCATCTTGACGGATTC (*Eco*RI)	1224
	Au_3446L′-a	GGGGTACCTTGCGTGTTTGG (*Kpn*I)	
	Au_3446R′-s	AACTGCAGCTAATCGAGATGTGC (*Pst*I)	1657
	Au_3446R′-a	CCCAAGCTTCCTTCAAAATGG (*Hin*dIII)	
	Au_3446-s	AAAGATGTTTCTCCCTTTGGCG	1167
	Au_3446-a	GAGGTCGCGGGCCTGATA	

	Au_3446L′-Au_3446-s	GGGGTACCATACACTCTCATCCTAC (*Kpn*I)	2502
	Au_3446L′-Au_3446-a	CTGCAGAACCAATGCATTGGCCGGTTCATGGCC (*Bst*XI)	
	Au_3446R′-s	CTGCAGAAAAAAAAGCTCGCTTTTGCAC (*Pst*I)	1012
	Au_3446R′-a	AAGCTTTGAACTTTTCTAGCCCGCCC (*Hin*dIII)	
POC*3446* deletion	POC*3446L*-s	GAATTCCCACAGATAGTATGCGAGTC (*Eco*RI)	1419
	POC*3446L*-a	TCTAGACAAACGCCAGAACATCACG (*Xba*I)	
	POC*3446R*-s	GTCGACTAGGGTGGTATTTGCGT (*Sal*I)	1128
	POC*3446R*-a	AAGCTTGGGCATCCGTATGATGTCG (*Hin*dIII)	
POC*8003* deletion	POC*8003L*-s	GAATTCGTCGTACAGTCAGGTAGGGA (*Eco*RI)	1085
	POC*8003L*-a	GGTACCAAGGAGGAGTGCAAGGAA (*Kpn*I)	
	POC*8003R*-s	CCTGCAGGTTGCCGAGGACAAGG (*Sbf*I)	1061
	POC*8003R*-a	AAGCTTCATCTGTGGACGGAGTAAAG (*Hin*dIII)	
	Au_8003-s	CGCCCACCACTCATTCTTTTAG	1063
	Au_8003-a	CGACCTGATCCGATGCTGTC	
POC*13192* deletion	POC*13192L*-s	GAATTCTAACTTCAAATCAGCGAGGA (*Eco*RI)	1224
	POC*13192L*-a	TCTAGAATACCCACGGACCCAAC (*Xba*I)	
	POC*13192R*-s	CCTGCAGGAGCAGCATCTTCGATTGG (*Sbf*I)	1077
	POC*13192R*-a	AAGCTTATTCTGGGACAGGAACTAG (*Hin*dIII)	
	Au_13192-s	AAGAACAAGCACGGACTGCC	1096
	Au_13192-a	CCTGCCTCGTCCAATACAGAT	

Continued

Table 3 Primers Used for *Au*3446 Gene and Gene Clusters Deletion and Complementation of *Au*3446—cont'd

Type	Full Name	Sequence: 5′–3′ (Restriction Endonuclease)	Product Size (bp)
POC*11565* deletion	POC*11565L*-s	GAATTCTTGTTGAGGCGTTTTGTG (*Eco*RI)	1058
	POC*11565L*-a	TCTAGAGTCATCGTGCGAGTAGG (*Xba*I)	
	POC*11565R*-s	CCTGCAGGCATCCGCAGTCATAGTAGT (*Sbf*I)	1018
	POC*11565R*-a	AAGCTTCGCCTCCACGTTG (*Hin*dIII)	
	Au_11564-s	CTCGTATTCGCTTTGGGTTGA	1106
	Au_11564-a	CCAGCATTGTCGGGTTCG	
POC*6298* deletion	POC*6298L*-s	GAATTCGCAGCCAGTTCCTTCCCTA (*Eco*RI)	1508
	POC*6298L*-a	GGATCCTTTTTACCCAAGCCCATC (*Bam*HI)	
	POC*6298R*-s	CCTGCAGGAGGGAAATACCGCCTCAAAAG (*Sbf*I)	1208
	POC*6298R*-a	AAGCTTAGCCTCCTCCTCGTTC (*Hin*dIII)	
	Au_6298-s	TTTGAGGCGGTATTTCCC	1038
	Au_6298-a	ACCTCCTTCTTCAGTCCCT	

Table 4 Plasmids Used for *Au*3446 Gene and Gene Clusters Deletion and Complementation of *Au*3446

Type	Serial Number/Full Name	Characteristics
Expression vectors	pGAPZαA	P_{GAP}, α-factor signal, myc epitope tag, C-terminal polyhistidine tag, T_{Aox1}, P_{Tef1}, P_{Em7}, *Sh ble* (Zeocin^r), T_{Cyc1}, pUC ori (ColE1 ori)
Cloning vectors	pMD18T	Derived from pUC18, pUC ori (ColE1 ori), O_{LacZ}, *bla* (Amp^r)
	pMD18-T-Simple	Derived from pMD18T, without multiple cloning site
	pCAMBIA1301	$P_{CAMV35S}$, *Gus*, $T_{Nos\ polyA}$, $P_{CAMV35S}$, *hptII* (HmB^r), $T_{CAMV35S\ polyA}$, pVS1 rep, pVS1 sta, pBR322 ori, pBR322 born, Kan^r
Deletion and complementation vectors	pUC18/P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}	Derived from pMD18T and pGAPZαA, pUC ori (ColE1 ori), O_{LacZ}, *bla* (Amp^r), P_{Tef1}, P_{Em7}, *Sh ble* (Zeocin^r), T_{Cyc1}
	pUC18/$P_{CAMV35S}$-*hptII*-$T_{CAMV35S\ polyA}$	Derived from pMD18T and pCAMBIA1301, pUC ori (ColE1 ori), O_{LacZ}, *bla* (Amp^r), $P_{CAMV35S}$, *hptII* (HmB^r), $T_{CAMV35S\ polyA}$
Genetic deletion and complementation system (gene *Au_3446*)	pUC18/*Au_3446L*	Derived from pMD18-T-Simple
	pUC18/*Au_3446R*	Derived from pMD18-T-Simple
	pUC18/*Au_3446L′*	Derived from pMD18-T-Simple
	pUC18/*Au_3446R′*	Derived from pMD18-T-Simple

Continued

Table 4 Plasmids Used for *Au*3446 Gene and Gene Clusters Deletion and Complementation of *Au*3446—cont'd

Type	Serial Number/Full Name	Characteristics
	pUC18/*Au_3446L′-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble-*T_{Cyc1}
	pUC18/*Au_3446L′-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}*-Au_3446R′*	Derived from pUC18/*Au_3446L′-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}
	pUC18/*Au_3446-Au_3446L′-*$P_{CAMV35S}$*-hptII-*$T_{CAMV35S\ polyA}$	Derive from pUC18/$P_{CAMV35S}$-*hptII-*$T_{CAMV35S\ polyA}$
	pUC18/*Au_3446-Au_3446L′-*$P_{CAMV35S}$*-hptII-*$T_{CAMV35S\ polyA}$*-Au_3446R′*	Derive from pUC18/*Au_3446-Au_3446L′-*$P_{CAMV35S}$*-hptII-*$T_{CAMV35S\ polyA}$
POC*3446* deletion	pUC18/POC*3446L*	Derived from pMD18-T-Simple
	pUC18/POC*3446R*	Derived from pMD18-T-Simple
	pUC18/POC*3446L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble-*T_{Cyc1}
	pUC18/POC*3446L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}*-*POC*3446R*	Derived from pUC18/POC*3446L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}
POC*8003* deletion	pUC18/*POC8003L*	Derived from pMD18-T-Simple
	pUC18/*POC8003R*	Derived from pMD18-T-Simple
	pUC18/POC*8003L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble-*T_{Cyc1}
	pUC18/POC*8003L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}*-*POC*8003R*	Derived from pUC18/POC*8003L-*P_{Tef1}*-*P_{Em7}*-Sh ble-*T_{Cyc1}

POC*13192* deletion	pUC18/POC*13192L*	Derived from pMD18-T-Simple
	pUC18/POC*13192R*	Derived from pMD18-T-Simple
	pUC18/POC*13192L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}
	pUC18/POC*13192L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}-POC*13192R*	Derived from pUC18/POC*13192L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}
POC*11565* deletion	pUC18/POC*11565L*	Derived from pMD18-T-Simple
	pUC18/POC*11565R*	Derived from pMD18-T-Simple
	pUC18/POC*11565L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}
	pUC18/POC*11565L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}-POC*11565R*	Derived from pUC18/POC*11565L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}
POC*6298* deletion	pUC18/POC*6298L*	Derive from pMD18-T-Simple
	pUC18/POC*6298R*	Derive from pMD18-T-Simple
	pUC18/POC*6298L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}	Derived from pUC18/P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}
	pUC18/POC*6298L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}-POC*6298R*	Derived from pUC18/POC*6298L*-P_{Tef1}-P_{Em7}-*Sh ble*-T_{Cyc1}

3. Add 1.25 mL PEG solution and mix gently by turning on its side and rotating it. Incubate at room temperature for 30 min.
4. Add 2 mL STC buffer, 6.5 mL melt SMM (about 45°C) and proper antibiotic into the mixture, and mix gently.
5. Quickly pour the 10 mL transformed mixture onto a 10-mL solid SMM (Zeocin^{+}, 1000 mg/L) in Petri dish and culture at 28°C for 3–5 days.

3.3.4 Notes

1. The protoplast should be prepared freshly for transformation.

3.4 Identification of Positive Transformants

After culturing the tranformants on the SMM medium containing Zeocin (1000 mg/L), the grown out colonies will be picked out to check whether the target gene (*Au3446*) has been successfully deleted using PCR amplification. If the *Au3446* gene cannot be amplified but the *Sh ble-s/a* gene can, a positive transformant is confirmed.

3.4.1 Procedure

1. Pick out the single colonies and purify if needed.
2. Extract gDNA of the single colony.
3. Confirm positive transformants by PCR using primers *Au3446*-s/a and *Sh ble*-s/a (Fig. 5C).

3.4.2 Notes

1. Pick at least six transformants for production of ophiobolin and drimane to make sure the yield of these compounds is stable.
2. Isolate the positive transformants quickly to avoid crosscontamination because of spores dispersal.

