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•Research article•

Genome mining of fungal globin-like enzymes for catalyzing the synthesis of linear terpenes

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[ABSTRACT] Genome mining for the search and discovery of two new globin-like enzymes, TriB from *Fusarium poae* and TutaA from *Schizophyllum commne*, are involved in the synthesis of two linear terpenes tricinonoic acid (**1**) and 2-butenedioic acid (**3**). Both *in vivo* heterologous biosynthesis and *in vitro* biochemical assays showed that these two enzymes catalyzed the C-C double bond cleavage of a cyclic sesquiterpene precursor (−)-germacrene D (**7**) and a linear diterpene backbone schizostain (**2**), respectively. Our work presents an unusual formation mechanism of linear terpenes from fungi and expands the functional skills of globin-like enzymes in the synthesis of terpene compounds.

[KEY WORDS] Genome mining; Globin-like enzyme; Linear terpene; C-C double bond cleavage; Fungi
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Introduction

Terpenes, charaterized by polycyclic frameworks, are synthesized from a series of linear, achiral C_{5n} (n = 2, 3, 4, *etc*.) isoprenoid diphosphates and cyclized by terpene cyclases (TCs) [1]. These compounds have drawn increasing attention from chemists and biologists due to their complex structures (such as multiple chiral centres and unusual C-C bond linkages) and diverse pharmacological activities (such as antitumor, antifungal, and anti-inflammatory effects), respectively [2]. In addition to the canonical multiply fused ring systems, some irregular terpene compounds have been identified [3]. Among them, linear terpenes are composed of a unique family compounds, with representative examples shown in Fig. 1A: (1) sesquiterpenes: farnesol from *Candida* sp. [4], fokienol from *Lentinellus* sp. [5], and tricinonoic acid (**1**) family compounds from *Trichoderma* sp. and *Fusarium* sp. [6]; and (2) diterpene schizostain (**2**) and its side chain tailoring derivative 2-butenedioic acid (**3**) from *Schizphyllum sp.* [7, 8]. Notably, in contrast to the canonical polycyclic terpene compounds [9, 10], the biosynthetic mechanism of these linear terpenes and related synthetic enzymes remain largely unknown.

We focused on these unusual linear terpenes, especially their biosynthetic process, based on our previous discovery of a new fungal globin-like enzyme PgMpaB during the biosynthesis of the first-line immunosuppressant mycophenolic acid [11]. PgMpaB contains a conserved DUF2236 domain and catalyzes the cleavage of the terminal olefin (Δ10′) of 6-farnesyl-5,7-dihydroxy-4-methylphthalide (FDHMP, **4**) to its aldehyde intermediate, which is then reduced or oxidized by the host to form **5** and **6**, respectively (Fig. 1B) [11]. PgMpaB represents a new clade of oxidase, which can specifically cleave the C-C double bonds of terpene scaffold [11]. Therefore, the globin-like enzymes from fungal sources (the homologue proteins of PgMpaB) may be responsible for the cleavage of the terpene scaffold, which is possibly the critical step involved in the formation of linear terpenes.

In the current study, a total of ten evolutionary branches of globin-like enzymes from fungi were identified using the sequence similarity network (SSN) analysis-based genome mining strategy. Through *in vivo* heterologous biosynthesis and *in vitro* biochemical assays, TriB from clade 2 was confirmed to catalyze the cleavage of the C_6-C_7 double bond of (−)-germacrene D (**7**) to generate **1**, while TutaA from clade 3 was demonstrated to be responsible for the synthesis of **3** through breaking the terminal olefin of **2**. Our work presents an unusual formation mechanism of fungal linear terpenes

