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Research Paper

Overproduction of geranylgeraniol in *Coprinopsis cinerea* by the expression of geranylgeranyl diphosphate synthase gene

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(*E*, *E*, *E*)-Geranylgeraniol (GGOH) is a valuable ingredient of many perfumes and a valuable precursor for synthesizing pharmaceuticals. In an attempt to increase the GGOH concentration in *Coprinopsis cinerea*, we demonstrated that the expression of geranylgeranyl diphosphate synthase (*ggpps*) gene isolated from *Taxus x media* could promote GGOH production. Furthermore, the concentrations of squalene and ergosterol were measured in the engineered strains. Expectedly, significant decreases of squalene and ergosterol levels were observed in those strains transformed with *ggpps* gene. This could be explained by the partial redirection of metabolic flux from squalene to GGOH, whose biosynthesis competes for the same precursor with squalene. This work suggested that the expression of *ggpps* in higher fungi was an effective method for bio-production of GGOH.

Keywords: *Coprinopsis cinerea* / Ergosterol / Geranylgeraniol / Geranylgeranyl diphosphate synthase / Squalene

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Introduction

(*E*,*E*,*E*)-geranylgeraniol (GGOH) is an acyclic diterpene alcohol which is known to be an important molecule in the synthesis of various terpenes and the geranylgeranylation of proteins. It has been used not only as one of the important ingredients in perfumes, but also as a valuable material for the chemical synthesis of pharmacological agents and hydrophobic vitamins such as 3-allylgeranylgeraniol, vitamin A and E [1].

Fungi produce many well-known bioactive secondary metabolites including terpenoids, which comprise a very large family with over 40,000 molecules identified till date [2–6]. The condensation of two five-carbon units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), is catalysed by prenyltransferases, which produce the linear prenyl diphosphate precursor for each class of terpenes: geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) for the mono-, sesqui- and diterpenes, respectively. For example, paclitaxel (Taxol), an extremely valuable chemotherapeutic agent especially for the treatment of lung, ovarian and breast cancer [7], which was first isolated from the Pacific yew (*Taxus brevifolia*), begins with formation of GGPP and putatively involves 19 additional steps [8]. The ink cap *Coprinopsis cinerea* (also known as *Coprinus cinereus*) is a model organism for studying fruiting body (mushroom) formation in homobasidiomycetes [9–11], which produce numerous bioactive and often cytotoxic sesquiterpenoid secondary metabolites [12, 13]. The availability of genome sequence led to discovery of several sesquiterpene synthase genes from *C. cinerea*. Six sesquiterpene synthases from *C. cinerea* were functionally characterized recently [6], of which only five showed activity. Cop6 is a high-fidelity enzyme whereas Cop4 is very promiscuous, isomerizing the C2–C3 π bond of (*E*,*E*)-FPP and generating multiple cyclization products [14]. In *C. cinerea* strain okayama7#130, of which the genome has been sequenced, a number of genes which encode terpenoid synthases have been detected and annotated [11, 15].

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Even though some open reading frames share sequence homology with genes involved in GGPP biosynthesis, no plausible GGOH has been isolated from *C. cinerea* so far.

Plants are one of the natural sources of terpenes, engineered microbial platforms may be the most convenient and cost-effective approach for large-scale production of terpene-based chemotherapeutics. Two of the most utilized microorganisms for expressing heterologous enzymes for the synthesis of isoprenoids are *Escherichia coli* and *Saccharomyces cerevisiae* [16, 17]. Genes encoding GGPP synthases have been isolated from various organisms. These GGPP synthases contain five conserved regions including the two aspartate-rich DDXX(XX)D and DDXXD motifs (where X is any amino acid) that are implicated in catalyzing the sequential addition of IPP to allylic prenyl diphosphates through Mg^{2+} ions [18].

A cDNA encoding GGPP synthase from *Taxus x media* has been isolated in our laboratory. In the present study, this gene was overexpressed in *C. cinerea* strain LT2 to examine the possibility for GGOH production in higher fungi.