3.5 Gene Complementation

To confirm the role of the deleted gene, the same gene is complemented back to the original location of the genome and its recovered phenotype is confirmed (Mohd-Zin et al., 2016; Penwell, Arivett, & Actis, 2012). We complemented *Au*3446 back into the mutant strain Δ*Au*3446 to get a mutant strain Δ*Au*3446::*Au*3446. In order that the complementation mutant can be easily observed, another resistant gene *hptII* of hygromycin is used. The yield of obtained complementary mutant, together with the wild strain, and the deletion mutant of *Au*3446 were obtained by HPLC analysis as described in Section 3.7. Ophiobolin production of the

complementary mutant recovered to near wild-type levels. The yield of sesterterpene decreased but was not abolished in the deletion mutant, which means that the *Au3446* gene is only partially involved in the biosynthesis of the sesterterpene ophiobolin and is not a key gene.

3.5.1 Procedure

1. Digest the pCAMBIA 1301 by *Sph*I (Fig. 5D) and amplify the hygromycin resistance gene *hptII* (2146 bp, together with the promoter pCaMV35S, shown in Fig. 5E) used as a selective marker for complementation with linear pCAMBIA 1301 with primers *hptII*-s/a (Table 3).
2. Clone *hptII* gene into pMD18T to produce pUC18-CaMV35S-*hptII*-CaMV35S polyA (Table 4) and checked by sequencing (Fig. 5F).
3. Amplify upstream fragment of *Au3446-Au3446L′* and downstream *Au3446R′* from *A. ustus* 094102 gDNA using primers *Au3446L′-Au3446*-s/a and *Au3446R′*-s/a, and clone them into pMD18-T-Simple by TA clone (Table 4) and check them by DNA sequencing.
4. Subclone *Au3446-Au3446L′* and *Au3446R′* to either side of *hptII* gene, respectively, to obtain pUC18-*Au3446L′-Au3446-CaMV35S-hptII-CaMV35S polyA-Au3446R′* (Table 4).
5. Digest pUC18-*Au3446L′-Au3446-CaMV35S-hptII-CaMV35S polyA-Au3446R′* by *Kpn*I and *Hin*dIII to obtain the DNA cassette *Au3446L′-Au3446-CaMV35S-hptII-CaMV35S*.
6. Transform the fragment into the protoplast of strain *A. ustus* 094102::Δ*Au3446* to obtain the complementary mutant strain by homologous recombination.
7. Select transformants on SMM with hygromycin B (150 g L^{-1}) and verify using PCR (Fig. 5G) using primers *Au3446*-s/a, *hptII*-s/a, and *Sh ble*-s/a (Table 3). *hptII* and *Au3446* gene can be amplified but *Sh ble* gene cannot be amplified among the positive mutants. Pick out the positive transformants 1 and 2 in Fig. 5G and detect the production of ophiobolin using the HPLC method as described in Section 3.7.

3.6 Gene Cluster Inactivation

In the biosynthetic pathway of filamentous fungal SM, many enzymes are involved in the process of catalysis, and the genes that encode these enzymes usually appear in clusters on the chromosomes (Keller & Hohn, 1997). A gene cluster deletion can be carried while the single gene deletion is not a sufficient evidence to confirm the function of a gene cluster. Such as the incomplete abolishment of ophiobolin production in *A. ustus* 094102::Δ*Au3446*

(Fig. 5H), we speculated that *Au3446* might not be the only gene involved in ophiobolin synthesis.

Methods of gene cluster localization include the in silico prediction and verification by experiment. The gene prediction may be carried by alignment of homologous gene protein sequence (Altschul, Gish, Miller, Myers, & Lipmen, 1990; Bromann et al., 2012) and the antiSMASH bioinformatic pipeline (http://antismash.secondarymetabolites.org) (Weber et al., 2015).

Since terpene biosynthesis includes steps such as isoprenyl chain elongation, cyclization, and postmodification (oxidation, hydroxylation, hydrolysis, etc.), a terpene biosynthesis gene cluster can be predicted based on the terpene synthase/cyclase or prenyl transferase genes, together with flanking genes encoding cytochrome P450 (CYP450), oxidoreductase, hydroxylase, hydrolase, and/or transcription regulators. Based on the structure of the ophiobolin (Fig. 1), the putative gene clusters named POC (Presumed Ophiobolin biosynthesis gene Cluster) can be predicted by in silico analysis of the regions flanking the terpene synthases (including the diterpene and "function unknown" group shown in Table 2). Based on this method, 15 POCs related to ophiobolins synthesis in strain 094102 were obtained. The method of gene cluster knockout is similar to that for single gene deletion. Primers and plasmids are shown in Tables 3 and 4, respectively.

3.7 HPLC Detection of Ophiobolins and Drimanes

Terpenoid extraction methods include steam distillation (Muzika, Campbell, Hanover, & Smith, 1990), solvent extraction (Bowman et al., 1997), headspace analysis (Tholl et al., 2006), supercritical fluid extraction (Pourmortazavi & Hajimirsadeghi, 2007), microwave method (Delazar, Nahar, Hamedeyazdan, & Sarker, 2012), ultrasound extraction (Liao, Zheng, & Qu, 2016), and the like. Commonly used extractants in the solvent extraction method are methanol, ethanol, *n*-hexane, ethyl ether, chloroform, and benzene.

Analytical methods for terpenoids include UV spectroscopy, gas chromatography-based analytical methods such as gas chromatography (Kappers et al., 2005), GC-MS (Fojtová, Lojková, & Kubán, 2008), two-dimensional capillary gas chromatography (Strunk & Bechtold, 1996), HPLC (Keinänen, Oldham, & Baldwin, 2001), liquid chromatography and tandem mass spectrometry, proton transfer reaction-mass spectrometry (Steeghs et al., 2004), and Fourier transform ion cyclotron resonance mass spectrometry (Vahur et al., 2012).

After obtaining the gene and gene cluster deletion mutant of *A. ustus* 094102, the yield of sesterterpene ophiobolins and sesquiterpene drimanes from *A. ustus* 094102 wild-type strain and its mutants can be checked by solvent extraction and the following HPLC analysis.

Five out of 15 POCs knockout mutants changed their ophiobolin yield (Fig. 6), which indicated their involvement in ophiobolin biosynthesis. Among those, the POC 8003 mutation abolished ophiobolin production, and the POC 6298 mutation increased, but POC 13192 and POC 11565 mutation decreased (Fig. 6B). These results indicated that sesterterpene ophiobolin biosynthesis involved multiple gene clusters (Chai et al., 2016).

3.7.1 Equipment

- Rotary evaporator (Vacuum controller V-850, BUCHI)
- HPLC (2998, Waters)
- Phenomenex Gemini column (C18, 250 × 4.6 mm, 5 μm)
- Photodiode Array detector

3.7.2 Materials and Reagents

- Medium: corn niblet 100 g, yeast extract 1.7 g, $MgSO_4$ 0.85 g, KH_2PO_4 0.5 g, sea salt 1.6 g, and tap water 40 g, for solid fermentation of fungus
- Methanol/acetone/ethyl acetate
- Microporous membrane (0.2 μm)
- Disposable sterile syringes (1-mL)

3.7.3 Procedure

1. Inoculate the hypha or spores of wild-type strain 094102 and its mutants in corn solid medium at 20°C for 18 days.
2. Add 30 mL 80% acetone into 20 g fermentation products and stay for 12 h.
3. Break it further by ultrasonication for 20 min.
4. Centrifuge at 8000 × *g* for10 min.
5. Remove acetone from the supernatant with rotary evaporator.
6. Extract the residua with a double volume of ethyl acetate and remove ethyl acetate using rotary evaporator.
7. Dissolve the crude extraction with 15 mL methanol for HPLC detection.
8. Filter fermentation crude extract dissolved with methanol using 0.22 μm microporous membrane and inject the filtrate into the chromatography bottle for HPLC detection.

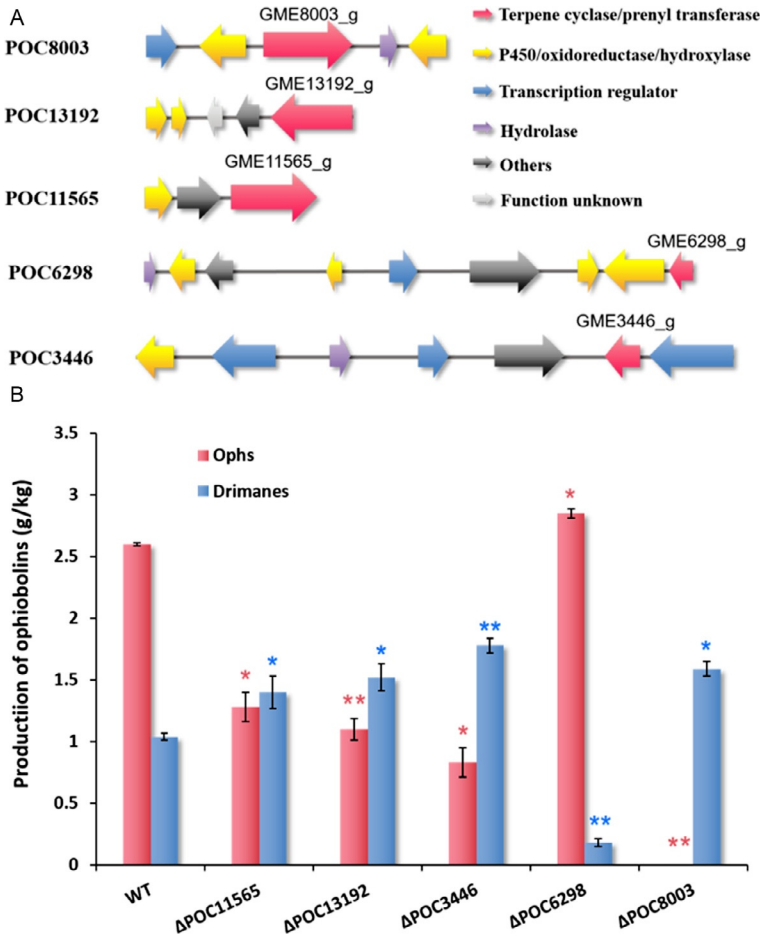

Fig. 6 The inactivation of gene cluster involved in the biosynthesis of ophiobolins. (A) Five gene clusters of *A. ustus* 094102 involved in ophiobolin synthesis. The clusters included the genes of terpene cyclase (or prenyl transferase) (in *red*), cytochrome P450 or oxidoreductase or hydroxylase (in *yellow*), transcription regulator (in *blue*), hydrolase (in *purple*), other function (in *black*), and function unknown (in *gray*). Numbers on or near the open reading frame are gene codes. (B) HPLC detection of five gene cluster mutants and wild-type strain in ophiobolin and drimane. Data are means ± SE of at least five independent measurements. $^{*}P < 0.05$, $^{**}P < 0.01$, vs wild-type strain. The production of ophiobolin was completely abolished in the ΔPOC8003 mutant. The yield of ophiobolin partially but significantly decreased in the ΔPOC3446 ($P < 0.01$), ΔPOC13192 ($P < 0.05$), and ΔPOC11565 ($P < 0.05$) mutants, but increased significantly in the ΔPOC6298 mutant ($P < 0.05$) compared with the wild-type strain. The yield of drimane significantly decreased in the ΔPOC6298 ($P < 0.01$) mutants but increased in ΔPOC3446 ($P < 0.01$), ΔPOC8003 ($P < 0.05$), ΔPOC11565 ($P < 0.05$), and ΔPOC13192 ($P < 0.05$) mutants.

