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Increased campesterol synthesis by improving lipid content in engineered *Yarrowia lipolytica*

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Abstract

Sterols attract increasing attention due to their important bioactivities. The oleaginous yeast *Yarrowia lipolytica* has large lipid droplets, which provide storage for the accumulated steroid compounds. In this study, we have successfully constructed a campesterol biosynthetic pathway by modifying the synthetic pathway of ergosterol in *Y. lipolytica* with different capacity of lipid synthesis. The results showed that the maximal campesterol production was produced in the engineered strain YL-$D^{+}M^{-}E^{-}$, as the optimal lipid content. Furthermore, we found that campesterol mainly exists in the lipid droplets. The campesterol production was further accumulated through the overexpression of two copies of *dhcr7*. Finally, the maximal campesterol production of 837 mg/L was obtained using a 5-L bioreactor in the engineered YL-$D^{+}D^{+}M^{-}E^{-}$, exhibiting a 3.7-fold increase compared with the initial strain YL-$D^{+}E^{-}$. Our results demonstrate that the proper promotion of lipid content plays an important role in campesterol biosynthesis in *Y. lipolytica*, and what we found provides an effective strategy for the production of hydrophobic compounds.

Key Points

- *Campesterol was biosynthesized by deleting erg5 and introducing heterologous dhcr7.*
- *Campesterol production elevated via promotion of lipid content.*
- *Campesterol was mainly found in lipid droplets.*
- *Promotion of lipid content is an effective strategy to produce hydrophobic compounds.*

Keywords Campesterol · Lipid droplets · Metabolic engineering · *Yarrowia lipolytica*

Ya Dan Qian and Si Yuan Tan contributed equally to this work.

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Introduction

Phytosterols, an important class of steroid compounds, play a crucial role in lowering total plasma cholesterol, anti-cancer, and anti-oxidation (Demonty et al. 2008). Phytosterols have been widely used in health foods, medicines, and cosmetics (Fernandes and Cabral 2007). However, the source of phytosterols is limited. Currently, phytosterols are mainly extracted from plant oils, and only a mixture of phytosterols is obtained (Liu et al. 2012). Mixed phytosterols include mostly sitosterol, stigmasterol, brassicasterol, and campesterol (Piironen et al. 2000). As a ubiquitous class of substances in phytosterols, campesterol is a key precursor of progesterone, pregnenolone, and hydrocortisone (Du et al. 2016). However, to separate campesterol from the mixed phytosterols is currently a challenge yet to be resolved.

Microorganisms, such as *Saccharomyces cerevisiae* (Lees et al. 2000), *Pichia pastoris* (Wriessnegger et al. 2008), and *Yarrowia lipolytica* (Du et al. 2016) are good producers of ergosterol. Ergosterol synthesis pathway can be converted to synthesize campesterol through metabolic engineering. Ergosterol is an essential structural and regulatory component of yeast cell membranes (Veen and Lang 2005). Campesterol and ergosterol share common precursor ergosta-5.7-dienol in the biosynthesis pathway. Therefore, it is feasible to synthesize campesterol by modifying the ergosterol synthetic pathway through genetic engineering technology. For example, *Saccharomyces cerevisiae* FY1679 was used as the host to synthesize campesterol derivatives by modifying the ergosterol synthetic pathway (Szczebara et al. 2003). The campesterol biosynthetic pathway was constructed by disrupting C-22 desaturase (*erg5*) and expression of the 7-dehydrocholesterol reductase (*dhcr7*) from different species in *Y. lipolytica* ATCC201249 (Du et al. 2016).

In the sterol synthesis pathway, C-24 sterol reductase (*erg4*) catalyzes ergosta-5.7.24-trienol conversion to ergosta-5.7-dienol. Then *erg5* converts ergosta-5.7-dienol to ergosterol. *dhcr7* is a membrane-embedded enzyme, which reduces the carbon-carbon double bonds in the seventh position of the ergosta-5.7-dienol to campesterol (Duport et al. 1998). Thus, disruption of *erg5* and expression of heterologous *dhcr7* are crucial to the production of campesterol from ergosterol in yeast.

The relationship between lipid content and campesterol accumulation is critical. *Y. lipolytica*, as an oleaginous organism, has large lipid droplets, which provide storage for the accumulated steroid compounds and relieve the adverse effect of cell membrane fluidity caused by excessive accumulation of sterols (Du et al. 2016). However, the biosynthesis of campesterol and fatty acids share some common precursors such as acetyl-CoA. That is, the intracellular lipids have both promoting and competing effects on the campesterol synthesis. How to make full use of the favorable factors for the synthesis of sterols by lipid accumulation to further increasing the campesterol content is a fundamental theoretical problem.

In this study, strains with different lipid yields were constructed to study the relationship between lipid content and campesterol accumulation. We first constructed the campesterol synthesis pathway in *Y. lipolytica*. Subsequently, we constructed strains with different lipid yields by knocking out multifunctional β-oxidation protein (*mfe1*) and peroxisomal biogenesis factor 10 (*pex10*). The relationship between lipid content and campesterol accumulation was studied. Furthermore, the multicopy plasmids of *dhcr7* were constructed and transformed to improve campesterol content. Finally, the fermentation of the optimum strain in a 5-L fermenter was studied. We have provided an effective strategy for promoting the amount of campesterol.

Materials and methods

Strains, media, and culture condition

All strains used in this study are listed in Table 1. The *Y. lipolytica* strain PO1f (ATCC No. MYA-2613) with a leucine and uracil auxotroph was used as the strain for all studies. The engineered *Y. lipolytica* strain was cultured at 29 °C in YPD liquid medium. After transformation, the recombinants were selected on an appropriate synthetic dropout (SD) medium (Gao et al. 2016). SD medium contained 20 g/L glucose, 1.7 g/L yeast nitrogen base (YNB) (without amino acids and ammonium sulfate), 5 g/L $(NH_4)_2SO_4$, and appropriate amino acid dropout mix. SD-LEU is the SD medium supplemented with 2 g/L amino acid dropout mix minus leucine (US Biological; Marblehead, USA), which was used to select the pJN44 plasmid and to derive the transformants. SD-URA is the SD medium supplemented with 2 g/L amino acid dropout mix minus uracil (US Biological; Marblehead, USA), which was used to screen recombinants of successful transformation-linearized pLoxp-ura-loxp plasmid. Lipid accumulation media consisted of 20 mg/L uracil, 80 g/L glucose, 1.7 g/L YNB, and 0.352 g/L $(NH_4)_2SO_4$.