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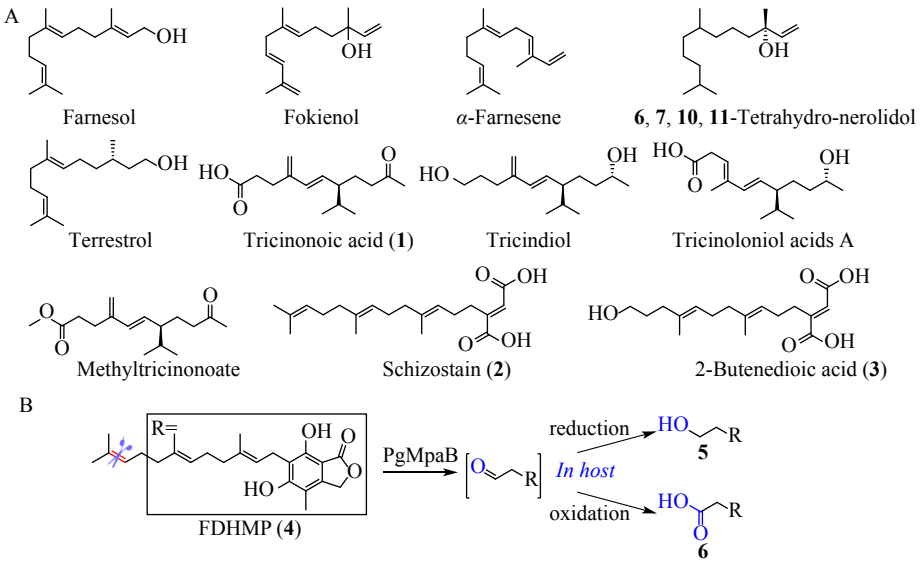

Fig. 1 Representative linear terpenes and function of a globin-like family enzyme PgMpaB. (A) Representative linear terpenes from fungi. (B) PgMpaB catalyzes the cleavage of FDHMP to form its aldehyde intermediate

and expands the functional skills of globin-like enzymes in tailoring of terpene scaffold.

Materials and Methods

Strains and culture conditions

Fusarium poae and *Schizophyllum commne* were obtained from China General Microbiological Culture Collection Center (CGMCC), which were grown at 25 °C for 7 days on PDA medium (2.6% potato dextrose water, 2% agar) for sporulation and on PDB medium for mycelial growth. *Aspergillus nidulans* was used as the host for heterologous expression of the *tri* cluster. *Saccharomyce cerevisiae* BJ5464-NpgA was used for heterologous expression of *tutaA* or *pgMpaB* gene, and for construction of *A. nidulans* overexpression plasmids through homologous recombination. *Escherichia coli* strain XL-1 was used for cloning.

Plasmid construction

The primers used in this study are listed in Table S1, Supporting Information. The plasmids are summarized in Table S2, Supporting Information.

In order to construct the plasmids for expression of *triA* and *triB* in *A. nidulans*, *triA* or *triB* gene with its terminator (−500 bp) was amplified from the gDNA of *F. poae* using primer pairs pANR-*triA*-F/pANR-*triA*-R, and pANU-*triB*-F/pANU-*triB*-R, respectively. In plasmid pIM 8032, *triA* was cloned into vector pANR with promoter *gpdA* through homologous recombination. In contrast, *triB* was cloned into vector pANU with promoter *glaA* to obtain pIM 8037. Plasmid pIM 3040 used for the expression of *tutaA* in *S. cerevisiae* BJ5464-NpgA was constructed by amplifying the corresponding open reading frames from the cDNA of *S. commne* using the primers pYEU-*tutaA*-F/pYEU-*tutaA*-R. Plasmid pIM 4010 for the expression of *pgMpaB* was from our previous research. All the plasmids were confirmed by DNA sequencing.

gDNA isolation and cDNA synthesis

F. poae was cultured in PDB medium (26 g·L^{-1} potato dextrose water) at 25 °C, 220 r·min^{-1} for 4 days. The mycelia were collected and used for gDNA extraction by the cetyltrimethylammonium bromide (CTAB) method (20 g·L^{-1} CTAB, 81.8 g·L^{-1} NaCl, 186.1 g·L^{-1} Na2EDTA·2H2O, and 0.1 mol·L^{-1} Tris-HCl pH 8.0). *S. commne* was cultivated in PDB medium at 25 °C for 4 days to obtain mycelia for RNA extraction by TRLZOL® Reagent (Ambion). RNA sample was then treated with *DNase*, followed by cDNA reverse transcription with the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Bioinformatic analysis