Materials and methods

Materials

Geranylgeraniol, squalene and ergosterol were obtained from the Sigma–Aldrich for calibration purposes. TaKaRa Ex Taq® DNA polymerase, Restriction enzymes, alkaline phosphatase from calf intestine (CIAP) and other modifying enzymes were purchased from Takara. Reagents for GC-FID and HPLC were chromatographically pure. All other chemicals were of analytic grade.

Organisms

E. coli strain DH5α (Invitrogen) was used for cloning of plasmids. Transformation of competent cells was conducted according to standard procedure [19]. Tryptophan auxotrophic *C. cinerea* strain LT2 and the plasmid pCc1001 containing tryptophan auxotroph complementary gene *trp1*$^+$ [19] were kindly provided by Professor Lorna A. Casselton at University of Oxford and Doctor Michael P. Challen at the Horticulture Research International of University of Warwick.

Construction of the expression vectors

The open reading frame of the geranylgeranyl diphosphate synthase gene (*ggpps*) from *T. x media* (GenBank accession number JQ029687) was amplified from plasmid pGEM-GGPP-M1(Full-length cDNA of *ggpps* subcloned into Promega pGEM-T Easy) using the primers 1eF (5′-GACTAGTCATGGCTTACACGGCAATGGCAG-3′) and 1eR (5′-CATGTACATCAGTTTTGCCTGAATGCAATGTAATC-3′), and *Spe*I and *Bsr*GI restriction sites (underlined) were designed for flanking the PCR product at the 5′- and 3′-terminus, respectively. The amplified DNA fragments were digested by *Spe*I and *Bsr*GI and then sub-cloned into pgLes-GGPPs containing the glycerol 3-phosphate dehydrogenase gene (*gpd*) promoter from *Lentinus edodes* and the manganese peroxidase isozyme 1 precursor gene (*MnP-1*) terminator (*Pcterm*) from *Phanerochaete chrysosporium*. The presence of the PCR products was verified by sequencing.

C. cinerea transformation

Protoplasts were prepared from the oidia of *C. cinerea* and performed as previously described [19] except that the Novozym 234 was replaced with Lywallzyme (Guangdong Institute of Microbiology, China) at 10 mg enzyme ml^{-1}. Briefly, the *C. cinerea* strain LT2 was cultivated on complete medium with 1 $mmol^{-1}$ tryptophan at 37 °C for 3 days to harvest oidias. Then the oidias were treated with enzyme for 90 min digestion. 1 μg of the pCc1001 for selection and 1 μg of pgLes-GGPPs were used in each transformation. Transformed protoplasts were inoculated on regeneration agar plates without tryptophan [19]. Putative transformants were picked out for further study onto complete medium.

Transformant analysis

The genomic DNA from the mycelium of *C. cinerea* transformants was extracted according to the methods described by Wang et al. [20]. Total RNA for RT-PCR was isolated using the E.Z.N.A. Fungal RNA Kit (Omega) by following the manufacturer's instructions. The RT reaction was conducted using PrimeScript RT-PCR Kit (Takara) according to the manufacturer's instructions. The *ggpps* gene was amplified by PCR using both genomic DNA and RT reaction products as the templates with 1eF and 1eR primers. All PCR products were sequenced to confirm their identities. Protein was extracted from lyophilized mycelium cultured for 2 weeks [21]. SDS–PAGE was performed on an 8% running gel [22] and resolved proteins were visualized by staining with Coomassie Brilliant Blue G 250. The non-transformed negative control was included in those analyses as well.

Preparation of cell extracts

The strains (LT2, Clg3, Clg4 and Clg5) were cultivated in 250 ml Erlenmeyer flasks in 100 ml of complete medium with slightly shaking at 37 °C for 12 days. At the end of each fermentation process, the mycelia were harvested and separated from the media by filtration, then washed

three times by distilled water. The filtered mycelia were dried by lyophilization for 24 h to determine the biomass. Isoprenoid diphosphates were extracted and hydrolyzed according to the method [23], and sterol preparations were conducted according to the methods previously described in detail [24, 25].