DNA manipulation and plasmids construction

Plasmids and primers used in this study are listed in Table 1 and Table S1. The upstream and downstream fragments of the knockout genes were PCR amplified from *Y. lipolytica* total DNA as the template with primers listed in Table S1.

Plasmid pLoxp-ura-loxp was used to gene knockout (Wang et al. 2016). To construct the knockout plasmid, the 5′ and 3′ flanking regions of corresponding genes were amplified from *Y. lipolytica* DNA with the primers listed in Table S1.

Table 1 Strains and plasmids used in this study

	Relative characteristics	Source
Strains		
E. coli DH5α	*endA1 hsdR17* [r-m+] *supE44 thi-1 recA1 gyrA* [NalR] *relA relA1* Δ[*lacZYA-argF*] *U169 deoR* [Ø80Δ (*LacZ*) M15]	Novagen
Y. lipolytica PO1f	MatA, leucine$^-$, uracil$^-$, xpr2-322, axp1-2	Madzak et al. (2000)
PO1f-E$^-$	MatA, leucine$^-$, uracil$^-$, xpr2-322, axp1-2, ERG5$^-$	This work
YL-D$^+$E$^-$	MatA, leucine$^-$, uracil$^-$, xpr2-322, axp1-2, ERG5$^-$ DHCR7$^+$	This work
PO1f-△*MFE1*	MatA, leucine$^-$, uracil$^-$, xpr2-322, axp1-2, MFE$^-$	Yan et al. (2020)
PO1f-△*MFE1*-△*PEX10*	MatA, leucine$^-$, uracil$^-$, xpr2-322, axp1-2, MFE$^-$, PEX10$^-$	Yan et al. (2020)
YL-D$^+$M$^-$E$^-$	PO1f-△*MFE1*, ERG5$^-$, DHCR7$^+$	This work
YL-D$^+$P$^-$M$^-$E$^-$	PO1f-△*MFE1*-△*PEX10*, ERG5$^-$, DHCR7$^+$	This work
YL-D$^+$D$^+$E$^-$	PO1f-E$^-$, DHCR7$^+$, DHCR7$^+$	This work
YL-D$^+$D$^+$M$^-$E$^-$	PO1f-△*MFE1*, ERG5$^-$, DHCR7$^+$, DHCR7$^+$	This work
YL-D$^+$D$^+$P$^-$M$^-$E$^-$	PO1f-△*MFE1*-△*PEX10*, ERG5$^-$, DHCR7$^+$, DHCR7$^+$	This work
Plasmids		
pJN44	Expression vector, leu$^+$, cEN 1-1, TEF, Introl, xpr2 terminator	Wang et al. (2016)
pJN43	TEF, Introl, xpr2 terminator	Wang et al. (2016)
pJN43-D7	P_{TEF}-*DHCR7*	This work
pJN44-D7	P_{TEF}-*DHCR7*	This work
pJN44-D7-D7	P_{TEF}-*DHCR7*	This work
pLoxp-ura-loxp	Knock out vector, loxp, ura3, ura3-testR1, ura3-F, loxp, AmpR	Wang et al. (2016)
p*ERG5*-up-ura3-loxp-down	*ERG5*-up, Loxp, ura3, ura3-testR1, ura3-F, loxp, *ERG5*-down, AmpR	This work

Plasmids pJN43 and pJN44 were used for gene episomal expression in *Y. lipolytica* (Wang et al., 2016). Plasmid pJN44 contains the leucine selection marker, whereas pJN43 does not. A fragment of *dhcr7* was designed as the *Xenopus laevis dhcr7* gene template, and the sequence of *dhcr7* was codon-optimized by Genscript (China). The nucleotide sequence of the codon-optimized *dhcr7* gene is presented in Table S3. For the construction of plasmid pJN43-*D7*, synthesized gene *dhcr7* fragment with a *Sal*I/*Hin*dIII digest and was inserted into plasmid pJN43. Plasmid pJN44 was digested with *Xba*I, and then the obtained leucine marker expression cassette was inserted into plasmid pJN43-*D7*, resulting in plasmid pJN44-*D7*. The *dhcr7* expression cassette was obtained from pJN43-*D7* with an *Xba*I/*Spe*I digest and was inserted into a pJN44-*D7* plasmid with a *Spe*I/Fast Alkaline Phosphatase (FastAP) digest to construct plasmid pJN44-*D7*-*D7*.

Strain construction

All knockout mutants were constructed by homologous recombination and marker delete, as previously described (Fickers et al. 2003; Wang et al. 2016). We first constructed the strain PO1f-E$^-$ through the deletion of *erg5* in the *Y. lipolytica* PO1f. Then, the strain YL-D$^+$E$^-$ was constructed through the episomal expression of pJN44-*D7* in PO1f-E$^-$ strain, utilizing a leucine selection marker. The strain YL-D$^+$M$^-$E$^-$ was obtained by knock outing the *erg5* and expressing pJN44-*D7* in strain PO1f-△*MFE1*. The gene *erg5* was disrupted, and plasmid pJN44-*D7* was expressed in PO1f-△*MFE1*-△*PEX10* strain, resulting in strain YL-D$^+$P$^-$M$^-$E$^-$. The plasmid pJN44-*D7*-*D7*was expressed in PO1f-E$^-$, PO1f-△*MFE1*, and PO1f-△*MFE1*-△*PEX10* strains, resulting in the creation of YL-D$^+$D$^+$E$^-$, YL-D$^+$D$^+$M$^-$E$^-$, and YL-D$^+$D$^+$P$^-$M$^-$E$^-$, respectively.