The enzyme function initiative-enzyme similarity tool (EFI-EST) was used to generate the sequence similarity networks (SSNs) of PgMpaB. Cytoscape software platform was adopted for visualizing the complex networks of PgMpaB. The Clustal programs were utilized for carrying out multiple sequence alignment of the globin superfamily protein (TriB, TutaA and PgMpaB) or TC (TriA) and other sequences obtained from NCBI database. Evolutionary analysis was conducted in MEGA7 software, while the phylogenetic tree was inferred using the neighbor-joining method. For gene cluster annotation of *tri* and *tuta*, 2ndFind program was used to predict the open reading frame and intron. BlastX was used to search the NCBI database to predict the function of related genes. DNAMAN8.0 software was used to analyze the sequence similarity of TriB, TutaA and PgMpaB.

Preparation of the protoplasts of A. nidulans

The spores of *A. nidulans* were inoculated in 40 mL CD liquid medium (10 g·L^{-1} glucose, 50 mL·L^{-1} 20 × nitrate salts and 1 mL·L^{-1} trace elements) containing 10 mmol·L^{-1} uridine, 5 mmol·L^{-1} uracil, 1 μg·mL^{-1} pyridoxine and 0.25 μg·mL^{-1} riboflavin at 37 °C for 9 h. After the germination of spores, the cultures were centrifuged at 4 °C, 3750 r·min^{-1} for 5 min to harvest the mycelia. The precipitate was washed twice with 15 mL osmotic buffer (1.2 mol·L^{-1} $MgSO_4·7H_2O$, and 10 mmol·L^{-1} sodium phosphate, pH 5.8) at 4 °C, 3750

r·min^{-1} for 5 min. Then, the precipitate was resuspended with 10 mL osmotic buffer containing 30 mg Lysing Enzymes (Sigma) and 20 mg Yatalase (Takara). The suspension was transferred into 50 mL Erlenmeyer flask, and cultured at 28 °C, 80 r·min^{-1} for 14 h. The culture fluid was directly poured in a sterile 50 mL centrifugal tube and overlaid gently with 10 mL of trapping buffer (0.6 mol·L^{-1} sorbitol and 0.1 mol·L^{-1} Tris-HCl, pH 7.0) before centrifugation at 4 °C, 5000 r·min^{-1} for 20 min. The protoplasm layer was transferred and fully scattered into 2 × STC buffer (1.2 mol·L^{-1} sorbitol, 10 mmol·L^{-1} $CaCl_2$, and 10 mmol·L^{-1} Tris-HCI, pH 7.5), before centrifugation at 4 °C, 5000 r·min^{-1} for 8 min. The supernatant was removed and STC buffer was added to resuspend the protoplasts for transformation.

Heterologous expression of tri cluster in A. nidulans

Briefly, 2 μL plasmids for heterologous expression of *triA* and *triB* (pIM8032 and pIM8037) were added to the 100 μL protoplasts of *A. nidulans*. After incubation on ice for 30 min, 600 μL of PEG solution (60% PEG 4000, 50 mmol·L^{-1} $CaC1_2$, and 50 mmol·L^{-1} Tris-HCl, pH 7.5) was added and placed at room temperature for 20 min. The mixture was cultured on the regeneration dropout solid medium (CD medium with 1.2 mmol·L^{-1} sorbitol, CD-SD medium) at 37 °C for two to three days. The transformants were transferred to solid CD at 37 °C for three to four days for sporulation. Then, the spores were incubated in solid CD-ST medium (20 g·L^{-1} starch, 20 g·L^{-1} casein hydrolysate (acid), 50 mL·L^{-1} nitrate salts, 1 mL·L^{-1} trace elements, and 20 g·L^{-1} agar) at 25 °C for 3.5 days. The products were extracted by ethyl acetate. The organic phase was dried *in vacuo*, and the products were dissolved in methanol for LC-MS analysis.

