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Improving monoterpene geraniol production through geranyl diphosphate synthesis regulation in *Saccharomyces cerevisiae*

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Abstract Monoterpenes have wide applications in the food, cosmetics, and medicine industries and have recently received increased attention as advanced biofuels. However, compared with sesquiterpenes, monoterpene production is still lagging in *Saccharomyces cerevisiae*. In this study, geraniol, a valuable acyclic monoterpene alcohol, was synthesized in *S. cerevisiae*. We evaluated three geraniol synthases in *S. cerevisiae*, and the geraniol synthase *Valeriana officinalis* (tVoGES), which lacked a plastid-targeting peptide, yielded the highest geraniol production. To improve geraniol production, synthesis of the precursor geranyl diphosphate (GPP) was regulated by comparing three specific GPP synthase genes derived from different plants and the endogenous farnesyl diphosphate synthase gene variants $ERG20^{G}$ ($ERG20^{K197G}$) and $ERG20^{WW}$ ($ERG20^{F96W\text{-}N127W}$), and controlling endogenous *ERG20* expression, coupled with increasing the expression of the mevalonate pathway by co-overexpressing *IDI1*, *tHMG1*, and *UPC2-1*. The results showed that overexpressing $ERG20^{WW}$ and strengthening the mevalonate pathway significantly improved geraniol production, while expressing heterologous GPP synthase genes or down-regulating endogenous *ERG20* expression did not show positive effect. In addition, we constructed an Erg20p(F96W-N127W)-tVoGES fusion protein, and geraniol production reached 66.2 mg/L after optimizing the amino acid linker and the order of the proteins. The best strain yielded 293 mg/L geraniol in a fed-batch cultivation, a sevenfold improvement over the highest titer previously reported in an engineered *S. cerevisiae* strain. Finally, we showed that the toxicity of geraniol limited its production. The platform developed here can be readily used to synthesize other monoterpenes.

Keywords Geraniol · Geranyl pyrophosphate · Geraniol synthase · Monoterpene · *Saccharomyces cerevisiae*

Introduction

Monoterpenes have been widely used as additives in the food and cosmetics industries. Some monoterpenes and their derivatives also exhibit antimicrobial or anti-carcinogenic properties or can be used as insecticides (Pattnaik et al. 1997). Recently, they have received increasing attention because of their potential application as advanced biofuels (e.g., pinene (Sarria et al. 2014), limonene (Duetz et al. 2003), and sabinene (Zhang et al. 2014)). Although metabolic engineering of larger terpenes has achieved significant yields in microbial hosts including *Saccharomyces cerevisiae*, monoterpene production is still relatively low (Kung et al. 2012).

Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol; $C_{10}H_{18}O$) is an acyclic monoterpene alcohol that is widely used in the perfume and flavor industries because of its pleasant odor (Chen and Viljoen 2010), and it is naturally produced at low yields by aromatic plants. It has also been developed as an anti-cancer drug, antimicrobial reagent, and biopesticide (Gietz and Woods 2002). Recently, the biosynthesis of the plant-derived monoterpene indole alkaloids (MIAs) intermediate strictosidine using engineered yeast strain has also

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attracted much attention because of its medicinal properties (Leggans et al. 2013; van Der Heijden et al. 2004), and geraniol is the primary precursor of strictosidine (Brown et al. 2015).

Monoterpenes, such as geraniol, contain 10 carbon atoms, and the precursor geranyl disphosphate (GPP) is synthesized from two common C5 intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), by geranyl disphosphate synthase (GPPS) (Withers and Keasling 2007); it is subsequently converted to monoterpenes by various monoterpene synthases (McGarvey and Croteau 1995). In plants, monoterpenes are synthesized in the plastid via the deoxyxylulose-5-phosphate (DXP) pathway. In contrast, sesquiterpenes are produced from the precursor farnesyl diphosphate (FPP) via the mevalonate (MVA) pathway in the cytosol. FPP is formed by the condensation of two molecules of IPP and one DMAPP molecule by farnesyl diphosphate synthase (FPPS), and it is subsequently used by sesquiterpene synthases to synthesize sesquiterpenes. In *S. cerevisiae*, the MVA pathway is used to synthesize ergosterol, which is essential for cell growth.