Extraction and quantification of sterols

Sample preparation One milliliter of fermentation broth was transferred into anaerobic tubes, and 0.1 mL 0.04 mg/mL of cholestanol (Solarbio; Beijing, China) was added as the internal standard; 2.5 mL of 3 M KOH-methanol solution was added and then the lid was screwed up. The mixture was saponified at 90 °C for 100 min. Two milliliters of H_2O and 2 mL of hexane were added to the saponification product, which was vortexed for 5 min at room temperature. When stratification occurs, the hydrophobic phase with the sample was transferred to a derivatizing reaction vial. Add 100 μL of derivatization reagent (99 μL BSTFA and 1 μL TMCS) to the vial and followed by reacted at 70 °C for 60 min (Jenner and Brown 2017). The resulting solution was analyzed using a gas chromatography-mass spectrometer (GC-MS).

Assay of campesterol Campesterol was determined by GC-MS as described previously with minor modifications (Tan et al. 2019). In brief, analyses of sterols were performed on an Agilent QP2010 Ultra GC-MS equipped with an RTX-5MS column (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies, USA). The 1.0-μL sample was injected at 300 °C with a split ratio of 10:1. Helium was used as carrier gas at a rate of 1.2 mL/min. The GC programs include an initiated temperature of 100 °C and increased to 280 °C at 30 °C/min for 15 min. The MS operating conditions include electron impact (EI) ionization voltage of 70 eV, ion source temperature of 230 °C, and interface temperature of 250 °C. When analyzing the sterol-mixed standards, SCAN mode was used to select characteristic ions. Subsequently, SIM mode was used for quantitative analyses, and the value of ions selected was m/z 75 for cholestanol, m/z 337 for ergosterol, and m/z 129 for campesterol. The NIST 2013 was employed for the interpretation of the sterols.

Analysis of lipid

Fatty acid content was determined using the method as previously described by Yan et al. (2020). One milliliter sample was transferred into screw-cap Pyrex culture tubes. One milliliter of 0.2 mg/mL of tridecanoic acid was added as the internal standard. The mixture was incubated at 85 °C, followed by adding 5.3 mL methanol and 0.7 mL 10 M NaOH solutions. Then the solution was reacted with 0.58 mL H_2SO_4; the resulting solution was vortexed at 85 °C for 15 min. Two milliliters of H_2O and 2 mL of hexane were added, and the mixture was eddied for 15 min. After extraction, the top hydrophobic phase containing fatty acid methyl esters were separated for GC-MS analysis. Samples were analyzed in a QP2010 Ultra GC-MS with a capillary column RTX-5MS (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies, USA). The program settings include an inlet temperature of 260 °C and a 1.0-μL sample with splitless mode. The GC temperature program is initiated at 120 °C (1 min), increased to 250 °C at 7 °C/min and increased at 8 °C/min to 295 °C for 7 min. As the internal standard, the addition of tridecanoic acid was carried through the entire analysis procedure.

Isolation of lipid droplets

Isolation of lipid droplets was carried out according to Matthaus et al. (2014). In short, cells representing 300 mg of DCW were harvested and were resuspended in 20 mL phosphate buffer (pH 7) containing 0.15 M KCl, 0.5 M sucrose, and 1 mM EDTA. The suspension was homogenized at 40 MPa and then was centrifuged at 1500×*g* for 5 min to remove cell debris. The supernatant was centrifuged for 1 h at 100,000×*g* in a Beckman Optima MAX-XP ultracentrifuge. The floating lipid droplet fraction, interphase, and membrane phase were separated to determine the campesterol content. The determination method was the same as described in the "Extraction and quantification of sterols" section.

Total RNA isolation and quantitative PCR analysis

A single colony of the *Y. lipolytica* target strain was incubated to extract total RNA, as previously described (Tai and Stephanopoulos 2013). RNA samples were reverse transcribed using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen, Beijing, China). qRT-PCR analyses were carried out using TransStrat Tip Green qPCR SuperMix Kit (Transgen, Beijing, China) and PIKOREAL 96 Real-Time PCR System, as previously described. The strain PO1f was used as the control, and relative quantification used *ACTIN* (YALI0D08272g) as the reference gene. All samples were analyzed in triplicates. The primers are listed in Table S2. The relative expression of the gene was analyzed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2000).

Bioreactor fermentations

Bioreactor fermentation was carried out in a 5-L bioreactor. The medium contained 40 g/L glucose, 3 g/L $(NH_4)_2SO_4$, 0.85 g/L YNB (without amino acids and ammonium sulfate), 1 g/L uracil, 2 g/L yeast extract powder, 12.5 g/L KH_2PO_4, and 2.5 g/L $MgSO_4{\cdot}7H_2O$. The fermentation conditions are based on reports by Qiao et al. (2015). The temperature was maintained at 28 °C and an initial agitation rate at 200 rpm/min. During the logarithmic growth phase, the agitation speed gradually increased to 900 rpm/min to maintain dissolved oxygen levels above 20%. The agitation was gradually reduced according to the dissolved oxygen level at the stationary phase. The pH of the culture was continuously controlled at 5.5 using 10 M KOH. The glucose content was maintained at not lower than 10 g/L by continuously adding a 50% glucose solution.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 (Chicago, IL). All experiments were repeated three times. Data in Figs. 3, 4, 5, 6, and 7 are shown as mean ± standard deviation. Data in Fig. 3, 4, 5, 6, and 7 were analyzed using the one-way analysis of variance (ANOVA), followed by Duncan's test to determine the significant difference between the means using SPSS18.0. Origin software 9.1 (Origin Lab; USA) was used for graph construction.

Accession numbers: codon-optimized *dhcr7* (MT340497) and *erg5* (YALI0A18062g).