In vitro characterization of TutaA and PgMpaB

The yeast of *BJ* harboring pIM 3040 or pIM 4010 was cultivated in uracil dropout medium overnight. The culture was transferred into 25 mL YPD medium at 28 °C, 250 r·min^{-1} for an additional 48 h to induce gene expression. Then, 25 mL of the cells were centrifuged at 4000 r·min^{-1} for 5 min. The resultant pellets were resuspended in 2 mL extraction buffer (50 mmol·L^{-1} Tris-HCl, pH 7.5, 1 mmol·L^{-1} EDTA, 0.6 mol·L^{-1} sorbitol, 1 % bovine serum albumin and 2 mmol·L^{-1} *β*-mercaptoethanol), to which glass beads (425−600 μm in diameter, Sigma) were added, and the cell wall was disrupted in a vortex shaker for 1 min with 1 min intervals on ice for a total of 12 min. Then, the cell extraction was centrifuged at 4 °C, 4000 r·min^{-1} for 5 min to remove the cellular debris. Then the cell free extraction was obtained by centrifugation at 4 °C, 16 000 r·min^{-1} for 40 min, and the resultant pellets were resuspended again using buffer C. *In vitro* characterization of TutaA and PgMpaB were performed in 100 μL reaction mixture containing 200 μmol·L^{-1} substrates. The reaction mixture was incubated at 25 °C for 24 h and quenched with 500 μL ethyl acetate. The samples were dried and resolved in CH_3OH for LC-MS analysis.

Chemical analysis of samples

LC-MS analysis was performed on a Waters ACQUITY H-Class UPLC-MS system coupled to a PDA detector and a SQD2 mass spectrometer (MS) detector with an ESI source. Chromatographic separation was performed at 35 °C using a C_{18} column (ACQUITY UPLC® BEH, 1.7 μm, 2.1 mm × 100 mm, Waters). LC-MS metabolite profiles were analyzed on a Waters UPLC-MS system using the method as follows: chromatographic separation was achieved with a linear gradient of 5% to 99% MeCN-H_2O (both with 0.02% *V/V* formic acid) in 12 min followed by 99% MeCN for 4 min. The MS data were collected in the *m/z* range 150−1500 at the positive and negative modes simultaneously.

*Purification and structural characterization of compound **1***

Compound **1** was produced by *A. nidulans*: pIM8032 + pIM8037 in liquid CD-ST medium 7.6 L at 25 °C, 220 r·min^{-1} for 3.5 days. The supernatant and the mycelia were extracted with ethyl acetate and acetone for three times, respectively. The extracts were combined and concentrated in a rotary evaporator. The residue (1.4 g) was separated by medium pressure liquid chromatography (MPLC) Reveleris® X2 (BUCHI, Inc) with a linear gradient of 40% to 90% MeOH in H_2O over 40 min. The fractions (46 mg) containing **1** was further purified by semi-preparation high performance liquid chromatograph (HPLC) using a Shimadzu LC-20AR Prominence UFLC system (column: YMC-Pack ODS-A, 5 μm, 10 mm × 250 mm; solvent: MeOH-H_2O, 70 : 30; flow: 2.5 mL·min^{-1}; detector: 210 nm; and t_R = 24.0 min) to yield **1** (15 mg).