Analysis of geranylgeraniol

Samples were analyzed by GC-FID for the determination of geranylgeraniol contents as described by Vallon et al. [23] with some modifications. Techcomp GC-7890II was fitted with a Phenomenex ZB-5 ms column (30 m × 0.25 mm, df 0.25 μm) and a nitrogen carrier. The sample volume was 1 μl in a splitless manner. The injector temperature was 250 °C. GC oven temperature was initially placed at 50 °C for 2 min, increased with a gradient of 30 °C min^{-1} until 150 °C. And then it was increased with a gradient 10 °C min^{-1} until 270 °C and held for 8 min.

Analysis of squalene and ergosterol

The concentration of squalene and ergosterol was determined by using RP-HPLC analysis as described by Baumann et al. [24] and Mantzouridou et al. [25]. The crude extracts were dissolved in methanol/dichloromethane (9:1, v/v). All samples were filtered through a 0.22 μm membrane filter just before HPLC. RP-HPLC analysis was performed using a Techcomp HPLC system LC2000 sampling from a Rheodyne 7725i sampler. Separation of squalene and sterols was achieved on a reversed phase Luna C18 column (particle size = 5 μm, 250 × 4.6 mm, Phenomenex) maintained at room temperature. The elution solvent was methanol/water (98:2, v/v), the flow rate was set at 1.0 ml min^{-1}, and the injection volume was controlled with a 20 μl loop. Detection and quantification of squalene and ergosterol were at 208 and 285 nm, respectively.

Statistical analysis

All results were submitted to one way analysis of variance (ANOVA) with the critical level $p \leq 0.05$ as the significant difference between the control and treated groups, followed by Duncan's Test and Pearson Correlation using the SPSS package (version 18.0, SPSS, Inc.) software.

Results

Generation and characterization of transformants

We attempted to generate a recombinant strain of *C. cinerea* expressing a *T. x media ggpps* gene encoding for GGPPS which competes for the naturally occurring FPP (Fig. 1). The genetic transformation was performed using the pgLes-GGPPs vector expressing the *ggpps* gene using the *gpd* promoter from *L. edodes* and the *Pcterm* terminator from *P. chrysosporium* (Fig. 2). In the first round of screening, colonies growing at 37 °C for 3–5 days on

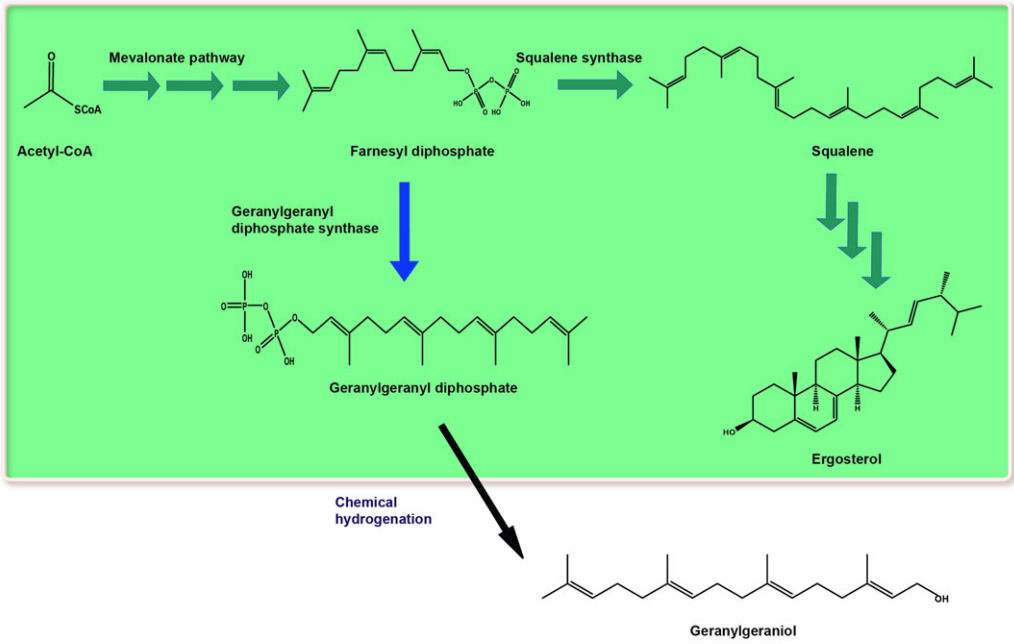

Figure 1. GGOH from chemical hydrogenation of GGPP produced by transgenic *C. cinerea*. The engineered microbe converts FPP into GGPP. Chemical hydrogenation of biosynthetic GGPP leads to GGOH.