Results

Campesterol was biosynthesized via disrupting *erg5* and introducing heterologous *dhcr7* in an engineered *Yarrowia lipolytica*

In the campesterol biosynthesis pathway, the conversion of ergosta-5.7.24-trienol to ergosta-5.7-dienol is catalyzed by C-24 sterol reductase (*erg4*). Ergosterol is synthesized from ergosta-5.7-dienol by C-22 desaturase, which is encoded by *erg5*. Blocking this desaturation may increase ergosta-5.7-dienol accumulation. The ergosta-5.7-dienol was used to synthesize campesterol by 7-dehydrocholesterol reductase (*dhcr7*), as shown in Fig. 1. To divert ergosta-5.7-dienol from the ergosterol synthetic pathway to campesterol biosynthesis, we knocked out the endogenous *erg5* in PO1f strain to generate the strain PO1f-E^{-}. Then, we inserted a heterologous *dhcr7* into PO1f-E^{-} to generate the campesterol-producing strain YL-D^{+}E^{-}.

We determined the fermentation products of strains PO1f, PO1f-E^{-}, and YL-D^{+}E^{-} to verify the compounds produced by each strain. Because the presence of auxotrophy causes an organism to grow slowly, we inserted an empty plasmid pJN44 into the leucine auxotrophic strains before performing shake flask fermentation. GC-MS data are presented in Fig. 2. The starting strain PO1f showed an ergosterol peak (1) at 15.370 min with its characteristic ion m/z 337 in the mass chromatogram (Fig. 2 (a1 and a2)). This result indicates that ergosterol is the only product accumulated in the starting PO1f strain. The strain PO1f-E^{-} was analyzed to identify the compounds produced after *erg5* deletion. Total ion chromatogram (TIC) in Fig. 2 (b1) showed one peak (3) for ergosta-5.7-dienol at 16.680 min with its characteristic ion m/z 365 in the mass chromatogram (Fig. 2 (b2)), indicating that the disruption of the *erg5* gene indeed increased ergosta-5.7-dienol accumulation. The result of fermentation products from the strain po1f-E^{-} also agrees with the theoretical result of modifying the ergosterol synthesis pathway to synthesize campesterol in *Y. lipolytica*. In recombinant strain YL-D^{+}E^{-}, peak detection was based on the retention time of 15.761 min (4) with its characteristic ion m/z 129 in the mass chromatogram (Fig. 2 (c1 and c2)), which identified the presence of campesterol. Moreover, the primary product was campesterol in fermentation products of strain YL-D^{+}E^{-}. Accordingly, these results indicate that the campesterol biosynthesis pathway was successfully constructed in *Y. lipolytica*, and campesterol-producing YL-D^{+}E^{-} strain was obtained.

The biosynthetic pathway of campesterol was successfully constructed by modifying the ergosterol synthesis pathway. We then attempted to increase lipid content and overexpress the heterologous *dhcr7* to increase campesterol accumulation in *Y. lipolytica* further (Fig. 1).

Campesterol content in strains with the different ability of lipid biosynthesis

Sterol is an essential structural component of the cell membrane, while excessive sterols will be stored in lipid droplets as esterification. The lipid droplets can provide storage for the campesterol enrichment and alleviate the negative impact of

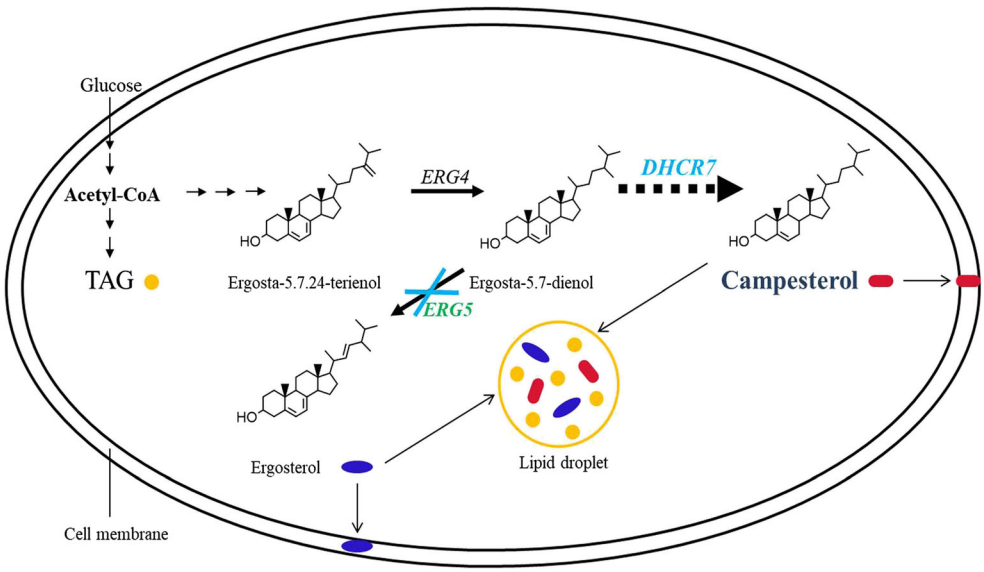

Fig. 1 A schematic diagram illustrates the construction of the campesterol biosynthesis pathway based on the ergosterol biosynthesis pathway in *Y. lipolytica* and the intracellular distribution of sterols in cells. Campesterol synthesis pathway was constructed by disrupting the C-22 desaturase gene (*erg5*) and expressing the heterologous 7-dehydrocholesterol reductase gene (*dhcr7*). Meanwhile, campesterol biosynthesis and lipid accumulation share a common precursor, acetyl-CoA. Sterol is an essential structural component of the cell membrane, and lipid droplets can provide storage for the campesterol accumulation