9. Set the program of HPLC: full wavelength (210–400 nm) scanning; isocratic elution with the mobile phase consisting of methanol and water (85:15, v/v); the injection volume, elution time, and flow rate are 10 μL, 30 min, and 1.0 mL/min, respectively.
10. Prepare ophiobolin G (>96% purity) in methanol at concentration of 0, 0.005, 0.010, 0.015, and 0.020 g L^{-1} for standard curve.
11. Peak areas of ophiobolins and drimanes were summed at 234 and 306 nm, respectively.
12. Determine the yield of ophiobolins and drimanes according to the standard curve.

3.7.4 Notes

1. Dilute the crude extract to a suitable fold if its concentration is too high.
2. If any precipitate appears in the filtrate, it should be filtered again.

4. IN VITRO FUNCTIONAL CHARACTERIZATION OF AU8003, AU13192, AND AU6298

Terpene synthases gene can be identified either using the in vivo gene (cluster) deletion and complementation in native host, or the in vitro biochemical reaction using the heterologously expressed proteins. While using in vitro approach, the prerequisite is to obtain the proteins of the presumed terpene synthases. *E. coli* is often used as a heterologous expression host, due to its pure background and accessibility to manipulate. Using *E. coli* heterologous expression system, the target proteins can be obtained (Ueda et al., 2015; Ye et al., 2015), and furthermore, SM such as terpenes can be produced directly since their precursors are available in *E. coli* (Bian et al., 2017; Jia, Potter, & Peters, 2016).

In this section, we describe the function verification of terpene synthase genes from *A. ustus* 094102 using the in vitro biochemical reaction. The process includes in silico analysis, target gene cloning, protein expression using *E. coli*, biochemical reaction, and product detection.

4.1 In Silico Analysis

Five terpene biosynthesis genes of *Au8003, Au13192, Au11565, Au6298,* and *Au3446*, in the five POCs which are selected by the in vivo gene cluster deletion (in Section 3.6), are further analyzed in silico. Phylogenetic analysis is performed using the amino acid sequences of the five genes' coding proteins, and their corresponding homologous proteins extracted from the

GenBank database. The neighbor-joining phylogenetic tree shows that the five proteins distributed in three clusters corresponding to three enzyme types: di(sester)terpene synthase, FPPS, and HexPPS, which demonstrate the functional diversity of these enzymes (Fig. 7A). Au8003 clustered closely with AcOS, an ophiobolin F synthase (Chiba et al., 2013). Au13192 clustered within the same di (sester)terpene synthase branch but not as closely as Au8003 (Fig. 7A). A multiple sequence alignment using BioEdit software also shows that Au8003, Au13192, and Au11565 each containing both TC and PT domains (Fig. 7B). The closest identified protein matching to Au6298 is the plant EpFPPS (*Euphorbia pekinensis* farnesyl diphosphate synthase), at an identity of 49%; and to Au3446 is the bacterial MlHexPPS (*Micrococcus luteus* heterodimeric hexaprenyl diphosphate synthase), at an identity of 32%. We therefore assume that proteins Au8003 and Au13192 are bifunctional terpene synthases, which are sesterterpene synthase and diterpene synthase, respectively, and that Au6298 may be a FPPS. Functions of genes *Au8003, Au13192,* and *Au6298* are verified further in vitro.

4.1.1 Software and Database

- GenBank database
- MEGA (version 5.0) for amino acid phylogenetic analysis
- BioEdit 7.0 for multiple sequence alignments

4.1.2 Procedure

1. Search the GenBank database by BLASTP (basic local alignment tool—protein) using amino acid sequences of Au8003, Au13192, Au11565, Au6298, and Au3446 as query sequences.
2. Select reference sequences of query proteins from BLASTP search results. Only those sequences of function confirmed by experiment were selected.
3. Use the BioEdit 7.0 software to perform multiple sequence alignments.
4. Use MEGA (version 5.0) to construct the phylogenetic analysis based on amino acid sequences.

4.1.3 Notes

1. The updated software and database are recommended for further use.

4.2 Target Gene Cloning

To study the function of target genes by heterologous expression, obtaining target genes from original strain is one of the most important steps.

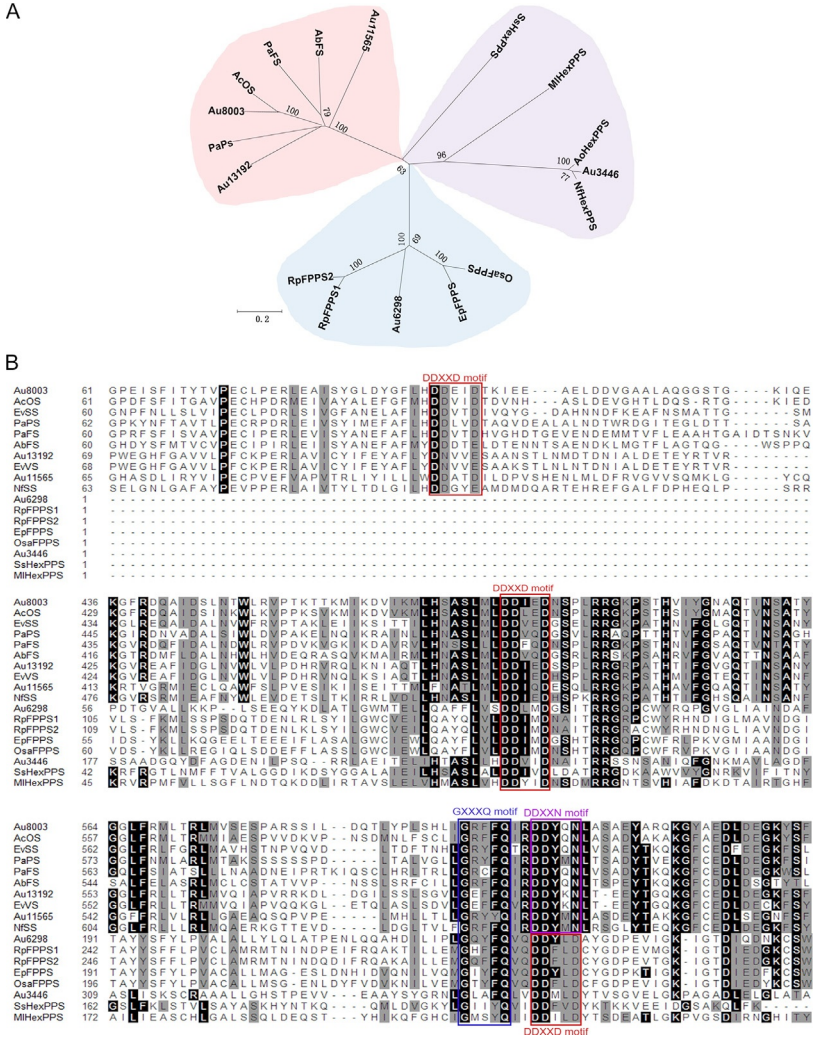

Fig. 7 In silico analysis of Au8003, Au13192, and Au6298. (A) Neighbor-joining phylogenetic tree based on amino acid sequences of bifunctional diterpene and sesterterpene synthases, farnesyl pyrophosphate synthases, and hexaprenyl pyrophosphate synthases. (B) Alignment of amino acid sequences of the five terpene synthases related to ophiobolin biosynthesis with function identified terpene synthases. The three DDXXD/E (terpene cyclase (TC) domain), DDXXD (prenyltransferases (PT) domain), GQ (PT), DDXXN (bifunction PT only) DDXXD (single function PT only) are showed in *colored frame*. Only sequence parts contain characteristic catalytic domain sequence are shown; other part of amino sequence is omitted.

The gDNA of eukaryotic fungi contains both exons and introns, therefore cDNA (complementary DNA from mRNA without introns) is employed as template while using PCR approach to get the target genes for heterologous expressing in prokaryotic hosts.

RT-PCR (reversible transcription PCR) technique is an effective method to acquire cDNA (Keilholz et al., 1998; Zheng, Qiu, & Han, 2015). This method consists of two steps: cDNA synthesis from mRNA by reverse transcription and amplification of PCR. It has been proved useful in amplifying very small amount of specific mRNA to obtain cDNA (Kusec, 1998). However, this approach can be limited by the availability of particular tissues or mRNA, making gDNA a desirable source for target gene cloning (Mitani, Nakayama, Harbers, & Hayashizaki, 2004). On this occasion, overlap PCR using gDNA as template is developed for individual exon amplification, using tagged primers with overlapping regions and their concatenations in additional rounds of PCR amplification. With these methods, most of the target genes can be obtained efficiently.

When the target gene sequences are derived from eukaryotic organisms, rare codons can be a problem on successful expression of these genes, especially for those have diad or triad rare codons structures (Kane, 1995). Under these circumstances, codon optimization of target sequences should be employed to increase their expression level and solubility (Utashima, Yamashita, Arima, & Masaki, 2017).