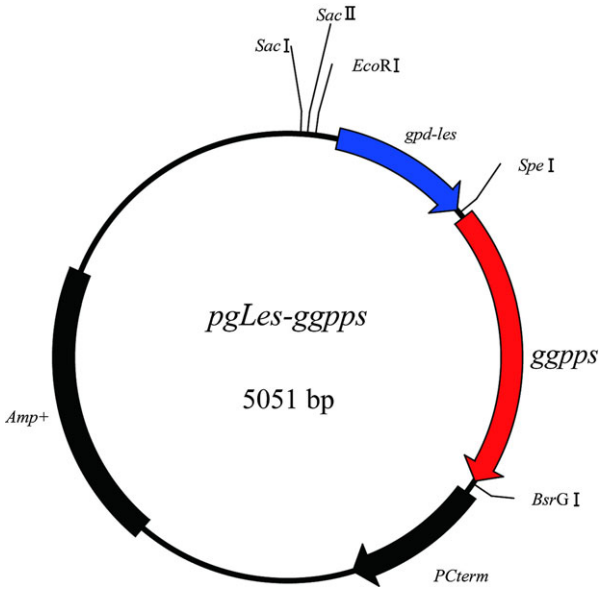

Figure 2. Vector for the expression of *ggpps* in *C. cinerea*. The *ggpps* gene from the *T. x media* was under control of the *gpd* promoter from *L. edodes*.

regeneration minimal agar plates without tryptophan were considered as putative transformants. PCR analysis was carried out to detect the *ggpps* in putative transformants. Positive transformants showed a bright band corresponding to the gene size (about 1,200 bp) (Fig. 3a). The determination of RNA expression levels of the introduced gene in transformants was performed with RT-PCR technique (data not shown). Three transformants were picked out for SDS–PAGE analysis. The result showed that cell extracts from transformants exhibited a clear band with a molecular weight about 43kDa that was the same size as estimated from the deduced amino acid sequence in *ggpps* (Fig. 3b), suggesting that GGPPS was successfully expressed in *C. cinerea* transformants.

GGOH analysis

Geranylgeraniol, a dephosphorylated alcohol of the GGPP, was quantified by GC-FID using authentic standard. Results showed that GGOH content increased in the *C. cinerea* transformants (Fig. 4b) when compared with the wild type strain LT2. The results suggested that the amount of GGOH in LT2 was not detected under our conditions, whereas that of three transformans Clg3, Clg4 and Clg5 were $2.19 \pm 0.09\,\mu g\,g^{-1}$, $1.76 \pm 0.18\mu g\,g^{-1}$ and $1.55 \pm 0.09\mu g\,g^{-1}$, respectively. According to the ANOVA analysis, GGOH production of transformant Clg4 was similar to that in Clg3 ($p = 0.127$) and Clg5 ($p = 0.408$). GGOH production of transformant Clg3 was significantly higher than that od transformant Clg5 ($p = 0.05$).

Squalene and ergosterol analysis

To determine whether intermediates of the mevalonate synthesis route are redirected for GGOH production, ergosterol and squalene were determined individually by RP-HPLC. The time course of ergosterol contents (Fig. 5c) showed that the wild-type strain accumulated nearly 7 μg ergosterol g^{-1} on 12th day, indicating the presence of a sufficient amount of substrate that could be utilized for terpenoid biosynthesis. According to the HPLC analysis, the content of squalene in LT2 was $93.72 \pm 10.66\mu g\,g^{-1}$, whereas that in three transformants Clg3, Clg4 and Clg5 were $59.31 \pm 4.89\mu g\,g^{-1}$, $50.27 \pm 6.10\mu g\,g^{-1}$ and $48.53 \pm 8.72\mu g\,g^{-1}$, respectively. The amount of ergosterol in LT2 was $7.24 \pm 0.97\mu g\,g^{-1}$, whereas that in Clg3, Clg4 and Clg5 were $4.82 \pm 0.88\mu g\,g^{-1}$, $4.16 \pm 0.39\mu g\,g^{-1}$ and $4.06 \pm 0.38\mu g\,g^{-1}$, respectively. These results suggested that squalene production of LT2 was significantly higher than those of all transformants ($p = 0.05$), while all transformants shared the same ability of squalene production ($p = 0.350$). Ergosterol production of LT2 was significantly higher than those of all transformants ($p = 0.05$), while all transformants shared the same