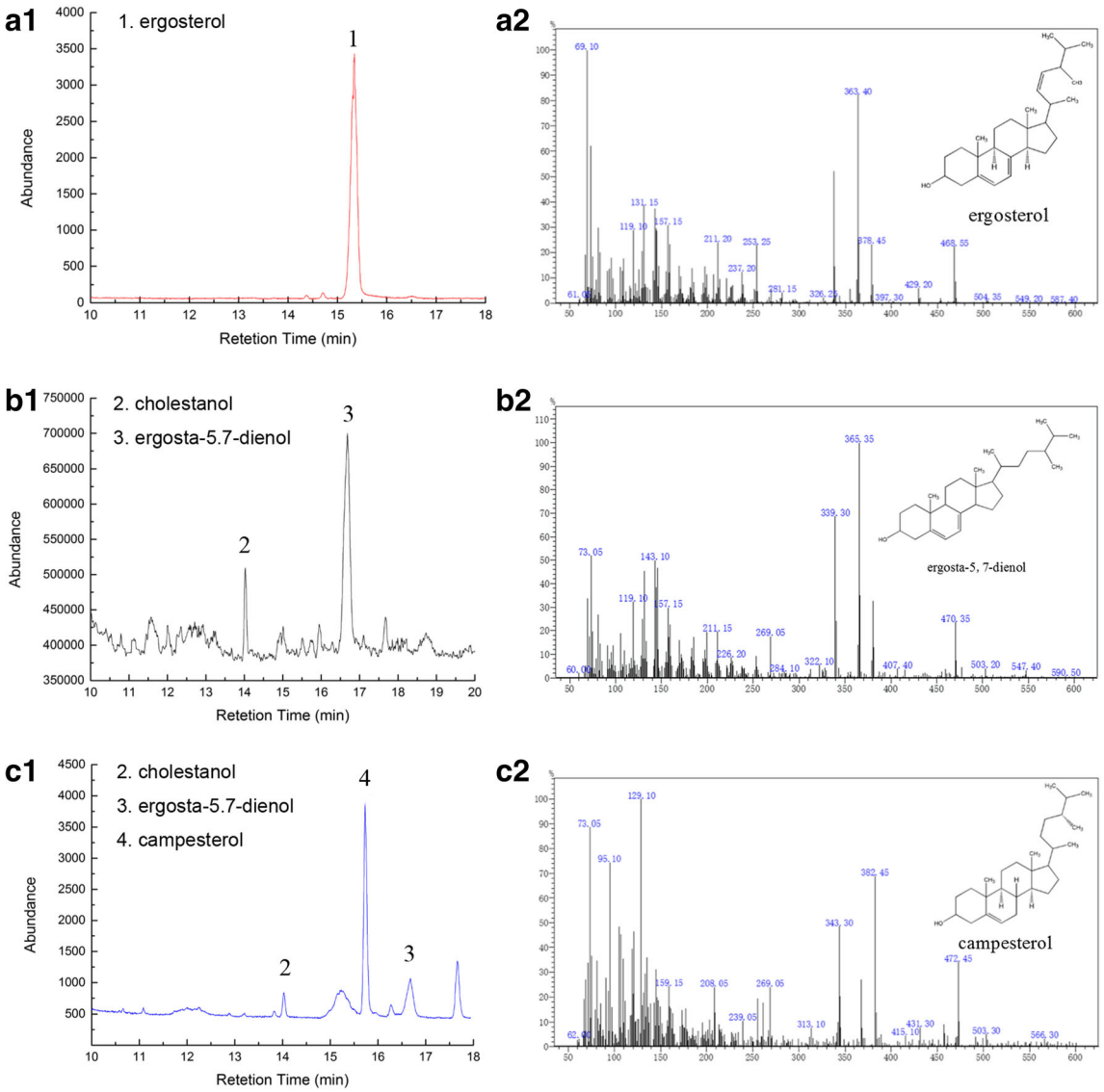

Fig. 2 Identification of fermentation products in recombinant strains by gas chromatography-mass spectrometry (GC-MS). GC-MS/SIM profile of the strain PO1f (**a1** and **a2**), campesterol-producing strain YL-D^{+}E^{-} (**c1** and **c2**), and *erg5* knockout strain PO1f-E^{-} (**b1** and **b2**). **a1**, **b1**, and **c1** represent mass chromatography of characteristic ions: m/z 337 for ergosterol (**a1**), m/z 365 for ergosta-5.7-dienol (**b1**), and m/z 129 for campesterol (**c1**). **a2**, **b2**, and **c2** represent the mass spectra of ergosterol (**a2**), ergosta-5.7-dienol (**b2**), and campesterol (**c2**)

redundant sterol accumulation on the cell membrane fluidity. However, the relationship between the biosynthesis of campesterol and lipid is competent since they share the precursor acetyl-CoA. Therefore, we explored the relationship between lipid content and campesterol production to synthesize more campesterol. The three strains PO1f, PO1f-Δ*MFE1*, and PO1f-Δ*PEX10*-Δ*MFE1* with the different ability of lipid synthesis were used as starting strains, in which the synthetic pathway of campesterol was constructed. We obtained the strain YL-D^{+}E^{-}, YL-D^{+}M^{-}E^{-}, and YL-D^{+}P^{-}M^{-}E^{-}, and campesterol content and lipid accumulation were measured separately. As shown in Fig. 3, the strain YL-D^{+}P^{-}M^{-}E^{-} produced the highest lipid content up to 114 mg/g and campesterol yield was 5.89 mg/g. The lipid content was produced at 67 mg/g in the strain YL-D^{+}M^{-}E^{-} and accumulated the highest campesterol production up to 7.57 mg/g. In the strain YL-D^{+}E^{-}, the lipid content was 42 mg/g and campesterol production was 4.8 mg/g. When the YL-D^{+}E^{-} and YL-D^{+}M^{-}E^{-} strains were compared, we found that the lipid content increased and the production of campesterol also improved. This result suggests that the increased intracellular lipids have promoting effects on the campesterol synthesis. With the further increase of lipid content, the campesterol production decreased in YL-D^{+}P^{-}M^{-}E^{-} strain. This decrease was primarily due to competing for the precursor acetyl-CoA in the biosynthesis of campesterol and lipid. These results indicate that the improvement of lipid content did not always lead to an increase in campesterol production. The

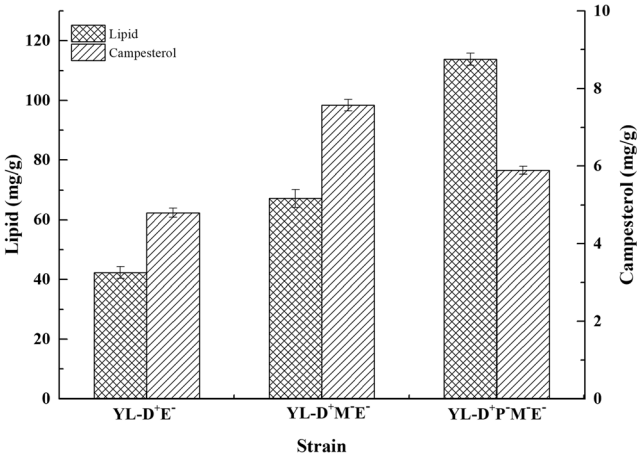

Fig. 3 Campesterol production in strains YL-D^+E^-, YL-$D^+M^-E^-$, and YL-$D^+P^-M^-E^-$, with different lipid contents. The campesterol production increased with the elevated lipid content within a specific range. Error bars represent standard deviations ($n = 3$)

campesterol production stops when the content of lipid increases to a certain extent.