*Purification and structural characterization of compound **2** and **3** from S. commune*

S. commune was cultured on rice at 25 °C for 30 days to produce compounds **2** and **3**. The culture was extracted with ethyl acetate/acetone (*V/V*, 3/1) for three times. The extracts were centrifuged to remove the cell pellet and the supernatant was evaporated to dryness under reduced pressure to afford a residue (22 g). The residue was initially separated by silica gel column chromatography to give fractions 1−12. Fraction 7 (1.643 g) containing **2** was separated by a MCI chromatography column with a linear gradient of methanol-water as the eluting solvent system. **2** was mainly concentrated in 100 % (*V/V*) methanol-water fractions through LC-MS analysis. The fraction was re-chromatographed over Sephadex LH-20 (150 mm × 10 mm), eluting with MeOH to afford compound **2** (5 mg). Fraction 9 (1.263 g) containing **3** was also separated by MCI with a linear gradient of methanol-water to give fractions 9.1−9.25. Fractions 9.18−9.19 (28 mg) containing **3** were separated by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μm, 10 mm × 250 mm; solvent: MeOH-H_2O, 63 : 37; flow: 2.5 mL·min^{-1}; and detector: 210 nm) to yield **3** (3 mg).

NMR spectra were recorded on Bruker AV-400 (400 HMz) spectrometers, with TMS as an internal standard at the Analysis and Testing Center of Southwest University.

Results and Discussion

SSN analysis identifies ten evolutionary clades of globin-like enzymes from fungi

To discover more fungal globin-like enzymes and confirm their roles in the biosynthesis of natural products, we used PgMpaB as the screen hit. With the aid of SSN analysis, a total of 957 fungal proteins containing DUF2236 domain were confirmed. As shown in Fig. 2, they are divided into ten evolutionary clades, which indicates that (1) fungi are indeed

the abundant natural sources for the discovery of new globin-like enzymes; and (2) except for the PgMpaB clade, the actural function of enzymes from other clades have not been identified, leaving the adequate spaces to explore their function *via in vivo* heterologous biosynthesis or *in vitro* biochemical assays.

*TriB catalyzes the cleavage of the C_6-C_7 double bond of (−)-germacrene D to form **1***

We first carefully analyzed the adjacent genes around the globin-like enzymes in each clade and found that (1) in clade 2, the globin-like enzyme (namely *triB* here) is the neighbor gene of a sesquiterpene cyclase gene (*triA*) in fungus *Fusarium poae* (Fig. 3A). (2) Coexisting of a regulator gene (*triC*) highly suggests that the *tri* cluster is responsible for the production of sesquiterpene compound(s) in *F. poae*, where TriB possibly acts as the tailoring enzyme for the critical conversion step after the formation of the sesquiterpene scaffold and is regulated by TriC. (3) Notably, further bioinformatic analysis found that the *triA-C* three-gene cassette is highly conserved in many clusters (Fig. S1, Supporting Information), which suggests that these three genes should be essential for the synthesis of one sesquiterpene in various fungi.

The sesquiterpene cyclase TriA showed 87/77 similarity/identity to the STC1 (FFUJ_00036), which was previously confirmed to be responsible for (−)-germacrene D (**7**) synthesis in *F. fujikuroi* [12]. We initially attempted to carry out the *in vitro* assay, using the purified TriA with farnesyl pyrophosphate (FPP), to solid the connection between TriA and the formation of **7**. Unfortunately, many attempts to express TriA as a soluble protein from *E. coli* were not successful, thereby precluding direct reaction using purified TriA. However, with respect to phylogenetic tree analysis together with the chemical structures of the cyclized products,-TriA and STC1 were clustered into the same clade (Fig. 3B), thus the product of TriA should be (−)-germacrene D.

Based on the above analysis, the globin-like enzyme TriB possibly catalyzes the cleavage of C_6-C_7 double bond of **7** to form the aldehyde intermediate, which is easy to undergo the oxidation by host to generate **1** (Fig. 3C). In this pathway, TriB independently breaks the C_6-C_7 double bond of **7**, although the previous proposal for the formation of **1** from **7** required a series of oxidases and hydrolases involved [13]. The enzymes for this conversion were previously proposed to be located outside the STC1 cluster, but they were not clarified in *F. fujikuroi* [12].