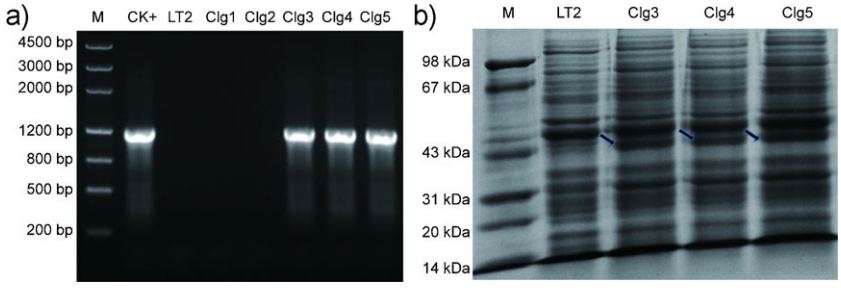

Figure 3. Screening and characterization of transformants. Three transformants (Clg3, Clg4 and Clg5) and nontransformed LT2 were performed both PCR and SDS–PAGE. (a) PCR amplification from genomic DNA with primers 1eF and 1eR. Lanes: M, DNA molecular size markers; Clg1 to Clg5: DNA isolated from putative transformants, respectively; LT2, DNA isolated from nontransformed negative control; CK+, vector pgLes-GGPPs as positive control. (b) SDS–PAGE analysis. Lanes: M, Marker (98, 67, 43, 31, 20 and 14 kDa); LT2, total protein isolated from nontransformed negative control; Clg3, Clg4 and Clg5, total protein isolated from three transformants, respectively. The arrows indicate the distinct protein bands.

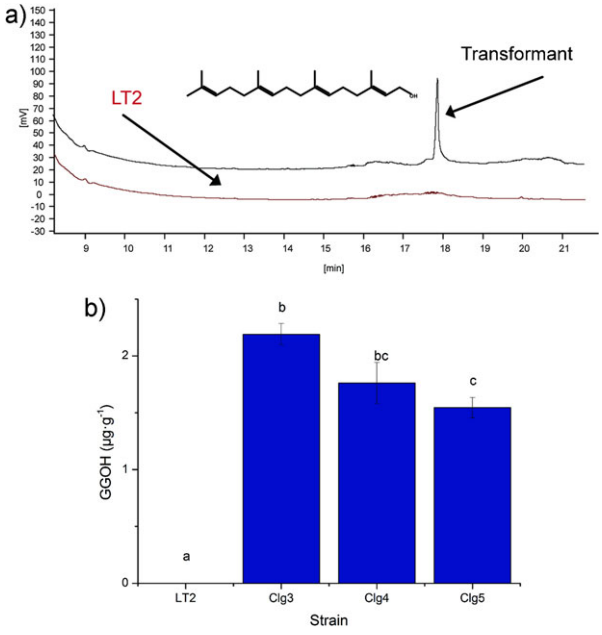

Figure 4. GC-FID detection of the GGOH production. (a) Total ion chromatograms of the samples extracted from LT2 and a randomly selected transformant. (b) GGOH was not detected in nontransformed LT2., significantly lower than transformants ($p = 0.05$). The error bars in the charts indicate standard deviation from the mean of each triplicate treatment. Data with the same superscript indicate no statistical difference, while data with different letters indicate a statistical difference ($p < 0.05$).

ability of ergosterol production ($p = 0.239$). Results of the current study showed that the levels of squalene (Fig. 5a, d) and ergosterol (Fig. 5b, e) decreased in the *C. cinerea* transformants., suggesting that rearrangement of FPP flux in *C. cinerea* resulted in the increase of GGOH level with the decrease of squalene level.