In recent years, metabolic engineering of *S. cerevisiae* has achieved significant progress in the production of many terpenes, such as artemisinin (Paddon et al. 2013), bisabolene (Foo et al. 2014), and miltiradiene (Zhou et al. 2012). Despite the achievement of significant yields of sesquiterpenes or larger terpenes, monoterpene production in yeast is much lower than that of larger terpenes. Therefore, it is necessary to develop a *S. cerevisiae* platform for monoterpene production. Only a few monoterpenes, such as sabinene (Ignea et al. 2014), linalool (Rico et al. 2010), and geraniol (Liu et al. 2013a), have been produced in *S. cerevisiae*, but their production was very low. For instance, 36 mg/L geraniol was obtained by engineering the MVA pathway (Liu et al. 2013a), and sabinene (17.5 mg/L) and linalool (0.132 mg/L) (Rico et al. 2010) production was also low. In *S. cerevisiae*, GPP and FPP are both synthesized via the MVA pathway by a single enzyme, Erg20p, which catalyzes the condensation of IPP and DMAPP to form GPP, followed by the addition of one molecule of IPP to form FPP (Fig. 1). GPP occurs exclusively as an intermediate of farnesyl diphosphate synthesis. Therefore, the available level of GPP that can be used for monoterpene synthesis is very low. Previous studies have demonstrated that the expression of Erg20p variants that have greater GPP synthase (GPPS) activity increased monoterpene titers (Fischer et al. 2011; Ignea et al. 2014; Liu et al. 2013a). However, the effect of regulating the GPP pool in *S. cerevisiae* has not been investigated extensively.

In this work, we optimized the geraniol biosynthesis pathway through efficient geraniol synthase expression and the regulation of GPP synthesis in *S. cerevisiae*. We selected different sources of geraniol synthases (GESs) and chose the best one from *Valeriana officinalis* (tVoGES) (Dong et al. 2013) for geraniol production. Different strategies to regulate GPP synthesis were then compared, including expressing heterologous GPPSs, expressing Erg20p variants that have high specificities for GPP synthesis, repressing the expression of endogenous Erg20p, and overexpressing key, limiting enzymes of the MVA pathway. Fusing Erg20p(F96W-N127W) and tVoGES and overexpressing a positive regulator of the MVA pathway were also used to further improve geraniol production. The best recombinant strain produced 293 mg/L geraniol in a fed-batch cultivation.

Materials and methods

Plasmid construction

Escherichia coli strain Trans5α was used for plasmid construction. The primers used in this study are listed in Table S1. The plasmids and strains constructed in this study are listed in Table S2. Ligations were performed using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) or by the Gibson method (Gibson et al. 2009). The DNA sequences of three geraniol synthase genes from *Lippia dulcis* (*LdGES*) (accession no. GU136162) (Dong et al. 2013), *Ocimum basilicum* (*ObGES*) (accession no. AY362553) (Iijima et al. 2004), and *V. officinalis* (*VoGES*) (accession no. KF951406) (Dong et al. 2013) were codon-optimized and synthesized (GENEWIZ, Suzhou, China). Full-length or truncated versions of the three GESs (*tLdGES* (bp 139–1775), *tObGES* (bp 133–1704), and *tVoGES* (bp 178–1785)) without their amino-terminal plastid-targeting sequence were ligated into the 2 μ plasmid pJFE3 (Shen et al. 2012) under the control of the *TEF1* promoter and the *PGK1* terminator to generate plasmids pZGL(f)/pZGL, pZGO(f)/pZGO, and pZGV(f)/pZGV, respectively. *IDI1* and *tHMG1* (a truncated version of *HMG1*) in the MVA pathway were amplified from the genomic DNA of *S. cerevisiae* CEN.PK102-5B and ligated into the 2 μ plasmid pIYC04 (Chen et al. 2013) under the control of the *PGK1* promoter and the *ADH1* terminator, and the *TEF1* promoter and the *CYC1* terminator, respectively. Three heterologous GPPS genes, including *AgGPPS2* from *Abies grandis* (accession no. AF513112) (Burke and Croteau 2002), *PaGPPS2* from *Picea abies* (accession no. ACA21458) (Schmidt and Gershenzon 2008), and *CrGPPS* from *Catharanthus roseus* (accession no. JX417185) (Rai et al. 2013), were also codon-optimized, and their amino-terminal plastid-targeting sequences were removed. Truncated versions of three GPPS genes (*tAgGPPS2*, *tPaGPPS2*, and *tCrGPPS*), as well as endogenous *ERG20* and the mutants $ERG20^{G}$ ($ERG20^{K197G}$) (Fischer et al. 2011) and $ERG20^{WW}$ ($ERG20^{F96W\text{-}N127W}$) (Ignea et al. 2014), were constructed