To prove the hypothesis that TriB is the only candidate for this conversion, we simultaneously expressed *triA* and *triB* genes in *A. nidulans* (Fig. S2, Supporting Information). After three days of culture followed by extraction with ethyl acetate, a new compound *m/z* 251 $[M-H]^-$ was produced in *AN-triAB* strain by liquid chromatography-mass spectrometry (LC-MS) analysis (Fig. 3D). This compound was subsequently purified from the large-scale fermentation of *AN-triAB* strain in CD-ST medium and further confirmed to be **1** by NMR analysis (Table. S4, Supporting Information and Fig. S4-S5, Supporting Information). Therefore, these results demonstrated that TriB indeed catalyzed the cleavage of the

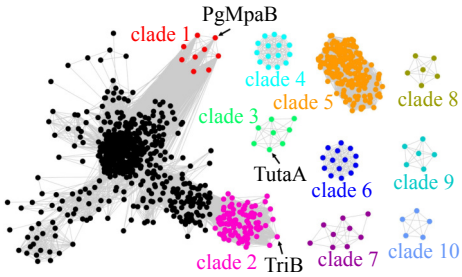

Fig. 2 Protein sequence similarity network (SSN) analysis of PgMpaB and other fungal globin-like family enzymes. The protein sequence number is 957 and the alignment score is 100

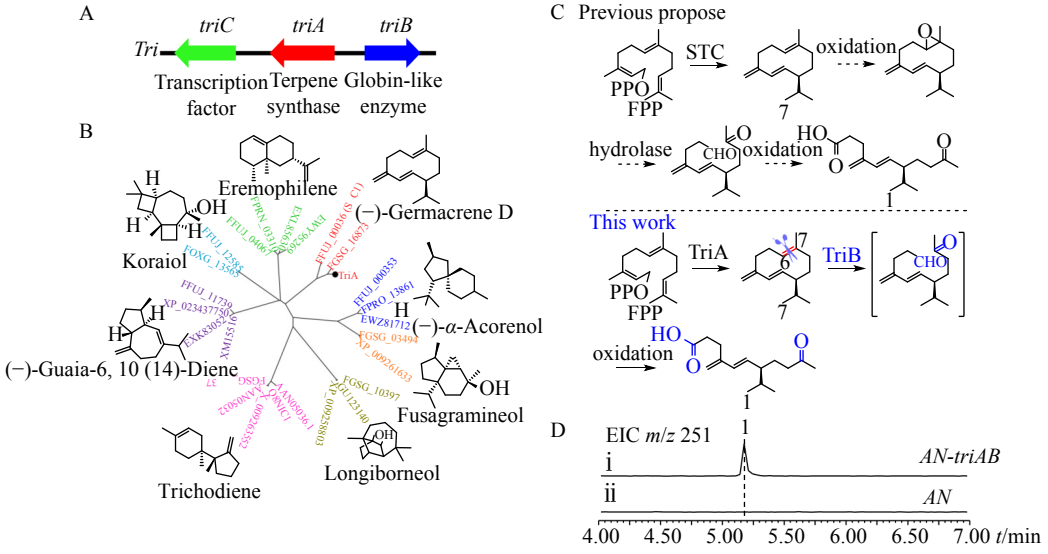

Fig. 3 *In vivo* biochemical assay of TriB. (A) The *tri* gene cluster in *F. poae*. (B) Phylogenetic analysis of fungal TC shows that TriA and STC1 cluster into the same clade. (C) Proposed biosynthetic pathway of tricinonoic acid (1). (D) LC-MS analysis of the *AN-triAB* transformant culture extracts. The EIC was extracted at *m/z* 251 $[M-H]^-$ for 1

C_6-C_7 double bond of (−)-germacrene D to form the linear terpene compound **1**. It is notable that TriB showed only −12% identity to PgMpaB. However, these two enzymes can break the C-C double bond, where the low sequence identity between them may be explained by different substrates. TriB selects cyclic terpene scaffold and PgMpaB prefers the linear terpene scaffold.