Correlation test

According to correlation analysis ($n = 12$, two-tailed test) of the primary intermediates in introduced pathway, GGOH had a significantly moderate negative correlation with squalene ($r = -0.731$, $p = 0.007$) and a significantly moderate negative correlation with ergosterol ($r = -0.785$, $p = 0.003$). At the same time, squalene was correlated positively and significantly with ergosterol ($r = 0.754$, $p = 0.005$). These observations suggest that higher amount of GGOH results in a significant decrease of squalene and ergosterol.

Discussion

C. cinerea has an intact isoprenoid and terpenoid biosynthetic pathway with a great pharmacological potential [26]. According to the Fungal Genome Initiative (http://www.broadinstitute.org/science/projects/fungal-genome-initiative), a number of genes involved in terpenoid biosynthesis have been detected and annotated [11, 15]. The isoprenoid biosynthetic pathway produces a series of isoprenoid diphosphate intermediates, some of which are incorporated post-translationally into proteins. GGPP exists in cells in equilibrium with their corresponding alcohols, namely GGOH. According to our results, it is sure that detectable pools of GGOH do not exist in wild-type *C. cinerea* cells (Fig. 4a). Recent efforts have been made concerning the engineering of microbes by introducing a combination of several genes. This yields GGOH in *S. cerevisiae* at titers of 134 mg L^{-1} in optimized jar fermentations and of 0.13 mg L^{-1} in *E. coli* [27, 28], respectively. When ergosterol and unsaturated fatty acids are added as supplement to the yeast cultures, squalene accumulates at a maximum yield of ~41 mg kg^{-1} dry weight [29]. In contrast, yield of squalene from *C. cinerea* was found to be ~93 mg kg^{-1} dry weight of cells when no ergosterol and unsaturated fatty acids were added. Similar work has been carried out to investigate the mushroom *Schizophyllum commune* for the production of sesquiterpenes, suggesting a production of 16 mg L^{-1} (+)-valencene by combination expression of valencene synthase and mutated RGS regulatory protein [30]. In the present study, it is concluded that a correlation exists between GGOH's increase and squalene's decrease. At the same time, the growth rate of fungal mycelium has been affected slightly (data not shown). These results implied that the ergosterol and squalene remaining should impair growth of the fungi.

C. cinerea is a model for mushroom-forming fungi with a relatively short life cycle (2 weeks) [9–11]. Genetic modification of this organism is relatively easy, efficient knockout technology is available. For instance, our lab has successfully used *C. cinerea* as a platform mushroom for the heterologous expression of functional proteins and peptides including multifunctional cellulase [31] and immunomodulatory protein [32]. Above all, *C. cinerea* has several abundant terpenes [12, 13]. Several genes have now been expressed in *C. cinerea*, however, more work needs to be done in improving the expression levels and scaling-up to industrial production.

Little is known about the mechanisms that govern the spatial distribution of the enzymes, cofactors and substrates within the fungal cells [23]. It is difficult to overproduce hydrophobic terpenoids including GGOH with metabolically engineered microbes [17, 34]. However, the prospects are bright because *C. cinerea* showed such impressive ability. Our results demonstrate that