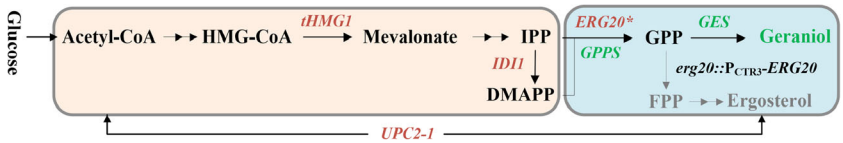

Fig. 1 The simplified mevalonic acid (MVA) pathway and geraniol biosynthetic pathway in *S. cerevisiae*. The genes in *red* are endogenous genes in *S. cerevisiae*, and the genes in *green* are heterologous genes. The native promoter of *ERG20* was replaced by the *CTR3* promoter. *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate

under the control of a synthetic $UAS_{CIT(3\times)}$-*TEF1* promoter (Blazeck et al. 2012) and the *CYC1* terminator. These cassettes were then respectively ligated into plasmid pZGV containing the *TEF1p-tVoGES-PGK1t* cassette, resulting in plasmids pZGV1–pZGV6. The sequences of the codon-optimized genes have been deposited to GenBank, cf. *ObGES* (accession no. KT894140), *LdGES* (accession no. KT894141), *VoGES* (accession no. KT894142), *AgGPPS2* (accession no. KT894143), *PaGPPS2* (accession no. KT894144), *CrGPPS* (accession no. KT894145).

All $ERG20^{WW}$ and *tVoGES* gene fusions were constructed by inserting a GGGS, GGGGS, or GSGGSG linker between the two genes by overlap extension polymerase chain reaction (PCR). The $ERG20^{WW}$-(GGGS)-*tVoGES*, $ERG20^{WW}$-(GGGGS)-*tVoGES*, $ERG20^{WW}$-(GSGGSG)-*tVoGES*, *tVoGES*-(GGGS)-$ERG20^{WW}$, *tVoGES*-(GGGGS)-$ERG20^{WW}$, and *tVoGES*-(GSGGSG)-$ERG20^{WW}$ gene fusions were ligated into pJFE3 under the control of the *TEF1* promoter and the *PGK1* terminator, respectively, generating plasmids pZGV6-EG1–pZGV6-EG3 and pZGV6-GE1–pZGV6-GE3. *UPC2-1* (Davies et al. 2005) was amplified from the genomic DNA of *S. cerevisiae* CEN.PK102-5B and inserted into the *TEF1* promoter and the *PGK1* terminator, and then the *TEF1p-UPC2.1-PGK1t* cassette was ligated into plasmid pZMVA3, which contains the *TEF1p-tHMG1-ADH1t* and *PGK1p-IDI1-CYC1t* cassettes, to generate pZMVA4.

Strain construction

S. cerevisiae strain CEN.PK102-5B (*MATa ura3-52 his3Δ1 leu2-3,112*) (Entian et al. 1998) was used as the parent strain for the construction of all yeast strains. The yeast strains used in this study are listed in Table S2, and yeast transformations were performed by the standard lithium acetate method (Gietz and Woods 2002). The YZG00 yeast strain harboring two empty plasmids, pJFE3 and pIYC04, was used as the control strain. The recombinant geraniol-producing strains were constructed by transforming various combinations of plasmids as described in Table S2. The replacement of the *ERG20* promoter with the *CTR3* promoter in strain YZG12 was accomplished as previously described (Hong et al. 2011). The *CTR3* promoter, from positions −1 to −734, was amplified from CEN.PK2-1C genomic DNA.

Medium and growth conditions

E. coli Trans5α strains were grown in Luria-Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, with/without 20 g/L agar) supplemented with 200 mg/L ampicillin at 37 °C. The yeast strain CEN.PK102-5B was cultured in yeast extract-peptone-dextrose (YPD) medium (20 g/L glucose, 20 g/L tryptone, and 10 g/L yeast extract) for transformations. Synthetic dextrose (SD) medium (20 g/L glucose, 1.7 g/L yeast nitrogen base, 5 g/L $(NH_4)_2SO_4$) with amino acids, but lacking uracil and histidine) (SD-Ura-His medium) was used for the selection of yeast transformants. A pre-culture was prepared by inoculating a single colony into 5 mL of SD-Ura-His medium for 24 h, and it was transferred into 10 mL of SD-Ura-His medium and grown for 10–12 h. The cells were then harvested and washed twice with sterile water for shake flask inoculations. The shake flask cultivations were conducted in 150-mL flasks containing 40 mL of SD-Ura-His medium at 30 °C, with shaking at 200 rpm. The initial optical density at 600 nm (OD_{600}) was adjusted to 0.1, and 10 % (*v*/*v*) dodecane was added to the medium to capture volatile geraniol. The cultures were sampled at 48 h for geraniol titer testing.