*TutaA catalyzes the cleavage of the terminal olefin of schizostain (**2**) to form 2-butenedioic acid (**3**)*

Next, we focused on clade 3, where globin-like enzymes in this clade are almost not clustered with any biosynthetic gene clusters of natural products. One of the enzymes (XP_003030903, namely TutaA here, Fig. 4A) from the eight proteins in clade 3 comes from *Schizophyllum commun* (*Schizophyllaceae*). Previous metabolite analysis showed that *S. commune* mainly produced seven secondary metabolites, which included cancer cell growth inhibitor schizine A/B and epischizine A [14], antitumor drug ergosterol peroxide [14, 15] and schizophyllan [16], squalene synthase inhibitor schizostain (**2**) [7] and 2-butenedioic acid (**3**) [8]. Further structural analysis indicated that **3** should be originated from the terminal olefin cleavage aldehyde intermediate of **2**, where TutaA possibly catalyzed this conversion (Fig. 4B).

To prove this hypothesis, we cultured the strain *S. commune* on solid rice media, purified **2** and **3**, and finally confirmed their structures by NMR analysis (Tables S5-S6, Supporting Information and Figs. S6-S17, Supporting Information). The cDNA of gene *tutaA* was commercially synthesized, which was then cloned and introduced into *S. cerevisiae* BJ5464 (*BJ-tutaA*) under the control of the ADH_2 promoter. Compared with *BJ* control, the microsomal fractions of *BJ-tutaA* were incubated with **2**, obtaining compound **3** (Fig. 4C, i and ii). It is interesting that the trace amounts of the carboxyl acid product **8** was observed (the ratio 3/8 : 13/1), which confirmed that the proposed aldehyde intermediate of **2** is more easily reduced by *S. cerevisiae* (Fig. 4B).

*TutaA has a broad substrate scope towards FDHMP (**4**)*

TutaA showed 45%/30% similarity/identity with PgMpaB (Fig. S3, Supporting Information). TutaA and PgMpaB catalyze the cleavage of terminal double bond of the linear terpene scaffold in C20 and C15 precursors, respectively (Fig. 4D). To confirm the substrate scopes of TutaA and PgMpaB, we first investigated the cleavage ability of PgMpaB on **2**. However, only an extreme trace amounts of **3** was detected (Fig. 4E, i), indicating a low efficiency of PgMpaB towards **2** (C20 terpene moiety). Alternatively, when TutaA was incubated with FDHMP (**4**) using PgMpaB as the positive control, the expected two products **5** and **6** were successfully observed in the TutaA-catalyzed assays (Fig. 4E, ii-IV). These results fully demonstrated that PgMpaB specifically breaks the terminal double bond of **4**; however, TutaA has a broad substrate scope, apart from **2**, it also accepts the C15-length linear terpene scaffold, it could be used as an ideal biocatalytic candidate for the C-C double bond cleavage in the future.

Conclusion

Cleavage of the C-C double bond by different types of enzymes (Fig. 5), such as ABM monooxygenase [17], *α/β*-hydrolase [18], heme dependent dioxygenases [19], non-heme Fe