Most of the campesterol accumulated in lipid droplets

To investigate the distribution of campesterol in cells, we performed sucrose gradient centrifugation experiment on the YL-$D^+M^-E^-$ strain. The lipid droplet, the membrane, and the interphase were separated, followed by extracting campesterol from each fraction. The campesterol distribution over the three fractions and the approximate volumes of each fraction are shown in Fig. 4. We find that campesterol mainly exists in the lipid droplet fraction but shows the lowest volume in the sucrose gradient. This result demonstrates that most campesterol was accumulated in lipid droplets and also explains why the maximum production of campesterol was

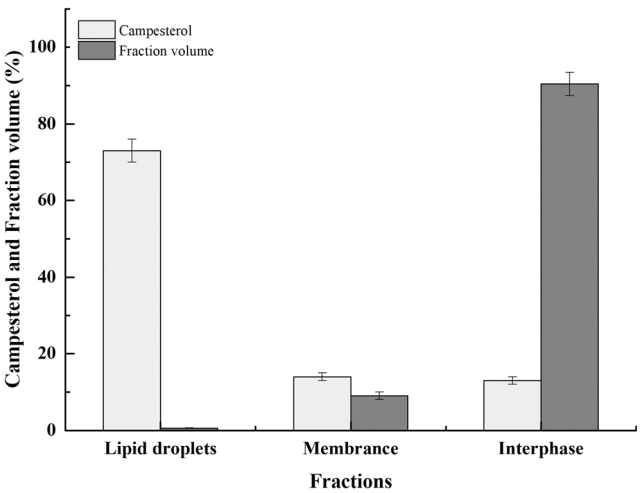

Fig. 4 Campesterol distribution and fraction volume in strain YL-$D^+M^-E^-$ after sucrose gradient centrifugation. Most of the campesterol are found in lipid droplets. Error bars represent standard deviations ($n = 3$)

obtained in strain YL-$D^+M^-E^-$. A small quantity of campesterol was detected in the membrane pellet and interphase, which suggests that the excessive campesterol accumulation in the membrane might affect the cell membrane fluidity. These results demonstrate that the storage capacity of campesterol increased for cells with a large number of lipid droplets. Therefore, the proper promotion of lipid content is beneficial to the storage of campesterol.

Overexpression of *dhcr7* elevated campesterol production

To further elevate the campesterol production capacity of the engineered strain, two copies of the *dhcr7* gene were overexpressed in strains PO1f-E^-, PO1f-Δ*MFE1*-E^-, and PO1f-Δ*PEX10*-Δ*MFE1*-E^-. The disruption of the *erg5* gene increased ergosta-5.7-dienol accumulation. However, abundant accumulation of ergosta-5.7-dienol tends to retard cell growth (Ma et al. 2018). We needed to reconstruct the metabolic balance between the upstream and downstream flux. Therefore, additional copies of the *dhcr7* were introduced into different engineered strains to decrease the accumulation of ergosta-5.7-dienol, and meanwhile, increase conversion of ergosta-5.7-dienol to campesterol. After overexpression of *dhcr7*, campesterol content was quantified using one copy of the *dhcr7* gene as the control (shown in Fig. 5). Based on the results in Fig. 5, the YL-$D^+D^+E^-$ strain was found to have higher campesterol, an increase of 16% compared with YL-D^+E^- strain. The YL-$D^+D^+M^-E^-$ was able to achieve the highest (10.62 mg/g DCW) campesterol production with a 1.4-fold improvement over the control YL-$D^+M^-E^-$. The strain YL-$D^+D^+P^-M^-E^-$, with two copies of *dhcr7* in the PO1f-Δ*PEX10*-Δ*MFE1*-E^- strain, exhibiting 10.43 mg/g DCW in campesterol content. These results demonstrate that the two copies of *dhcr7* increased campesterol accumulation in these three strains. Thus, overexpression of the *dhcr7* most probably increased the expression of 7-dehydrocholesterol reductase in the campesterol synthesis pathway, which promoted higher flux to campesterol.

mRNA analysis of *dhcr7* and *erg4* in strains with different campesterol yields

To investigate the expression of key genes in the campesterol synthetic pathway, we evaluated the relative mRNA level of *erg4* and *dhcr7* in strains with varying yields of campesterol. The expression of *erg4* involves the conversion of ergosta-5.7.24-trienol into ergosta-5.7-dienol, and then the *dhcr7* (7-dehydrocholesterol reductase) catalyzes the synthesis of ergosta-5.7-dienol to campesterol. RT-qPCR was used to analyze the mRNA of two key genes (Fig. 6). The actin gene was used as an internal reference and the mRNA of *erg4* in PO1f was set as 1.0.