Batch and fed-batch cultivations

The best geraniol-producing strain, YZG13-GE1, was used for batch and fed-batch cultivations. A pre-culture was prepared by inoculating a single colony into 5 mL of medium for 24 h, and then the culture was transferred into a 300-mL flask containing 50 mL of medium and grown for 10–12 h. Then, the seed culture was inoculated into a 1-L Infors-HT fermenter (Infors AG, Bottmingen, Switzerland) containing 0.8 L of SD-Ura-His medium in batch cultivations at an initial OD_{600} of 0.15. The batch cultivations were conducted at 30 °C, with shaking at 600 rpm and an airflow rate of 1 vvm. The pH was maintained at 5.0 by automatically adding 2.5 M NaOH. Dissolved oxygen was monitored and kept above 30 % saturation with an agitation cascade. An organic layer of dodecane (Sigma-Aldrich, St. Louis, MO, USA) was added aseptically to 20 % (*v*/*v*) after 12 h of cultivation. The cultures were sampled at 36, 48, and 60 h for geraniol titer testing. For fed-batch cultivation, strains were first grown in a batch culture at 30 °C, with shaking at 600 rpm, an airflow rate of

1 vvm, pH 6.0, and an initial volume of 400 mL. A feed solution, containing 400 g/L glucose, 100 g/L $(NH_4)_2SO_4$, 34 g/L yeast nitrogen base, 1.3 g/L CSM-Ura-His, and 2 g/L leucine, was fed to the fermenter by controlling the specific feed rate to 0.1 h^{-1} after the glucose and ethanol were consumed. The cultures were sampled every 12 h for geraniol titer testing from 36 h. Independently, duplicate cultivations were conducted for each strain.

Metabolite analysis by high-performance liquid chromatography

Samples from shake flasks or batch cultivations were centrifuged at 12,000 rpm for 5 min and filtered through a 0.45-μm syringe filter. Glucose, glycerol, ethanol, and acetic acid concentrations were analyzed by high-performance liquid chromatography (HPLC). The separation column was an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA, USA) operating at 45 °C with a mobile phase of 5 mM H_2SO_4 at a flow of 0.6 mL/min. The peaks were detected by refractive index (RI) and ultraviolet (UV) detectors.

Cell mass determination

OD_{600} was measured by a spectrophotometer (Eppendorf AG, 22331 Hamburg, Germany). The dry cell weight (DCW) was measured by filtering 10 mL of culture through a 0.45-μm nitrocellulose filter. The filters were dried at 105 °C until their weight stabilized, and then they were re-weighed. The DCW was calculated according to the relationship between the OD_{600} and the DCW (1 g/L biomass = 0.246 × (OD_{600}) − 0.0012).

Geraniol extraction, identification, and quantification

One hundred microliters of the organic layer (dodecane/medium mixture) was sampled and centrifuged at 13,000 rpm for 5 min, and then 50 μL of the dodecane layer was collected and stored at −20 °C for analysis. Geraniol was identified by a gas chromatography-mass spectrometry (GC-MS) system (Shimadzu Co., Kyoto, Japan) equipped with an AOC-20i auto-injector, a QP-2010 mass detector, and an Rtx-Wax capillary column (30 m × 250 μm, i.d., 0.25-μm film thickness). One microliter of the dodecane sample was analyzed in a capillary column with a split ratio of 10 using helium as the carrier gas at a flow rate of 0.78 mL/min. The temperatures of the injector and the detector were set at 260 and 280 °C, respectively. The initial oven temperature was adjusted to 60 °C for 2 min and then gradually increased to 150 °C at a rate of 10 °C/min, held for 10 min, and finally increased to 230 °C at a rate of 20 °C/min and held for 5 min. The concentration of a geraniol standard (Sigma-Aldrich) was calculated using linear calibration curves.

Squalene extraction and quantification

The extraction of intracellular squalene was performed as previously described (Rodriguez et al. 2014), with minor modifications. Eight milliliters of yeast cell culture was collected, centrifuged, and washed twice with deionized water. The cell pellets were suspended in 0.4 mL of an alcoholic KOH solution (KOH (20 % (wt/vol)) in 50 % ethanol) and boiled for 10 min. After cooling to room temperature, 0.4 mL of dodecane was added to the samples and vortexed for 10 min for squalene extraction. The dodecane layer was collected for quantification analysis by a GC-MS system (Shimadzu Co.) as described previously (Rodriguez et al. 2014). A squalene standard (Sigma-Aldrich) was used for quantification.

Geraniol toxicity measurements

CEN.PK102-5B cells were cultured in YPD medium overnight and harvested by centrifugation. The cell pellets were washed three times with steriled H_2O. The OD_{600} of the cells was adjusted to 1.0, and the cells were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} of the OD_{600}). Four microliters of each dilution of the samples was spotted on plates containing 0 to 200 mg/L geraniol, and the cultures were grown at 30 °C for 2 days.