After obtaining the target genes, they are linked to cloning vectors for high-efficient cloning of DNA fragments. Cloning methods can be divided into two classes, depending on whether or not ligase is required (Zhou, Clark, & Gomezsanchez, 1995). TA cloning is a method that ligase is not required and often used when constructing cloning vectors. It is a convenient and quick way to clone PCR product, taking advantage of Taq DNA polymerase's terminal transferase activity. For PCR product of Pfu DNA polymerase, vector kit based on DNA topoisomerase I may be a good choice (Shuman, 1994).

In case of strain 094102, genes of *Au8003* and *Au6298* can be obtained by mRNA extraction and RT-PCR, but for gene *Au13192*, overlap PCR using gDNA as template is used. After that, target genes are cloned to pEASY-blunt vector and transformed to *E. coli* by heat shock for further cloning.

4.2.1 Equipment

- Honor HNY-1 incubator shaker
- Mortar and pestle

- BIO-RAD C1000TM Thermal Cycler
- Thermo Nano Drop 2000C
- BIO-RAD gel electrophoresis system
- SYNGENE gel image analyzing system

4.2.2 Buffers and Reagents

- 0.1% DEPC (diethyl pyrocarbonate) treated H_2O: 1 mL high-purity (more than 97%) DEPC, dilute with ddH_2O to 1 L, stir until it is fully dissolved.
- QIAGEN RNeasy Mini Kit includes Buffer RLT, Buffer RW1, and Buffer RPE.
- Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, includes oligo (dT) 18, 5× reaction buffer, Ribo Lock RNase Inhibitor, RevertAid RT, and dNTP Mix.
- NEB pfu DNA polymerase kit, includes Pfu DNA polymerase and 5× NF buffer.
- OMEGA Gel Extraction Kit, includes Binding Buffer (XP2), SPW Wash Buffer and Elution Buffer.
- Transgen pEASY-Blunt vector kit.
- Maikelaibo plasmid extraction kit, includes Buffer 1, Buffer 2, Buffer 3, Buffer D, Buffer W, and Buffer E.

4.2.3 Procedure

4.2.3.1 RNA Extraction From Strain *Aspergillus* sp. 094102

Extraction of the total RNA from mycelia of strain 094102 is carried out with the German company QIAGEN RNeasy Mini Kit (50) Kit (stock # 74104), do as the following:

1. Culture strain 094102 in fungal II liquid medium at 28°C, 220 rpm for 2–3 days.
2. Take 50 mg of hyphae at one time for microextraction.
3. Grind the sample into a powder with liquid nitrogen, transfer the powder into a 1.5-mL plastic centrifuge tube.
4. Add 450 μL Buffer RLT (lysis buffer) in the centrifugal tube (add 10 μL β-ME per 1 mL Buffer RLT before use), oscillate on the oscillator for 1–3 min, make sure that the solution at the bottom of the tube is fully oscillated.
5. Centrifuge at 13,000 × *g* for 2 min at 4°C, transfer the supernatant to a clean 1.5-mL plastic centrifugal tube. Avoid touching or drawing the middle and lower organic phase, which contain DNA, proteins, and other impurities.

6. Add 0.5 volume of anhydrous ethanol, fully reverse the mix till it is thoroughly mixed.
7. Transfer the mixed solution to a centrifugal adsorption column and centrifuge at 4°C, 8000 × *g* for 15 s, discard the filtrate.
8. Add 700 μL Buffer RW1 (wash buffer) into centrifugal adsorption column and centrifuge at 4°C, 8000 × *g* for 15 s, discard the filtrate.
9. Move the centrifugal column to a new collection tube (no RNA enzymes), add 500 μL Buffer RPE (wash buffer), and centrifuge at 4°C, 8000 × *g* for 15 s, discard the filtrate.
10. Add 500 μL Buffer RPE (wash buffer, add 4 times volume of ethanol before use). Centrifuge for 2 min at 4°C, 13,000 × *g*.
11. Move the centrifugal column to a new collection tube (no RNA enzymes). Centrifuge for 2 min at 4°C, 13,000 × *g*.
12. Move the centrifugal column to a new 1.5-mL plastic centrifugal tube (no RNA enzymes), add 50 μL RNase-free water, centrifuge at 4°C, 8000 × *g* for 15 s.
13. Transfer 40 μL RNA sample to a new 1.5-mL plastic centrifugal tube (no RNA enzymes), add 5 μL each of DNase I and DNase I buffer, incubated at 37°C for 2 h.
14. Add 5 μL of 50 m*M* EDTA, incubated at 65°C for 10 min, preserved at −80°C.

4.2.3.2 Reverse Transcription

America's Revert of Thermo Fisher Scientific Company Aid First strand cDNA synthesis kit (model K1621) was used for reverse transcription, the steps are as follows:

1. Load 2 μg of the prepared RNA samples into a PCR tube, add 1 μL of 10 μ*M* oligo (dT) 18.
2. Add RNase-free water up to 12 μL, incubate at 65°C for 5 min.
3. Cool for 5 min on ice, centrifuge at 4°C, 13,000 × *g* for 2 min.
4. Add 4 μL of 5 × reaction buffer, 1 μL of Ribo Lock RNase Inhibitor, 1 μL of RevertAid RT, 2 μL of 10 m*M* dNTP Mix, and RNase-free water up to 20 μL.
5. Incubate at 42°C for 60 min, then at 70°C for 5 min.
6. Store the cDNA product at −20°C.

4.2.3.3 Amplification of Au8003 and Au6298

1. Design primer pairs of *Au8003* and *Au6298* according to the genomic sequence of strain 094102 (Table 5).

Table 5 Primers Used for Target Gene Cloning

Primer Name	Primer Sequence (5′–3′)
Au8003-F	**CATATG**ATGGAGTATAAGTACTCGACC (*Nde*I)
Au8003-R	**AAGCTT**TCAAACCTTCAGCAGCTCCA (*Hin*dIII)
Au6298-F	CC**CATATG**ATGTCTTCACCGCGTGCC (*Nde*I)
Au6298-R	CC**AAGCTT**TTATTTGGTGCGCTTGTAAA(*Hin*dIII)
Au13192-E1-S	**GCGGCCGC**ATGCCACAAACACAATCC (*Not*I)
Au13192-E1-A	GCCGACTCGACAACATTATCATACAGAAACGCATACTCAAAGATAT AACAAATAA
Au13192-E3-A	CATGAGCATGTCGACGAAG GGGGCGCCGGTATCGAT
Au13192-E4-S	CTTCGTCGACATGCTCATG
Au13192-E4-A	GCCGGGGCGAGGAGGATCTCGTCTCCTAGCTGCAC
Au13192-E5-S	ATCCTCCTCGCCCCGGC
Au13192-E5-A	AGTCTTCGATGTCGTCGAGCATGAGCGAGGCATTGTGTA
Au13192-E6-S	CTCGACGACATCGAAGACT
Au13192-E6-A	CTGCACCATCAACCTCGTAAGCAACCGGAACAACCCACCGGTCTT CTGCCTAACCATCTCCAAAT
Au13192-E8-S	TTACGAGGTTGATGGTGCAGATTGCGCCGGTGCGGCGGAAAGATC TCGACGGCATCTTATCAT
Au13192-E8-A	CCCTTTTGGCCGGTGTACTCTTCAGTTAGGTTCTTGTAGT
Au13192-E9-S	AGTACACCGGCCAAAAGGG
Au13192-E9-A	**TCTAGA**TACACCTTCAACCTCTGCAC (Xba I)

2. Prepare reaction system: 1 μL of Pfu DNA polymerase, 21 μL of sterilized deionized water, 1 μL of Pfu DNA polymerase 5 × NF buffer, 2.5 μL (10 μ*M*) each of forward and reverse primer, and 2 μL of cDNA template.
3. Start PCR, the reaction conditions: initial denaturation at 95°C for 3 min; denaturation at 95°C for 30 s; anneal at 53°C for 20 s; elongation at 72°C for 70 s; cycle for 35 times; elongation at 72°C for 10 min.
4. Verify PCR products by agarose gel electrophoresis (Fig. 8A and B).

4.2.3.4 Amplification of Au13192

Since there are seven introns in *Au13192* and the gene cannot be amplified from cDNA, we carried out overlap PCR using gDNA of *A. ustus* 094102 as template (Fig. 8C). The primers are designed as shown in Table 5 and Fig. 8. The primers for short fragments are designed with partially overlap of the