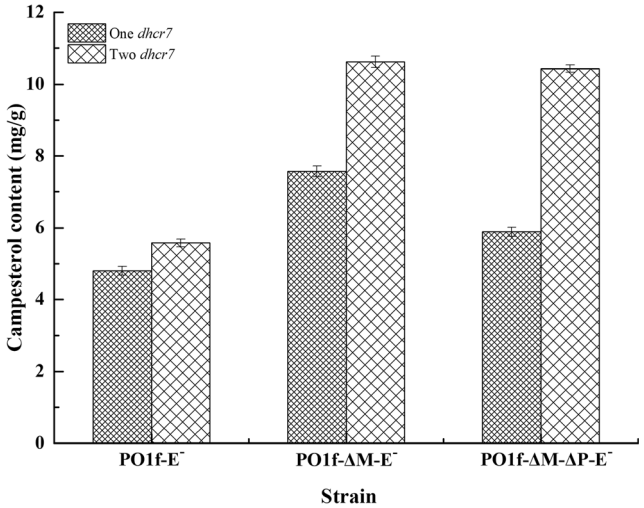

Fig. 5 Campesterol content of strains with *dhcr7* overexpression in PO1f-E$^-$, PO1f-Δ*MFE1*-E$^-$ (PO1f-Δ*M*-E$^-$), and PO1f-Δ*PEX10*-Δ*MFE1*-E$^-$ (PO1f-Δ*P*-Δ*M*-E$^-$). The *dhcr7* overexpression increased campesterol production by 16 to 77%. Error bars represent standard deviations ($n = 3$)

In the campesterol-producing strains expressing one copy of the *dhcr7* gene, the relative expression of the *erg4* was about 1.0, as shown in Fig. 6a. Compared with PO1f, the mRNA of the *erg4* gene decreased in three strains with different campesterol yields. This result suggests that disruption of *erg5*, *mfe1*, and *pex10* gene hurts the host cell metabolism, which reduces the expression of some genes in the synthetic pathway. As compared with that in the strain YL-D$^+$E$^-$, the mRNA of *dhcr7* was increased 2.1- and 1.1-fold in YL-D$^+$M$^-$E$^-$ and YL-D$^+$P$^-$M$^-$E$^-$, respectively. The mRNA of *erg4* and *dhcr7* genes was the highest in YL-D$^+$M$^-$E$^-$ strain, which led to the higher campesterol production in expressing one copy of *dhcr7* gene strains.

Two copies of the *dhcr7* gene were expressed in the strains with different campesterol production and exhibited an increase in the mRNA of *erg4* compared with the strain PO1f (Fig. 6b). This result indicates that the overexpression of the *dhcr7* gene elevated the mRNA level of *erg4* or other upstream genes in the campesterol synthetic pathway. The mRNA of *dhcr7* in both YL-D$^+$D$^+$E$^-$ and YL-D$^+$D$^+$P$^-$M$^-$E$^-$ were lower than that in YL-D$^+$D$^+$M$^-$E$^-$ strain. The highest mRNA of *erg4* and *dhcr7* were obtained in the YL-D$^+$D$^+$M$^-$E$^-$ strain, which may lead to the most campesterol content. Upregulation of *erg4* expression enhanced ergosta-5.7-dienol accumulation, which can be used to synthesize campesterol catalyzed by the *dhcr7*. Therefore, the strain YL-D$^+$D$^+$M$^-$E$^-$ was selected to be the candidate for the subsequent bioreactor fermentation experiment.

Campesterol was biosynthesized by the optimal strain in a 5-L fermenter

To explore campesterol accumulation and further monitor the growth characteristics of the optimal strain YL-D$^+$D$^+$M$^-$E$^-$, we conducted the fermentation experiment using a 5-L bioreactor with glucose as the sole carbon source. The fermentation experiment was performed three times, and the results show that the fermentation has much-improved repeatability. We chose one of the fermentation results for further analysis, as shown in Fig. 7.

After the initial 40 g/L glucose was depleted at 72 h, glucose was fed continuously into the medium to keep its concentration not lower than 10 g/L. We found that the exponential growth phase of strain YL-D$^+$D$^+$M$^-$E$^-$ was 24–108 h, and the stationary phase was 108–144 h. The campesterol gradually accumulated with increasing fermentation time. Ultimately, a titer of 837 mg/L campesterol yield was obtained after 144 h of culture. Compared with the initial strain YL-D$^+$E$^-$, the optimal strain YL-D$^+$D$^+$M$^-$E$^-$ increased campesterol production from 223 to 837 mg/L, a 3.7-fold increase (Fig. S1).

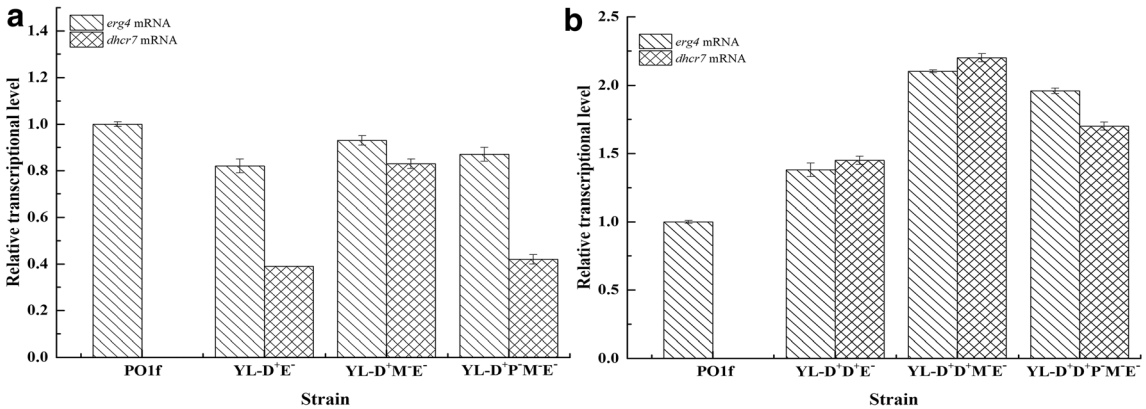

Fig. 6 Real-time PCR analysis of *dhcr7* and *erg4* in strains with different campesterol yields. Overexpression of the *dhcr7* elevated mRNA level of *dhcr7* and *erg4*. **a** The campesterol-producing strains expressing one copy of the *dhcr7* gene. **b** The campesterol-producing strains expressing two copies of *dhcr7* gene. Error bars represent standard deviations ($n = 3$)