Results

Selection of geraniol synthases

To produce geraniol in *S. cerevisiae*, we first focused on the expression of GESs in *S. cerevisiae*. The plant GESs that produced the geraniol with the highest fidelities in vitro were selected, including LdGES (Dong et al. 2013), ObGES (Iijima et al. 2004), and VoGES (Dong et al. 2013). These three GESs and their truncated versions (tGESs) without the peptide for plastid targeting were expressed in *S. cerevisiae*, and their geraniol production was compared. The control strain that harbored empty plasmid did not produce geraniol, while all of the GES-expressing strains produced geraniol (Fig. 2a b). We found that the strains expressing tGESs showed higher geraniol production than the strains expressing full-length GESs (Fig. 2c). Previous studies only investigated the performance of ObGES in *S. cerevisiae* and a relatively low geraniol production was obtained (Fischer et al. 2011; Oswald et al. 2007). In our study, the strain expressing *ObGES* produced

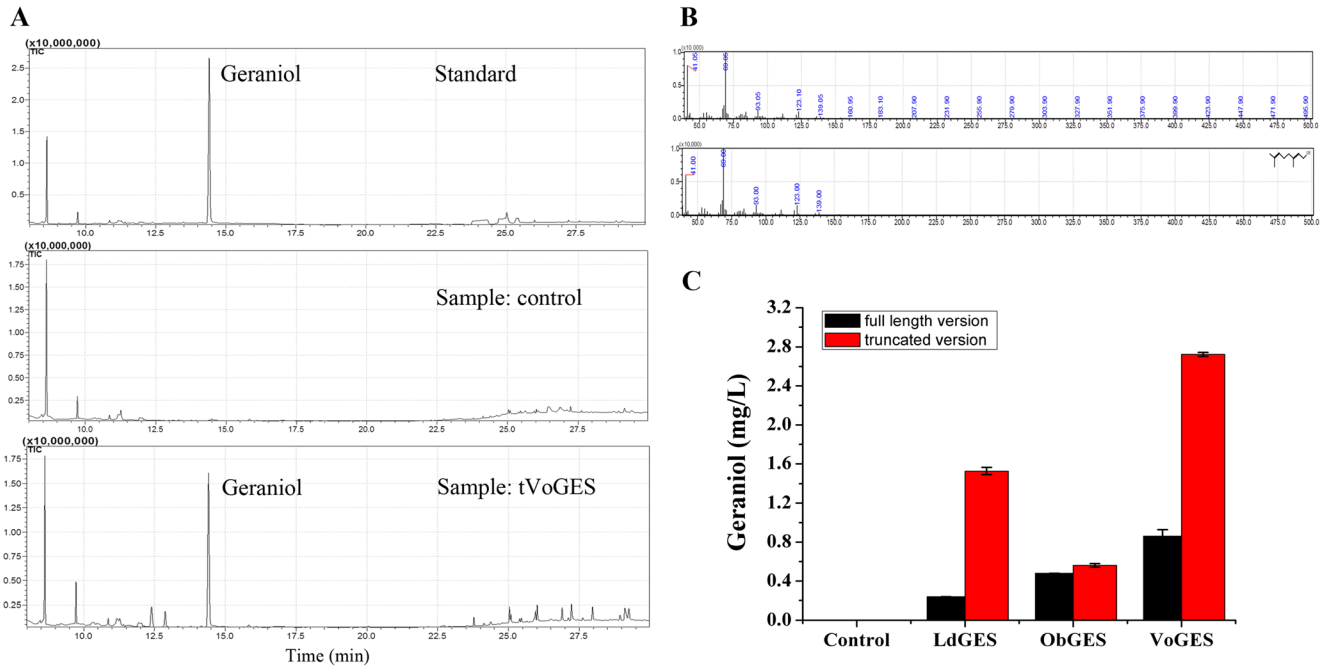

Fig. 2 Geraniol production of strains overexpressing geraniol synthases from different sources in *S. cerevisiae*. **a** Gas chromatography–mass spectrometry results for the geraniol standard, and extracts from the control strain (YZG00) and *tVoGES*-overexpressing strain (YZG03). **b** Mass spectra of the geraniol standard and the geraniol peak of the *tVoGES*-overexpressing strain. **c** Geraniol production in yeast strains overexpressing full-length (*black bars*) and truncated versions (*red bars*) of GES from different sources. The values shown are average values from triplicates, and the *error bars* represent standard deviations

0.56 mg/L geraniol, which was similar to a previous report (Liu et al. 2013a). Interestingly, the strains expressing *tLdGES* and *tVoGES* had much higher geraniol production than the strain expressing *ObGES*. Importantly, the expression of *tVoGES* alone in *S. cerevisiae* yielded 2.7 mg/L geraniol, a 4.2-fold increase compared with that of previous reports (Fischer et al. 2011; Liu et al. 2013a; Oswald et al. 2007). Therefore, *tVoGES* was employed in the engineering of geraniol-producing *S. cerevisiae* strains.