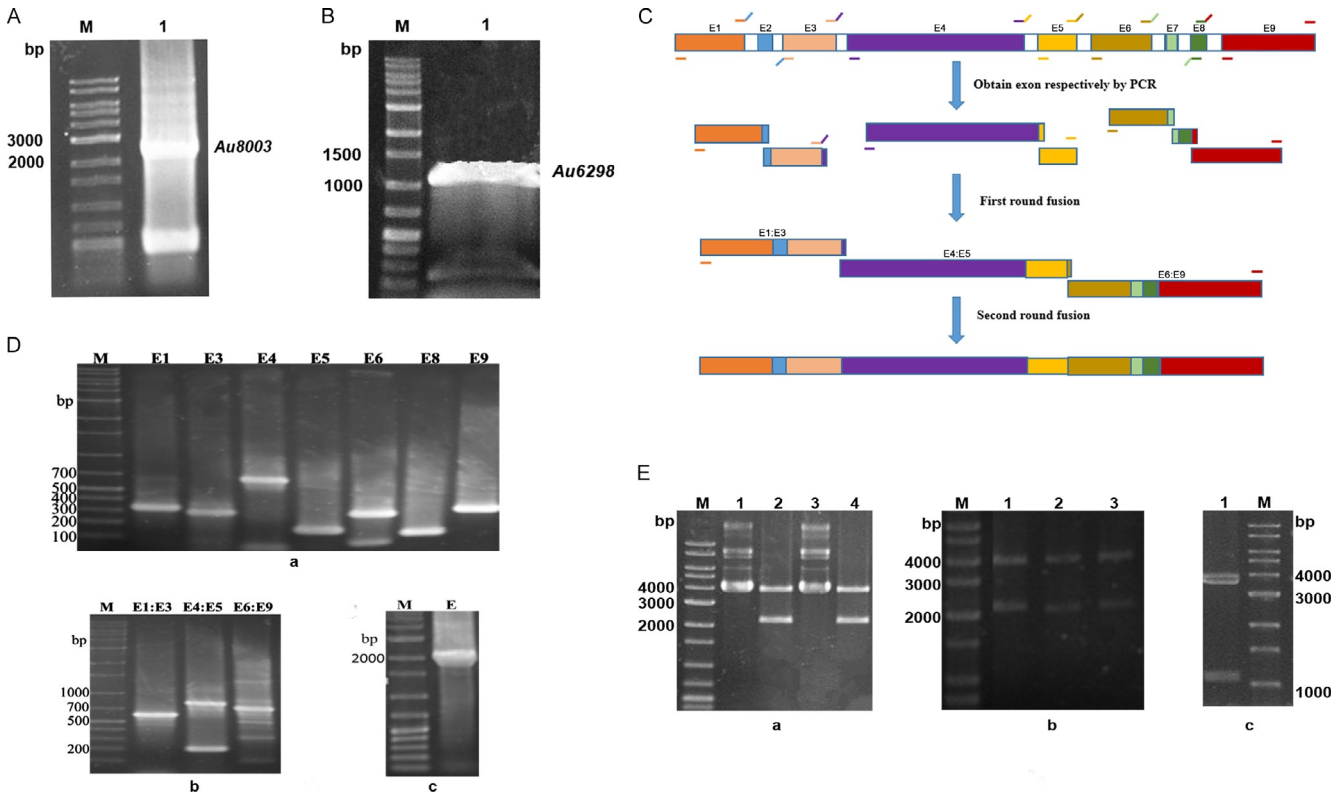

Fig. 8 See figure legend on opposite page.

long fragments, such as fragments of E2 were designed in primers *Au13192*-E1-A and *Au13192*-E3-A. Detailed steps are listed below:

1. First round PCR, obtain seven exon fragments: E1, E3, E4, E5, E6, E8, and E9 (Fig. 8D(a))
2. First round fusion PCR to get E1:E3, E4:E5, and E6:E9 fusion fragments, verify PCR products using agarose gel electrophoresis (Fig. 8D(b)).
3. Second round fusion PCR to fuse the three fragments obtained from step 2 and get full-length *Au13192* cDNA.
4. Verify PCR product by agarose gel electrophoresis (Fig. 8D(c)).

4.2.3.5 Recovery of Target DNA

After the electrophoresis of the PCR product, the target DNA was recovered from the agarose gel according to the Gel Extraction Kit (100) (Series No. D2500-01) supplier (OMEGA, Germany) as following:

1. Cut the target DNA fragment under UV light from the agarose gel to minimize gel size and improve DNA recovery. Absorb liquid on the gel surface using paper towel.
2. Chop the gel block to decrease the gel melting time and improve DNA recovery.
3. Weigh the gel block. Add binding buffer at the ratio of 400 μL per 100 mg agarose gel, incubate at 50–60°C for 10 min (mix every 2 min till all the gel melt).
4. Insert a HiBind DNA Mini Column in a 2-mL collection tube. Transfer less than 700 μL DNA/agarose melt solution to the column, centrifuge at 10,000 × *g* for 1 min at room temperature. Discard the filtrate.

Fig. 8 Target gene cloning of *Au8003*, *Au13192*, and *Au6298*. (A) Electrophoretic analysis of *Au8003* (2350 bp) PCR product. Lane M: marker; lane 1: *Au8003* PCR product. (B) Analysis of *Au6298* (1102 bp) PCR product. Lane M: marker; lane 1: *Au6298* PCR product. (C) Strategy of *Au13192* multiple exon fusion by overlap extension PCR. (D) Electrophoretic analysis of fusion fragments in the process of *Au13192* cDNA (2608 bp) fusion, respectively. (a) PCR product of each exon fragments. M: marker; E1, E3, E4, E5, E6, E8, and E9 represent for corresponding exon fragment. (b) Fusion PCR product after first round fusion. M: marker; E1:E3, E4:E5, and E6:E9 represent for fusion exon fragment E1 & E2, E4 & E5, and E6 & E8–9. (c) *Au13192* cDNA after second round fusion PCR. M: marker; E: *Au13192* cDNA. (E) Double digestion of cloning vectors pEASY-Blunt-*Au8003*, pEASY-Blunt-*Au13192*, pEASY-Blunt-*Au6298*. (a) Double digestion of pEASY-Blunt-*Au8003*. M: marker; lanes 1 and 3: pEASY-Blunt-*Au8003* plasmid; lanes 2 and 4: pEASY-Blunt-*Au8003* double digestion product. (b) Double digestion of pEASY-Blunt-*Au13192*. M: marker; lanes 1–3: pEASY-Blunt-*Au13192* double digestion product. (c) Double digestion of pEASY-Blunt-*Au6298*. M: marker; lane 1: double digestion product of pEASY-Blunt-*Au8003*.

5. Reuse the column, repeat step 4 till all the melt gel are transferred to the column.
6. Add 300 μL XP2 to the adsorption column, centrifuge at room temperature, 10,000 × *g* for 1 min, discard the filtrate, and reuse the collection tube.
7. Add 700 μL SPW wash buffer to the adsorption column, centrifuge at room temperature, 10,000 × *g* for 1 min, discard the filtrate, and reuse collection tube.
8. Add 300 μL SPW wash buffer to the adsorption column, centrifuge at room temperature, 10,000 × *g* for 1 min, discard the filtrate.
9. Centrifuge the HiBind DNA Mini Column at room temperature, maximum speed for 2 min to dry the column matrix.
10. Transfer the adsorption column to a new 1.5-mL tube. Add 30–50 μL elution buffer or sterilized water on the center of the column membrane.
11. Keep at room temperature for 2–5 min, centrifuge at maximum speed at room temperature for 1 min.
12. Check the recovered product with 1% agarose gel electrophoresis.

4.2.3.6 Target DNA Cloning

The recovered DNA is ligated to the pEASY-Blunt vector at 16°C for 1 h. The resultants are transformed to *E. coli* TOP 10 cells and cultured overnight. The positive colonies are selected and checked using rapid testing methods. The procedures are as following:

1. Pick out about 20 clones from the transformation agar plate with a sterile toothpick, streak onto the Luria-Bertani (LB) agar plates containing benzyl ammonia (100 μg/mL), incubate at 37°C for 8 h.
2. Add 30 μL sodium chloride–Tris–EDTA (STE) solution (20 m*M* Tris–HCl, 25 m*M* EDTA, 75 m*M* NaCl) in 1.5-mL plastic centrifugal tube, transfer the single colony individually to the plastic centrifugal tubes with toothpick, vortex 2 min till mixed well.
3. Add equal volume of chloroform:phenol:isoamyl alcohol (v:v:v = 24:25:1), shake the mix, centrifuge at 10,000 × *g* for 5 min at room temperature.
4. Check the supernatant using 1% agarose gel electrophoresis, to determine initially whether the plasmid containing the inserted exogenous fragments.

5. Extract the plasmid containing small foreign fragment using plasmid extraction kits (Wuhan Maikelaibo Technology, Ltd). The procedure is as following:
   (1) Transfer the single colony to ampicillin (100 μg/mL)-resistant LB medium incubated overnight at 37°C, 220 rpm.
   (2) Transfer 2 mL culture to a 2-mL plastic centrifugal tube, centrifuge at 10,000 × *g* for 1 min at room temperature. Discard liquid.
   (3) Add 250 μL Buffer 1, vortex to make the cells suspend thoroughly.
   (4) Add 250 μL Buffer 2, invert the centrifuge tube 5–6 times mildly, till the solution turn to clear.
   (5) Add 350 μL Buffer 3, invert the centrifugal tube 3–5 times mildly to mix thoroughly.
   (6) Centrifuge 12,000 × *g* for 10 min at room temperature.
   (7) Transfer the supernatant carefully into the collection tube adsorption column.
   (8) Centrifuge at 12,000 × *g* for 1 min at room temperature. Pour the liquid out of the collection tube. Put the column back into the collection tube.
   (9) Add 500 μL Buffer D, centrifuge at 12,000 × *g* for 1 min at room temperature. Pour the liquid out of the collection tube, put the column back into the collection tube.
   (10) Add 600 μL Buffer W, centrifuge at 12,000 × *g*, for 1 min at room temperature. Pour the liquid out of the collection tube, put the column back into the collection tube.
   (11) Add 630 μL Buffer W, centrifuge at 12,000 × *g* for 1 min at room temperature.
   (12) Transfer the column to a clean 1.5-mL plastic centrifuge tube, add 50–100 μL Buffer E to the adsorb membrane at the center, standing for 1 min at room temperature, centrifuge at 12,000 × g, for 1 min at room temperature to wash off the cloning plasmid.
   (13) Store the obtained plasmid at −20°C.

4.2.3.7 Verification of Cloning Vectors

1. Digest Plasmid pEASY-Blunt-*Au8003* and pEASY-Blunt-*Au6298* with *Hin*dIII and *Nde*I, in reaction conditions of following: 2 μL cutsmart buffer, 0.5 μL *Hin*dIII, 0.5 μL *Nde*I, 1 μL each plasmid, and 16 μL ddH_2O, incubate at 37°C for 1 h.

2. Digest pEASY-Blunt-*Au13192* with *Hin*dIII and *Not*I, in reaction conditions of following: 2 μL cutsmart buffer, 0.5 μL *Hin*dIII, 0.5 μL *Nde*I, 1 μL each plasmid, and 16 μL ddH_2O, incubate at 37°C for 1 h.
3. Check the digested products with 1% of agarose gel electrophoresis to determine whether the DNA fragment has cloned in plasmid (Fig. 8E(a–c)).
4. Submit 1 mL of culture containing the cloned plasmid of target DNA for sequencing to confirm the target DNA has been successfully cloned.

4.2.4 Notes

1. RNA extraction is supposed to be carried out in an enclosure space without RNase to prevent degradation. The pipettor, pipette tip, and EP tubes being used should have no RNase, and the mortars and pestles should be soaked overnight with 0.1% DEPC ddH_2O and then autoclaved for 40 min.
2. DEPC is toxic, 0.1% DEPC ddH_2O should be prepared in fuming cupboard, and avoid skin and respiratory exposure.
3. In Table 5, bases showed in bold are restriction enzyme cutting sites. Take care that the enzyme cutting sites should not exist in target gene sequence.