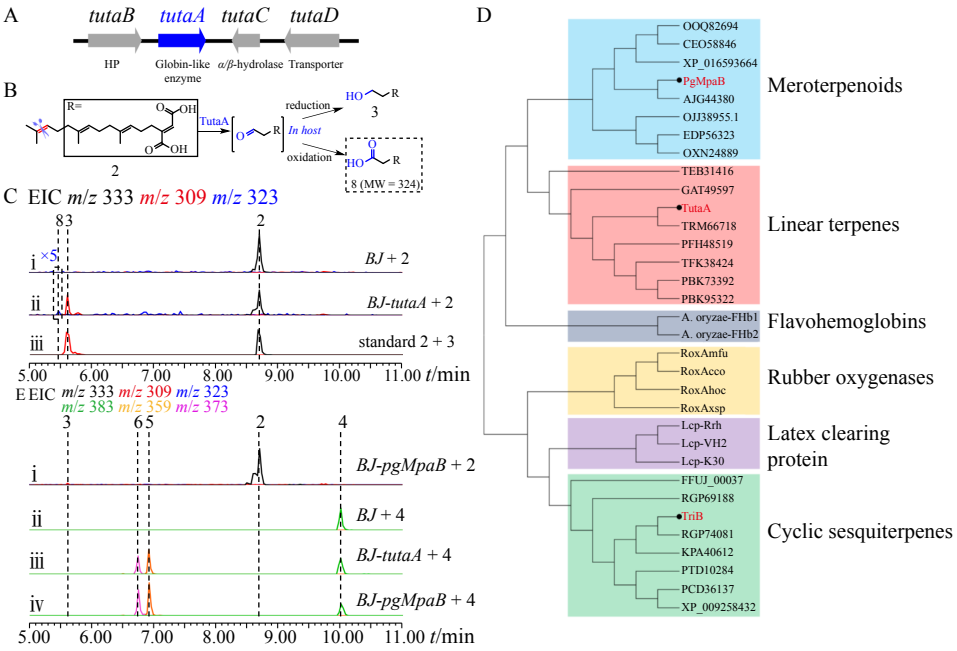

Fig. 4 Biochemical confirmation of the function of TutaA *in vitro*. (A) The *tuta* cluster in *S. commune*. (B) Proposed biosynthetic pathway of 3 from 2. (C) TutaA catalyzes the cleavage of the terminal C-C double bond of 2 to form 3. (D) Phylogenetic analysis of globin-like enzymes indicates C20- and C15-precursors towards TutaA and PgMpaB, respectively. (E) The substrate tolerance of TutaA. The EICs were extracted at *m/z* 333 $[M-H]^-$ for 2, *m/z* 309 $[M-H]^-$ for 3, *m/z* 323 $[M-H]^-$ for 8, *m/z* 383 $[M-H]^-$ for 4, *m/z* 359 $[M-H]^-$ for 5, *m/z* 373 $[M-H]^-$ for 6

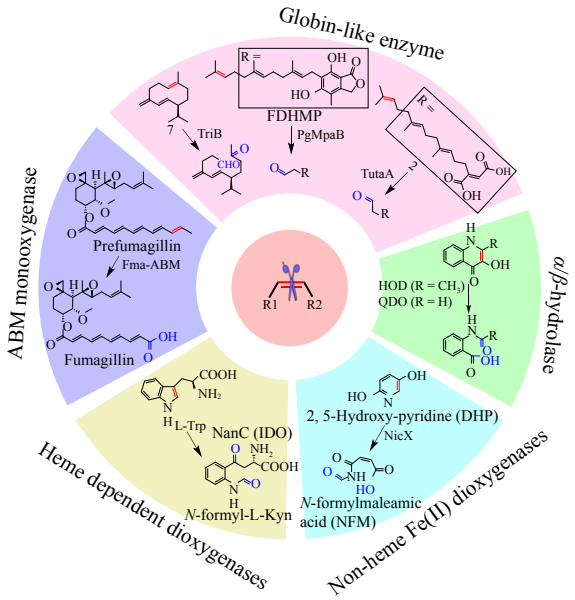

Fig. 5 Five types of enzymes that break the C-C double bond during the biosynthesis of natural products

(II) dioxygenases [20], and globin-like enzymes here, is the essential step for the reconstruction and reformation of the structural scaffolds during the biosynthesis of natural products. In this work, through SSN analysis-based genome mining strategy, ten evolutionary clades of globin-like enzymes from fungi were identified. We confirmed that two new globin-like enzymes, TriB from clade 2 catalyzes the cleavage of the C_6-C_7 double bond of (−)-germacrene D to generate tricinonoic acid; TutaA from clade 3 is responsible for the synthesis of 2-butenedioic acid through breaking the terminal olefin of schizostain. Our work presents an unusual formation mechanism of fungal linear terpenes and deepens our understanding of the biosynthesis of globin-like enzymes in fungi.

Supporting information

Supporting information can be requested by emailing the corresponding author.

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