Overexpression of the rate-limiting enzymes in the mevalonate pathway

HMG-CoA reductase was identified as a rate-limiting enzyme, and expressing a truncated *HMG1* (*tHMG1*) improved terpenoid production (Dai et al. 2013; Ro et al. 2006; Zhou et al. 2012). In addition, *IDI1* encodes IPP isomerase, which converts IPP to DMAPP and plays an important role in the distribution of GPP and FPP fluxes. Therefore, we overexpressed *tHMG1* and *IDI1* to improve GPP synthesis. As shown in Fig. 3, *IDI1* overexpression increased geraniol production by 51 %, and *tHMG1* overexpression enhanced geraniol production by 83 %. The strain expressing both *IDI1* and *tHMG1* produced 7.9 mg/L geraniol, which was 2.7-fold higher than that of the control strain (Fig. 3A). Squalene is derived from FPP by squalene synthase, and it can be used as an indicator of the flux of isoprene intermediates through the sterol pathway (Jennings et al. 1991). We also detected the squalene accumulation in these strains (Fig. 3B), and *IDI1* overexpression did not increase squalene accumulation significantly, but *tHMG1* overexpression enhanced its accumulation by 34.6-fold compared with that of the control

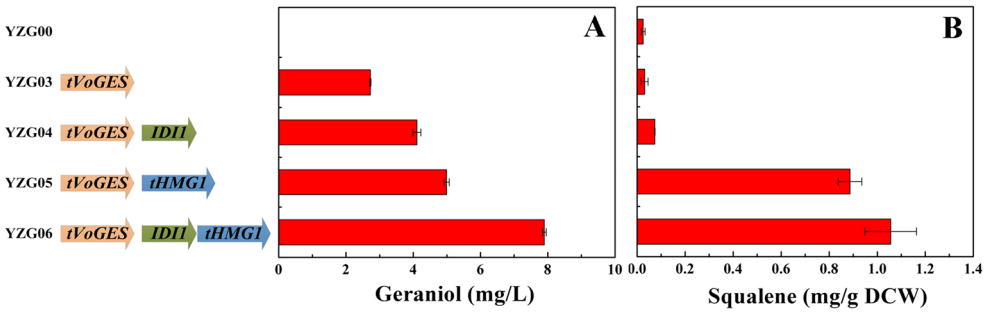

Fig. 3 The geraniol and squalene production of the strains overexpressing the key rate-limiting enzymes of the mevalonic acid (*MVA*) pathway. **a** Geraniol production. **b** Squalene production. The values shown are the average values from triplicates, and the *error bars* represent standard deviations

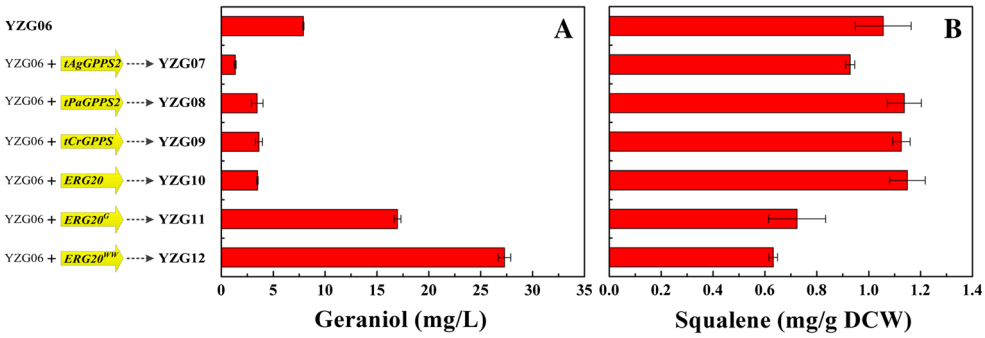

Fig. 4 Geraniol and squalene production in strains overexpressing geranyl disphosphate synthases (*GPPSs*) from different sources. **a** Geraniol production. **b** Squalene production. The values shown are the average values from triplicates, and the *error bars* represent standard deviations

strain. This indicated that *tHMG1* can increase the MVA flux, while *IDI1* mainly improves GPP synthesis.