4.3 Protein Expression and Purification

For heterologous expression of target genes in *E. coli*, the pET expression system developed by Novagen is a common choice (Mierendorf, Morris, Hammer, & Novy, 1998). Target genes cloned in pET plasmids are controlled by strong bacteriophage T7 transcription and translation signals. T7 promoter is so efficient that target protein can comprise more than 50% of the total cell protein after a few hours of induction (Studier & Moffatt, 1986). Besides, this system is able to maintain target genes transcriptionally silent in the uninduced state, only active when transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control, and induced by the addition of IPTG. In addition, the His-T7-tag fusion proteins in pET system facilitate the target protein purification by using affinity nickel resin, as the Ni^{2+} can combine with 6 × His-tag.

A most used host of pET expression system is *E. coli* BL21 (DE3). It is a type B strain which is deficient in the *lon* protease *omp*T and lacks the *omp*T outer membrane protease that can degrade proteins during purification (Grodberg & Dunn, 1988). Taking advantage of these properties, target protein can stay more stably in strain BL21 (DE3).

E. coli BL21 (DE3) containing pET28a (+)-*Au8003*, pET28a (+)-*Au6298*, and pET28a (+)-*Au13192* are constructed to produce the His-T7-tag fusion protein using each gene cloned in Section 4.2, respectively. After elution with imidazole, desalination, and concentration, the target proteins are obtained.

4.3.1 Equipment

- Honour HNY-1 incubator shaker
- Beckman J2-MI centrifuge
- Scientz JY92-IIN Ultrasonic Cell Disruptor
- Novagen Ni-NTA His Bind Resin Nickel column
- Standard SDS-PAGE equipment
- Ultra-15 Centrifugal Filter Devices
- GE Healthcare PD-10 desalting column

4.3.2 Buffers and Reagents

- NEB restriction enzymes
- Thermo T4 DNA Ligase
- HEPES lysis buffer: 20 m*M* HEPES, 300 m*M* NaCl, 10% glycerol, 5 m*M* iminazole
- Elution buffer: 20 m*M* HEPES, 300 m*M* NaCl, 10% glycerol, 50–500 m*M* iminazole
- SDS-PAGE electrophoresis buffer: Tris 3 g, glycine 14.4 g, SDS 1 g, dilute with ddH_2O to 1 L, adjust pH value to 8.3
- 5 × SDS-PAGE loading buffer: glycerol 5 mL, SDS 1 g, 2-mercaptoethanol 0.5 mL, 1 *M* Tris–HCl (pH 6.8) 3.15 mL, 1% bromophenol blue 1 mL
- Coomassie brilliant blue staining solution: isopropanol 250 mL, Coomassie brilliant blue R-250 1 g, glacial acetic acid 100 mL, ddH_2O 650 mL
- Coomassie brilliant blue destaining solution: ethyl alcohol 50 mL, glacial acetic acid 100 mL, ddH_2O 850 mL
- HEPES desalination buffer: 20 m*M* HEPES, 150 m*M* NaCl, 25% glycerol

4.3.3 Procedure

4.3.3.1 Recovery of Target DNA Fragments and pET28a Fragment

1. Digest recombinant vectors pEASY-Blunt-*Au8003*, pEASY-Blunt-*Au6298*, and pET28a with *Hin*dIII and *Nde*I, in reaction conditions of

following: 5 μL cutsmart buffer, 2.5 μL *Hin*dIII, 2.5 μL *Nde*I, 2 μg each plasmid, add ddH_2O up to 50 μL, incubate at 37°C for 3 h.
2. Digest pEASY-Blunt-*Au13192* and pET28a with *Hin*dIII and *Not*I, in reaction conditions of following: 5 μL cutsmart buffer, 2.5 μL *Hin*dIII, 2.5 μL *Nde*I, 2 μg of each plasmid, add ddH_2O up to 50 μL, incubate at 37°C for 3 h.
3. Recover target DNA fragments and pET28a fragment after 1% agarose gel electrophoresis, using OMEGA Gel Extraction Kit.
4. Store the DNA fragments at −20°C.

4.3.3.2 Ligation of Target DNA and pET28a Fragments

1. Ligate target DNA fragments and pET28 fragment using T4 DNA ligase overnight, at a temperature of 4°C.
2. Transform ligation resultants into *E. coli* TOP 10 cells.
3. Positive colonies were selected using rapid testing methods after overnight culture, as described in Section 4.2.3.6.

4.3.3.3 Verification of Expressing Vectors

1. Extract plasmids pET28a-*Au8003*, pET28a-*Au13192*, and pET28a-*Au6298* using plasmid extraction kit (Wuhan Maikelaibo Technology, Ltd).
2. Digest pET28a-*Au8003* and pET28a-*Au6298* with *Hin*dIII and *Nde*I, respectively; reaction conditions are as described in Section 4.2.3.7.
3. Digest pET28a-*Au8003* and pET28a-*Au6298* with *Hin*dIII and *Not*I; reaction conditions are as described in Section 4.2.3.7.
4. Check digested products with 1% of agarose gel electrophoresis to determine whether the DNA fragments have been cloned in expressing plasmid successfully (Fig. 9).
5. Submit 1 mL of culture containing the expressing plasmid of target DNA to Qingke Company, China, for sequencing to confirm whether the vectors have been successfully constructed.

4.3.3.4 Expression Quality Analysis

Heterogeneous expression of eukaryotic fungi genes in prokaryotic *E. coli* may come across some problems, such as no gene expression and produce inclusion body. On this account, before purification of target proteins, an assay to detect the expression quality is necessary. Steps are listed below:

1. Add 10 μL of the recombinant plasmids pET28a-*Au8003*, pET28a-*Au13192*, pET28a-*Au6298*, and pET28a to 100 μL *E. coli* BL21

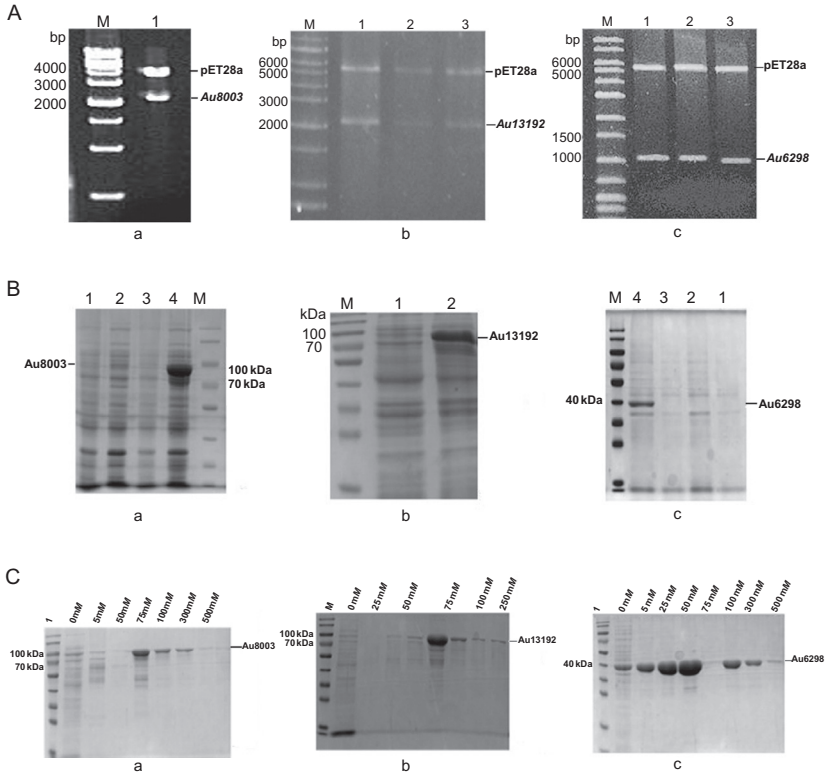

Fig. 9 Expression and purification of Au8003, Au13192, and Au6298. (A) Double digestion of cloning vectors pET-*Au8003*, pET-*Au13192*, pET-*Au6298*. (a) Double digestion of pET-*Au8003*. M: marker; lane 1: pET-*Au8003* double digestion product. (b) Double digestion of pET-*Au13192*. M: marker; lanes 1–3: pET-*Au13192* double digestion product. (c) Double digestion of pET-*Au6298*. M: marker; lanes 1–3: double digestion product of pET-*Au6298*. (B) SDS-PAGE electrophoresis result of expression detection of Au8003, Au13192, and Au6298. (a) Overexpression of *Au8003* gene in *E. coli* BL21 (DE3). Lane M: marker; lane 1: uninduced (without IPTG); lane 2: induced (with IPTG) bacterial protein carrying plasmid pET28a; lane 3: uninduced bacterial protein carrying *Au8003* gene; lane 4: induced bacterial protein carrying *Au8003* gene. (b) Overexpression of *Au13192* gene in *E. coli* BL21 (DE3). Lane M: marker; lane 1: uninduced bacterial protein carrying the 2.1 kb *Au13192* gene; lane 2: induced bacterial protein carrying the 2.1 kb *Au13192* gene. (c) Overexpression of *Au6298* gene in *E. coli* BL21 (DE3). Lane M: marker; lane 1: uninduced bacterial protein carrying plasmid pET28a; lane 2: induced bacterial protein carrying plasmid pET28a; lane 3: uninduced bacterial protein carrying *Au6298* gene; lane 4: induced bacterial protein carrying *Au6298* gene. (C) SDS-PAGE electrophoresis result of different imidazole concentration elutions of Au8003, Au13192, and Au6298. (a) Au8003 purification with different concentration of imidazole. Lane 1: marker; 0, 5, 50, 75, 100, 300 and 500 m*M* represent for corresponding imidazole concentration elutions. (b) Au13192 purification with different concentration of imidazole. Lane M: marker; 0, 25, 50, 75, 100, and 250 m*M* represent for corresponding imidazole concentration elutions. (c) Au13192 purification with different concentration of imidazole. Lane 1: marker; 0, 5, 25, 50, 75, 100, 300, and 500 m*M* represent for corresponding imidazole concentration elutions, respectively.