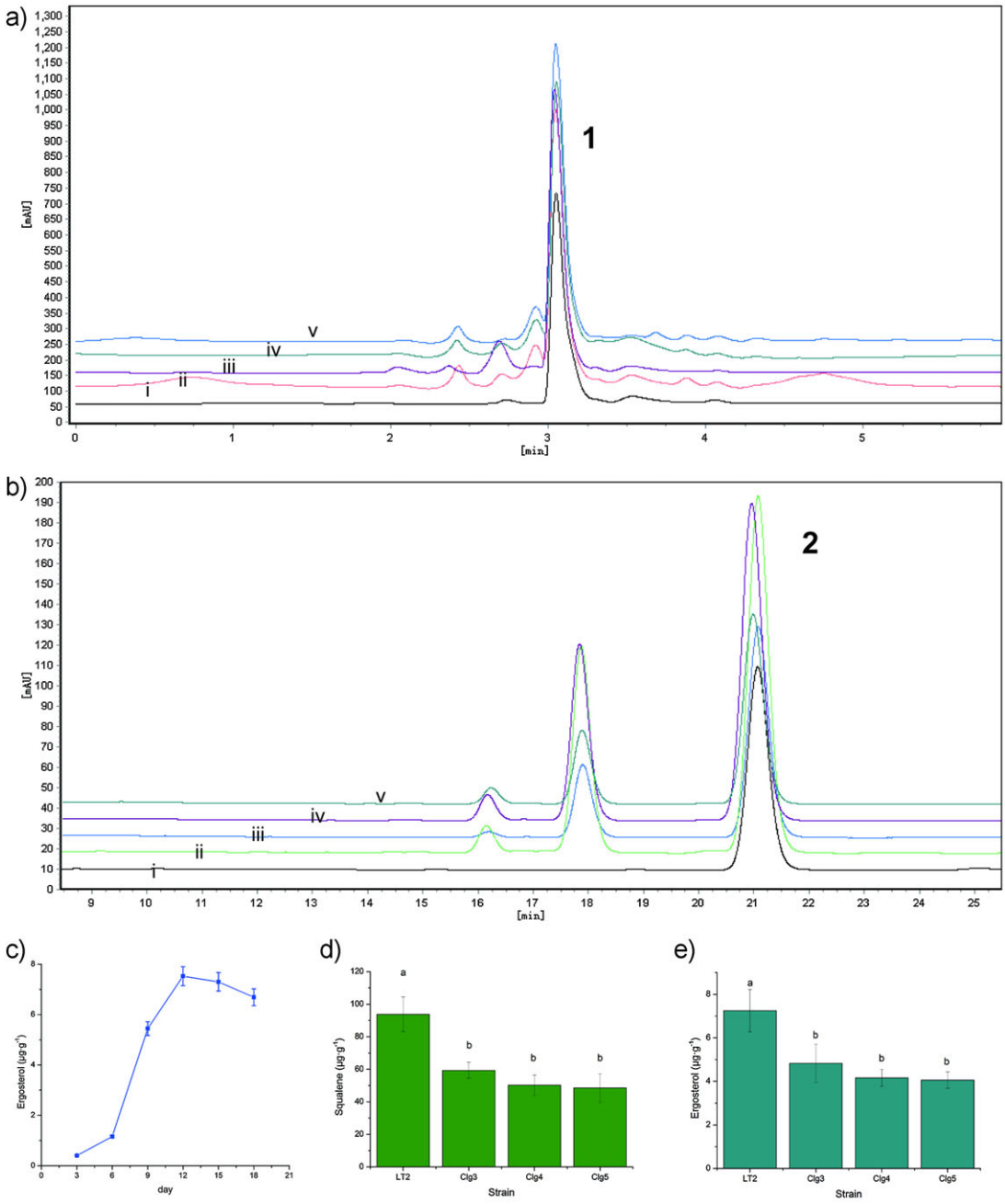

Figure 5. Analysis of squalene (a, d) and ergosterol (b, c, e) in transformants Clg3, Clg4 and Clg5. TIC of the samples: peak a(1) and peak b(2) refers to authentic standards (line i); the others refer to the samples extracted from transformants (Clg3, iii; Clg4, iv; Clg5, v) and LT2 (ii). (c) Time courses of ergosterol production of the strain LT2 cell line cultured for 18 days in the production medium. (d) Squalene production of LT2 was significantly higher than those of all transformants ($p = 0.05$). (e) Ergosterol production of LT2 was significantly higher than those of all transformants ($p = 0.05$). The error bars represent the standard deviation of three replicates. Data with different letters indicate a statistical difference ($p < 0.05$).

bioengineering of the spatial organization of metabolic enzymes around a branch point is a potential means for diverting FPP away from the sterol biosynthesis toward other biosynthetic pathways of interesting isoprenoids.

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Conflict of interest statement

We declared that all authors of this manuscript have no conflicts of interest to this work.

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