Redistribution of GPP and FPP synthesis

Although the overexpression of *IDI1* and *tHMG1* can increase GPP synthesis, the GPP pool is still very low compared with that of FPP because of the lack of a specific GPPS in *S. cerevisiae*. The reaction of DMAPP with IPP to form GPP and the conversion of GPP to FPP are sequentially catalyzed by the farnesyl diphosphate synthase Erg20p. Although Erg20p has both GPP and FPP synthase activities, it releases very little GPP from its catalytic site (Fischer et al. 2011). Therefore, to synthesize free GPP, we expressed three specific GPPS genes that are derived from different plants, including *A. grandis GPPS2*, *P. abies GPPS2*, and *C. roseus GPPS*, which produced only GPP without any byproduct in vitro. Since GPPSs are endogenously expressed in plant plastids, we also removed their plastid-targeting peptides. In addition, endogenous Erg20p and the Erg20p mutants (Erg20p(K197G) and Erg20p(F96W-N127W)), which showed more GPPS activity than FPPS activity, were also overexpressed in geraniol-producing strains (*tVoGES*-, *IDI1*-, and *tHMG1*-expressing strains) (Fig. 4a). Surprisingly, the expression of heterologous GPPSs decreased the geraniol yield, and only 3.5 mg/L geraniol was produced in these three strains (Fig. 4a), although a previous report showed that PaGPPS2 slightly increased sabinene production (Ignea et al. 2014). The squalene accumulation in these strains was similar to that of the control strain YZG06, indicating that the introduction of heterologous GPPSs did not obviously change the flux distribution (Fig. 4b). In contrast, strains overexpressing $ERG20^{G}$ and $ERG20^{WW}$ exhibited significantly improved geraniol production, and their geraniol titer was increased by 2.1- and 3.5-fold, respectively, compared with the control strain YZG06 (Fig. 4a). The squalene accumulation in these *ERG20* mutant strains decreased by 31 and 40 %, respectively, indicating that the flux to squalene was reduced along with the improvement in geraniol production (Fig. 4b). We found that although the tested GPPSs from different plants had GPPS activity in vitro, they did not increase the GPP pool when overexpressed in *S. cerevisiae*, and they even reduced the geraniol yield, while the endogenous *ERG20* mutants $ERG20^{G}$ and $ERG20^{WW}$ significantly improved geraniol production.

To further increase the GPP pool, it is necessary to decrease FPP synthesis. Therefore, we investigated the effect of down-regulating the expression of *ERG20* in the $ERG20^{WW}$-overexpressing strain (YZG12). The native promoter of *ERG20* was replaced with the copper-repressing *CTR3* promoter to control *ERG20* transcription. However, the *CTR3* promoter decreased geraniol production even without $CuSO_4$ addition, although the geraniol yield increased by approximately 15 % (Table 1) compared with that of the YZG12 strain. The addition of $CuSO_4$ further reduced geraniol production. We found that both up-regulating and down-regulating the expression of native *ERG20* decreased geraniol production (Fig. 4a and Table 1), indicating the endogenous Erg20p was necessary to maintain GPP synthesis to a certain extent.

Table 1 The effects of $CuSO_4$ addition on the cell mass and geraniol production of strain YZG12-pCTR3

Strains[a]	Media	Geraniol production (mg/L)	Geraniol yield (mg/g DCW)	Dry cell weight (g/L)
YZG12	SD-Ura-His	27.57 ± 1.32	12.89 ± 0.68	2.14 ± 0.07
YZG12-pCTR3	SD-Ura-His	21.81 ± 0.04	14.79 ± 0.47	1.47 ± 0.06
YZG12-pCTR3	SD-Ura-His +50 μM $CuSO_4$	12.33 ± 0.97	8.20 ± 0.65	1.49 ± 0.07
YZG12-pCTR3	SD-Ura-His +100 μM $CuSO_4$	11.15 ± 1.02	7.79 ± 0.21	1.43 ± 0.03
YZG12-pCTR3	SD-Ura-His +150 μM $CuSO_4$	10.47 ± 0.78	7.56 ± 0.66	1.38 ± 0.05

[a] Three replicates were performed for each strain; the average values and standard deviations are shown

Global regulation of the mevalonate pathway

UPC2 is a global transcription factor that regulates the transcription of genes involved in the biosynthesis of sterols, including ergosterol, in *S. cerevisiae* (Davies et al. 2005). UPC2-1 is a semi-dominant mutant (G888D) that enhances UPC2 activity and increases aerobic sterol uptake. The overexpression of UPC2-1 (YZG13) resulted in a 32 % increase in geraniol production compared with that of its parent strain YZG12, and the titer reached 39.3 mg/L (Fig. 5).