(DE3) broth on ice for 30 min, respectively. Heat at 42°C for 45–60 s. Keep on ice for 2 min.

2. Inoculate each of the engineered *E. coli* BL21 (DE3) strains containing recombinant plasmid in 5 mL kanamycin (50 μg/mL)-resistant LB medium, culture at 37°C, 220 rpm, overnight.
3. Inoculate each of the fresh culture to another 5 mL LB broth of kanamycin (50 μg/mL) resistant with 1% inoculation. Culture at 37°C for 2–3 h, till the OD_{600} reaches to 0.4–0.6 at 37°C.
4. Take out 1 mL each cultures, then add IPTG to the rest of culture to concentration of 1 m*M*. Continue the inducted culture for 3–5 h.
5. Take 200 μL of each culture of before and after induction, add 5 × SDS-PAGE buffer and boil for 10 min, centrifuge at 12,000 × *g* for 10 min, at room temperature check the supernatant with SDS-PAGE (dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis).

The results show that, comparing with the control, new protein bands appear after induction (Au8003: 82 kDa; Au13192: 80 kDa; Au6298: 39 kDa), which agree with the target proteins' size, indicating that the target proteins are successfully expressed in *E. coli* BL21 (DE3) (Fig. 9).

4.3.3.5 Purification of Expressed Proteins

In order to get enough quantity of target protein for in vitro analysis, the qualified engineering *E coli* strain is cultured in 500 mL LB broth to accumulate cells and then for protein extraction and purification. The process is as following:

1. Inoculate engineered *E. coli* BL21 (DE3) strains containing recombinant plasmid individually in 5 mL LB broth of kanamycin (50 μg/mL) resistance. Cultivate at 37°C, 220 rpm, overnight.
2. Inoculate each fresh culture to 500 mL LB medium containing kanamycin (50 μg/mL), cultivate at 37°C, 220 rpm. Culture at 37°C for 2–3 h, till the OD_{600} reaches to 0.4–0.6 at 37°C.
3. Add IPTG to a final concentration of 0.1 m*M* to induce the protein expression at 18°C for 16–18 h.
4. Centrifuge at room temperature, 5000 × *g* for 5 min to collect *E. coli* cells, wash the cells with ddH_2O twice.
5. Resuspend the *E. coli* cells with 10 mL HEPES buffer and ultrasonicate on ice (ultrasonicate 5S, 5S interval, power 300 W) for 30 min.
6. Centrifuge at 4°C, 10,000 × *g* for 20 min, filer the supernatant with 0.45 μm filter membrane to remove insoluble substances.
7. Add 2 mL of nickel beads onto the column, let the anhydrous ethanol fully flow out of the column under gravity.

8. Wash column with 20 mL aseptic ddH_2O and balance the column with 20 mL HEPES buffer.
9. Add supernatant containing each target protein to the nickel column, collect effluent liquid, detect with SDS-PAGE.
10. Elute nickel column with iminazole elution at concentrations of 0, 5, 25, 50, 75, 100, 250, 300, 500 m*M*, collect the elution and detect with SDS-PAGE (Fig. 9).
11. Combine iminazole fractions containing target proteins together. Concentrate to approximately 2.5 mL with Ultra-15 Centrifugal Filter Devices.
12. Balance GE Healthcare PD-10 desalting column with 10 mL balance buffer, discard the filtrate. Repeat 4 times.
13. Load the 2.5 mL concentrated protein solution onto the desalting column till it is fully soaked in the column. Discard the filtrate.
14. Add 3.5 mL HEPES desalination buffer. Collect the elution which contain the purified protein. Deposit at −80°C for further use.

4.3.4 Notes

1. To improve the ligation efficiency, the total quantity of target DNA fragments and pET28a fragment should be calculated when carrying out ligation; the ratio of target DNA fragment and plasmid fragment should be 6:1 to 7:1.
2. The pH value of HEPES lysis buffer, HEPES desalination buffer, and elution buffer should be adjusted to an appropriate threshold far away from the isoelectric point of the protein, otherwise it will precipitate. Also, according to the properties of different proteins, protease inhibitor (phenylmethylsulfonyl fluoride) or reductive agents (β-ME and dithiothreitol (DTT)) can be added to buffer to ensure protein activity.
3. Low temperature (4°C) is required during the purification process to keep the protein stable.

4.4 In Vitro Enzymatic Reaction

In vitro enzymatic reaction is a valid method to study functions of terpene synthetase (Anthony et al., 2009; Bian et al., 2017; Chiba et al., 2013). This method provides a simple background, and many influence factors are eliminated, which is helpful to identify the function of target protein.

An in vitro reaction system for terpene synthetase catalization including substances as following: DMAPP, GPP, FPP, GGPP, and IPP are substrates (Chiba et al., 2013; Toyomasu et al., 2007); $MgCl_2$ is needed to provide Mg^{2+} for reaction as a cofactor (Laskovics & Poulter, 1981).

Low concentration (<10 m*M*) of DTT is added to protect target proteins against inactivation by oxidation on disulfide bond (Aros et al., 2012; Lin et al., 2014).

We describe the in vitro biochemical reaction of the purified proteins Au8003, Au13192, and Au6298 in this section, which are examples of the sesterterpene, diterpene, and FPP synthases, respectively.

4.4.1 Equipment

- Grant GD120 water bath

4.4.2 Buffers and Reagents

- Substrates: IPP, DMAPP, GPP, FPP, GGPP, from Sigma-Aldrich.
- HEPES desalination buffer: 20 m*M* HEPES, 150 m*M* NaCl, 25% glycerol.

4.4.3 Procedure

4.4.3.1 Au8003 in vitro Reaction

Au8003 is predicted to be a chimeric sesterterpene synthetase. It is supposed that four kinds of substrates, including DMAPP, GPP, FPP, and GGPP, can be catalized by Au8003 on adding IPP to produce GFPP, and then cyclized by the same protein to produce ophiobolin F. The in vitro biochemical reactions are carried out as following:

1. Take five 1.5-mL tubes as reaction vessels, and label them with numbers 1–5.
2. Add four groups of substrates: 50 μ*M* DMAPP with 50 μ*M* IPP, 50 μ*M* GPP with 50 μ*M* IPP, 50 μ*M* FPP with 50 μ*M* IPP, and 50 μ*M* GGPP with 50 μ*M* IPP, into tubes Nos. 1–4, respectively.
3. Add 50 μ*M* DMAPP and 100 μ*M* IPP into the tube No. 5 as a control.
4. Add His-T7-tag-Au8003 recombinant protein (6.1 μ*M*) into tubes Nos. 1–4, add the same volume boiled protein into tube No. 5.
5. Add HEPES reaction buffer, which contains 20 m*M* HEPES, 10% glycerol, 5 m*M* DTT, and 5 m*M* $MgCl_2$ into all of the five tubes, to the final volume of 200 μL.
6. Mix the components by vortex oscillation. Centrifuge at 3500 × *g* for 1 min at room temperature.
7. Incubate at 30°C for 3 h. Add equal volume of *n*-hexane to terminate the reaction.

4.4.3.2 Au13192 in vitro Reaction

Au13192 is a hypothetical diterpene synthetase; in this reaction, three groups of substrates (DMAPP with IPP, GPP with IPP, FPP with IPP) are subjected to test its catalytic activity. Steps are listed below:

1. Take four 1.5-mL tubes as reaction vessels and label them with numbers 1–4.
2. Add three groups of substrates, 50 μ*M* DMAPP with 50 μ*M* IPP, 50 μ*M* GPP with 50 μ*M* IPP, and 50 μ*M* FPP with 50 μ*M* IPP into tubes Nos. 1–3, respectively.
3. Add 50 μ*M* DMAPP and 50 μ*M* IPP into tube No. 4 as a control.
4. Add His-T7-tag-Au13192 recombinant protein (6.1 μ*M*) into tubes Nos. 1–3, add the same volume boiled protein into tube No. 4.
5. Add HEPES reaction buffer, which contains 20 m*M* HEPES, 10% glycerol, 5 m*M* DTT, and 5 m*M* $MgCl_2$ into all of the four tubes, to the final volume of 200 μL.
6. Mix the components by vortex oscillation. Centrifuge at 3500 × *g* for 1 min at room temperature.
7. Incubate at 37°C for 3 h, add equal volume of *n*-hexane to terminate the reaction.

4.4.3.3 Au6298 in vitro Reaction

Au6298 is predicted as a FPP synthetase, and using DMAPP and IPP as substrates, the reaction steps are as following:

1. Take two 1.5-mL tubes, label them with Nos. 1 and 2.
2. Add 50 μ*M* DMAPP and 50 μ*M* IPP into both of the tubes.
3. Add purified His-T7-tag-Au6298 recombinant protein (4.2 μ*M*) into tube No. 1, and add the same volume boiled protein into tube No. 2 as a control.
4. Mix the components by vortex oscillation. Centrifuge at 3500 × *g* for 1 min at room temperature.
5. Add HEPES reaction buffer which contains 20 m*M* HEPES, 10% glycerol, 5 m*M* DTT, and 5 m*M* $MgCl_2$ up to 600 μL into both of the tubes.
6. Incubate at 30°C for 5 h and add equal volume of H_2O saturated *n*-butanol to end the reaction.

4.4.4 Note

1. After adding protein to the reaction system, be careful to observe whether the protein is precipitate to avoid negative result caused by protein denaturation.

4.5 GC-MS Analysis

GC-MS is a rapid method that provides rich information on natural products analysis and is used to analyze complex organic and biochemical mixtures (Hussain & Maqbool, 2014). Taking advantage of this method, in vitro products of purified terpene synthetase and in vivo products by heterologous expression can be analyzed rapidly and conveniently (Chai et al., 2016; Chiba et al., 2013; Matsuda et al., 2015; Okada et al., 2016).

Cyclic terpenes products of the in vitro reaction can be detected directly by GC-MS. But for the polyprenyl pyrophosphate end product of the farnesol synthase, it should be dephosphorized to the corresponding alcohol, then detected by GC-MS. Acid phosphatase is recommended in this case (Fujii, Koyama, & Ogura, 1982).