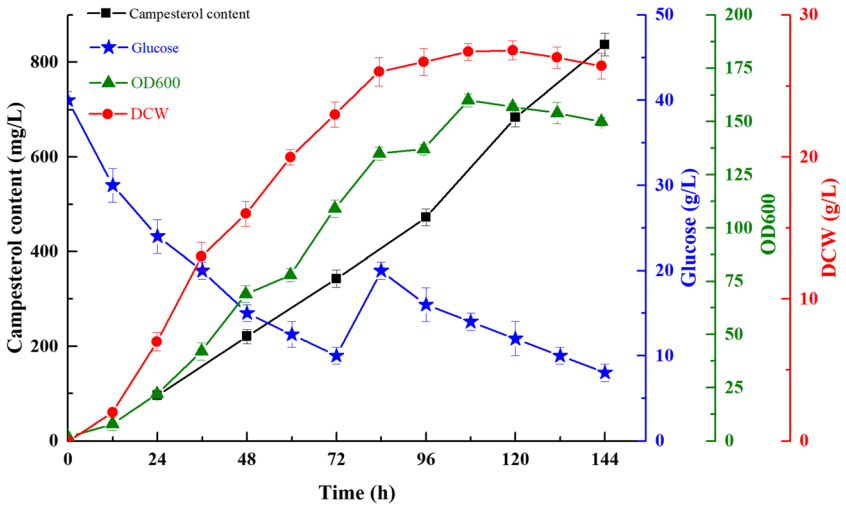

Fig. 7 Fermentation characteristics of the optimal strain YL-D^{+}D^{+}M^{-}E^{-}

Discussion

Phytosterols have important bioactivities and are widely used in health foods, medicines, and cosmetics. Increasing the production of phytosterols to meet the market demand has become more urgent. To make full use of the favorable factors for storing campesterol by lipid accumulation, the relationship between lipid and campesterol content is critical to further increase campesterol production in engineered *Y. lipolytica*.

Proper promotion of lipid content is beneficial to the storage of campesterol Sterols are vital components of eukaryotic cell membranes, which modulate the fluidity and permeability of phospholipid bilayer structure (Caspeta et al. 2014). The *Y. lipolytica* can produce a big lipid body that can store large amounts of hydrophobic compounds, such as lycopene and campesterol (Mlickova et al. 2004; Matthaus et al. 2014). In this work, we found most of the campesterol accumulated in lipid droplets. Therefore, we hypothesize that the content of lipid plays a crucial role in the campesterol yields. The campesterol-producing pathway was constructed in strains with different lipid contents by deleting *erg5* and inserting one copy of the *dhcr7*. We found that increasing levels of campesterol can be attributed to the elevated lipid accumulation, which provided storage for more campesterol. When the lipid content continues to increase, we found that the decrease in campesterol production, which was due to the accumulation of lipid leading to competition for acetyl-CoA. Therefore, it is necessary to increase the precursor supply and achieve a rebalance between campesterol and lipid biosynthetic pathways to maximize the production of the target product, campesterol (Ma et al. 2018). The engineered strain YL-D^{+}E^{-} produces lower lipid content because of a stronger capacity for metabolizing and degrading lipid. The metabolism and degradation of lipid can cause acetyl-CoA accumulation, which provides a precursor for the tricarboxylic acid cycle and campesterol biosynthesis. The result shows that the campesterol production was lower in the strain YL-D^{+}E^{-}. We found that the mRNA level of key genes *erg4* and *dhcr7* was low in the campesterol synthesis pathway. This result indicates that key point conversion steps are limited when producing campesterol in the strain YL-D^{+}E^{-}. Consequently, the accumulation of acetyl-CoA did not result in increased campesterol production. We supposed that the acetyl-CoA might be used to synthesize a by-product, such as citric acid (Abdel-Mawgoud et al., 2018). Thus, strengthening the key genes of the synthetic pathways is also an effective strategy to increase campesterol production in the engineered strain.

Further improvement of campesterol content through strengthening the synthetic pathway in the corresponding engineered strain

The *erg5* deletion blocks downstream metabolic pathways and increases ergosta-5.7-dienol accumulation. Excessive accumulation of ergosta-5.7-dienol was shown to affect cell growth (Ma et al., 2018). Hence, we expressed one copy of the *dhcr7* in the *erg5* deletion strains, which catalyzes the synthesis of ergosta-5.7-dienol to campesterol. However, the mRNA level of *erg4* in strains expressing one copy of the *dhcr7* was decreased compared with the control strain PO1f. The explanation for this observation is that cells hinder the ergosta-5.7-dienol accumulation by reducing the expression of *erg4* in order to avoid damage caused by ergosta-5.7-dienol excessive accumulation. We hypothesized that the overexpression of *dhcr7* increases the downstream flux, and more intermediate ergosta-5.7-dienol convert to campesterol. Subsequently, we further improve campesterol production by strengthening the synthetic pathway. Two copies of the *dhcr7* gene were expressed in the strains with different campesterol productions. The result shows that the mRNA

level of *erg4* and *dhcr7* was all increased, which resulted in a further increase in campesterol production. There is an implicit requirement that cells need to avoid an excessive cellular burden caused by an accumulation of cytotoxic intermediates (Li et al. 2017). In the shake flask experiment, the optimal strain increased campesterol content from 4.8 to 10.6 mg/g DCW. Moreover, the maximal campesterol production of 837 mg/L was obtained by the fermentation, which is a 3.7-fold increase in campesterol production compared with the initial campesterol-producing strain.

In brief, we found that the proper promotion of lipid content is crucial for elevating the production of campesterol in *Y. lipolytica*. Moreover, strengthening the expression of key genes in the synthetic pathway also is a critical strategy in metabolic engineering. Thus, the engineered *Y. lipolytica* has the potential to produce campesterol on a large scale through additional metabolomic engineering manipulations and optimizing fermentation conditions.

Data availability statement All datasets obtained for this study are included in the manuscript/Supplementary material.

Author contributions ST, GD, and YM designed the study. GD, ST, and YN carried out the experiment. YQ, GD, ST, YN, CH, and YM analyzed the data and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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