In this section, we show the process of GC-MS analysis of each reaction product of Au8003, Au13192, and Au6298. The peaks show on the gas chromatograph of those molecules that their corresponding MS data are in agreement with the prediction of a sesterterpene ophiobolin, a diterpene variediene, and a farnesol (Fig. 10).

4.5.1 Equipment

- Miulab NK200-1B digital dry bath
- Varian 450-GC-320TQ GC-MS
- Varian FactorFour VF-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness)

4.5.2 Buffers and Reagents

- *n*-Hexane for extraction
- *n*-Butanol saturated by ddH_2O
- Acetate buffer (pH 6.0): sodium acetate 54.6 g, 1 *M* acetic acid 20 mL, add H_2O to 500 mL
- Methanol for washing syringes
- Potato acid phosphatase, purchased from Sigma-Aldrich

4.5.3 Procedure

4.5.3.1 Sample Preparation of Au8003 and Au13192 in vitro Reaction Products

1. For Au8003 and Au13192 in vitro reaction products, extract the resultants using *n*-hexane at the same volume for 3 times after the reaction, respectively.
2. Evaporate the organic solvent by dry bath.
3. Add 50 μL *n*-hexane to dissolve the products and filter the solution.
4. Load the filtered *n*-hexane solutions into chromatography bottles.

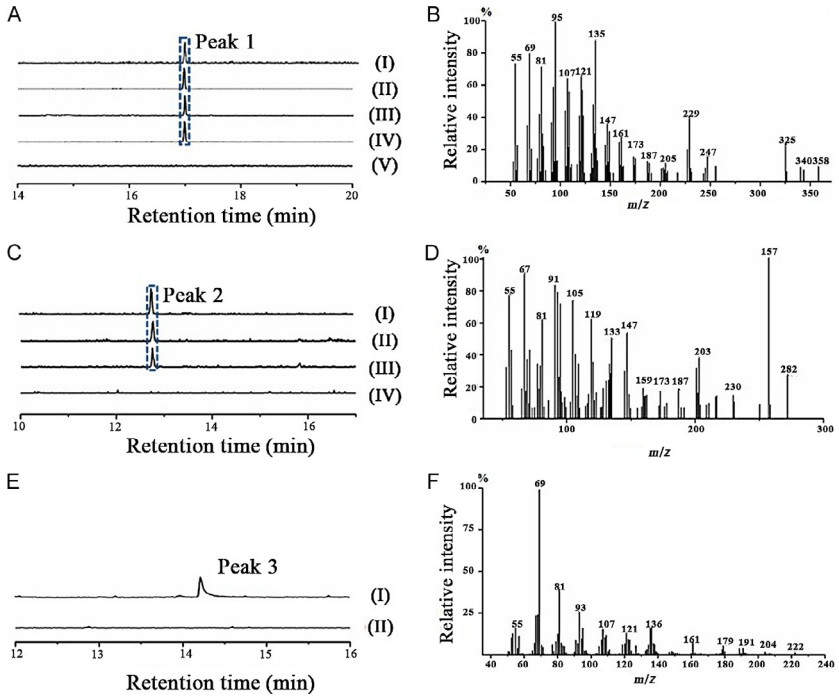

Fig. 10 GC-MS profile of Au8003, Au13192, and Au6298 reaction products. (A) The chromatograms of Au8003 reaction product ophiobolin F extracted at *m/z* 358: (I) GGPP + IPP, (II) FPP + IPP, (III) GPP + IPP, (IV) DMAPP + IPP, and (V) Control, and MS Spectra of peak 1 (B); (C) Chromatograms of Au13192 reaction product variediene extracted at *m/z* 272: (I) FPP + IPP, (II) GPP + IPP, (III) DMAPP + IPP, (IV) Control, and MS Spectra of peak 2 (D); (E) Chromatograms of phosphatase hydrolysate of Au6298 reaction product FPP extracted at *m/z* 222: (I) DMAPP + IPP, (II) Control, and MS Spectra of peak 3 (F).

4.5.3.2 Sample Preparation of Au6298

1. For Au6298 reaction product, after the synthesis reaction, extract the resultant using water saturated *n*-butanol at the same volume for 3 times.
2. Evaporate the organic solvent by dry bath.
3. Add 5 μL acetate buffer, 0.05 μL Triton X-100, 30 μL methanol, 2 μL potato acid phosphatase to a 1.5-mL tube, then add ddH_2O to 50 μL.
4. Mix the components by vortex oscillation. Centrifuge at 3500 × *g* for 1 min at room temperature.
5. Incubate at 37°C for 3 h.
6. Extract the resultant using *n*-hexane at the same volume for 3 times after the synthesis.
7. Evaporate the organic solvent by dry bath.
8. Dissolve the products with 50 μL ethyl acetate and then filtered to chromatography bottle.

4.5.3.3 Preparation for Running GC-MS

1. Before sample injection, the microsyringe should be washed by methanol.
2. Inject 1 μL *n*-hexane to check baseline of GC-MS.

4.5.3.4 GC-MS Conditions

1. For in vitro reaction products of Au8003 and Au13192, inject each sample into the column at 60°C in the splitless mode. After a 2-min isothermal hold at 60°C, increase the column temperature to 150°C at 30°C min^{-1}, from 150°C to 180°C at 10°C min^{-1}, from 180°C to 210°C at 2°C min^{-1} with a 5-min isothermal hold at 210°C. Set the helium carrier gas flow rate at 0.66 mL min^{-1}.
2. For farnesol analysis (dephosphorized product of Au6298), inject the sample into the column at 80°C in the splitless mode. After a 2-min isothermal hold at 80°C, increase the column temperature to 290°C at 10°C min^{-1}, with a 5-min isothermal hold at 290°C. Set the helium carrier gas flow rate at 0.66 mL min^{-1}.

4.5.4 Note

1. Samples for GC-MS analysis should have no high concentration substances, no sediment, or no water; otherwise GC-MS instrument will be damaged.
2. Organic solvents used to extract products can be adjusted according to the polarity of terpenes.
3. GC temperature programming will influence the retention time of sample, so the temperature programming should keep the same while running one specific product.

5. DISCUSSION AND CONCLUSION

With the development of genome sequencing, more and more SM-related unknown enzyme will be identified. The first step is to predict the function of an interested gene or gene clusters. A high quality genome sequence is required, and the software that helps to identify the SM gene clusters from the genome sequence is important. The bioinformatics analysis for terpene synthases is relatively immature, particularly for chimeric fungal terpene synthases, but can be carried out through local alignment methods, for instance, the extensively used BLAST algorithm (Altschul et al., 1990; Yamada et al., 2015). AntiSMASH, a powerful software for SM searching

(Weber et al., 2015), in our case gave only 11 terpene gene clusters. But using "terpene synthase" or "terpene cyclase" as the key word to search against the local gene annotation data base, and confirmed by using the typical conserved sequences of aspartate-rich DDXX(D/E) motif and the NSE/DTE triad, (N/D)DXX(S/T)XX(K/R)(D/E), 27 terpene synthase genes were selected. During the past decade, more and more chimeric terpene synthases were discovered, with fantastic catalytic characteristics (Bian et al., 2017; Chai et al., 2016; Chiba et al., 2013; Minami et al., 2009; Qin et al., 2016; Toyomasu et al., 2007; Ye et al., 2015). Hence, the combination of software and manually local database searching is recommended on thoroughly searching of terpene synthases from a fungal genome. Furthermore, a thorough analysis of terpene synthases on genome scale, resulted in the discovery of multiple gene clusters involved in the biosynthesis of sesterterpene ophiobolin in *A. ustus* 094102 (Chai et al., 2016). This network biosynthesis may be explained by the characteristics of chimeric terpene synthase enzymes (i.e., Au8003 and Au13192) whose PT domain can catalyze chain elongation from each substrate of DMAPP, GPP, FPP, or GGPP. These precursors could be a pool for these enzymes in the fungal strain and affect the end terpene product synthesis.

The method of in vivo gene functional identification for fungi may encounter the challenge of constructing mutant strains owing to protoplast preparation and choice of resistance genes for yielding correct transformations (Pudake, Kumari, Sahu, & Sultan, 2017). The other methods employed in fungi for disrupting a gene/cluster or blocking its expression are TALENs and CRISPR/Cas, RNAi, ribozymes, etc. (Wang et al., 2017). For *Aspergillus* species, the methods for mutant alleles can be constructed by PEG-mediated protoplast fusion, electroporation, shock waves, and biolistic transformation fusion such as *A. tumefaciens* (Pudake et al., 2017). If the object of interest is a nonmodel organism, the process of targeting specific gene/cluster is possibly difficult. In this case, a CRISPR–Cas9-based system facilitates in the genetically manipulating of filamentous fungi (Nødvig, Nielsen, Kogle, & Mortensen, 2015).

The in vitro method of enzyme function verification needs to obtain the purified enzyme of interest first and carry the corresponding reaction subsequently. The common hosts for protein expression are *Saccharomyces cerevisiae* (Cacho & Tang, 2016; Liu, Martínez, Liu, Petranovic, & Nielsen, 2014) and *E. coli* (Rosano & Ceccarelli, 2014). In this chapter, the function of the enzymes Au8003, Au13192, Au6298 were revealed by this means via expression and purification from *E. coli* BL21 (DE3)

and verified their function by the in vitro reactions. It should be kept in mind that the real function and the physiological activity of the enzyme in the native host may be different from the in vitro biochemical reaction. Another method for functional characterization is cloning the corresponding coding gene in proper heterologous hosts, such as Aspergilli (Anyaogu & Mortensen, 2015; Lv et al., 2017), and thus revealing the function via comparing product profiles.

In conclusion, this chapter systematically introduces the methodology for the elucidation of terpene synthases and hopes to boost the development of unknown terpene synthase, in particular, chimeric synthase, since there are many novel terpene compounds discovered from marine fungi (Ebel, 2010; Elissawy, El-Shazly, Ebada, Singab, & Proksch, 2015). Major breakthroughs in other fields, such as the characterized crystalline structure of a chimeric synthase PaFS (Chen, Chou, Toyomasu, Cane, & Christianson, 2016) and maturing synthetic biology platform (Gruchattka, Hädicke, Kayser, Klamt, & Schütz, 2013), weighed well in broadening the applications of this chapter.

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