Fusion of Erg20p(F96W-N127W) and tVoGES

It has been demonstrated that adjacent active centers of consecutive enzymes in a metabolic pathway are helpful for effective substrate utilization (Kühner et al. 2009; Menon et al. 2009). Therefore, we investigated the impact of the Erg20p(F96W-N127W) and tVoGES fusions on geraniol production. We constructed multiple combinations of Erg20p(F96W-N127W) and tVoGES fusions using three different linkers (GGGS, GGGGS, GSGGSG). As shown in Fig. 5, most of the Erg20p(F96W-N127W) and tVoGES fusions obviously increased geraniol production. Among the six combinations, the tVoGES-GGGS-Erg20p(F96W-N127W) fusion protein exhibited the highest increase, achieving 66.2 mg/L geraniol. This was 1.7-fold higher than that of the strain overexpressing Erg20p(F96W-N127W) and tVoGES separately. This geraniol production was 23.5-fold higher than that of the YZG03 strain that only overexpressed the *tVoGES* gene.

Batch and fed-batch cultivations

We performed batch and fed-batch cultivations using the best geraniol-producing strain, YZG13-GE1. Compared with the YZG00 strain that does not produce geraniol, strain YZG13-GE1 showed a higher maximum specific growth rate (0.35 vs. 0.31/h, respectively) (Fig. 6a, b), which may result from a higher MVA pathway flux. The strain produced 197 mg/L geraniol in batch cultivation. However, the fed-batch cultivation only yielded 293 mg/L geraniol, and the growth of the strain was also poor when the geraniol titer reached 293 mg/L (Fig. 6b, c). It has been reported that monoterpenes are highly toxic to many microorganisms, including *S. cerevisiae*; therefore, we determined the toxicity of geraniol using qualitative growth assays and found that 150 mg/L geraniol slightly inhibited growth and 200 mg/L geraniol completely inhibited cell growth (Fig. 6d). Although dodecane was added to the culture to extract geraniol, the phase toxicity of geraniol may still inhibit growth. Therefore, the toxicity of monoterpenes is another barrier to increasing their production; thus, the geraniol titer cannot be further enhanced by fed-batch cultivations.

Discussion

Terpenoids are a highly diverse group of natural products with applications ranging from medicine, agriculture, and energy production to the chemical industry. Recently, a great improvement in the production of larger terpenes was achieved by employing either *E. coli* or *S. cerevisiae* as cell factories. For example, the sesquiterpenes artemisinic acid (25 g/L) (Paddon et al. 2013), bisabolene (900 mg/L) (Peralta-Yahya et al. 2011), and santalene (163 mg/L) (Tippmann et al. 2015) were produced at high levels in engineered *S. cerevisiae* strain. However, the metabolic engineering of monoterpene production has mainly been done in *E. coli*, and most monoterpenes were produced at low yields. Thus far, the synthesis of a few monoterpenes has been reported in yeast, but their production was also very low. In this study, we developed yeast strains for efficient geraniol production and identified potential barriers to geraniol production. The platform developed here can be readily incorporated for the synthesis of other monoterpenes.

Although a few winemaking strains have been verified to produce less than 5 μg/L monoterpenes (Carrau et al. 2005), including (S)-linalool and α-terpineol, wild-type *S. cerevisiae* strains do not naturally produce monoterpenes in the absence

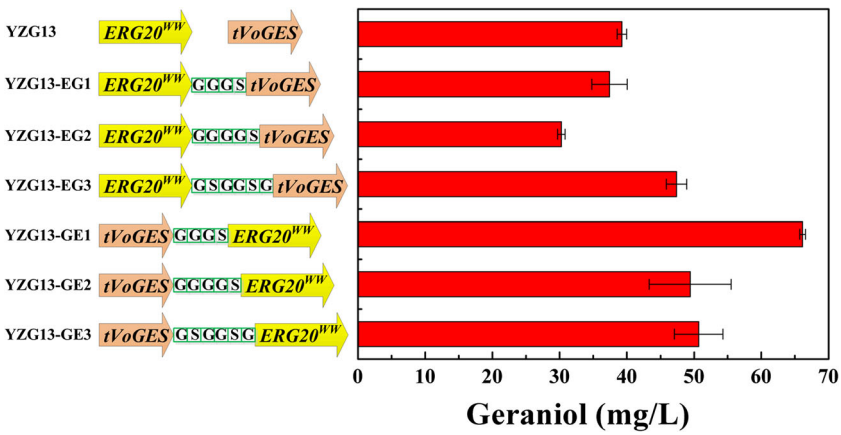

Fig. 5 Geraniol production of strains overexpressing different fusions of Erg20p(F96W-N127W) and tVoGES. YZG13: strain expressing *tVoGES*, *ERG20*^{WW}, *IDI1*, *tHMG1*, and *UPC2-1*. The values shown are the average values from triplicates, and the *error bars* represent standard deviations

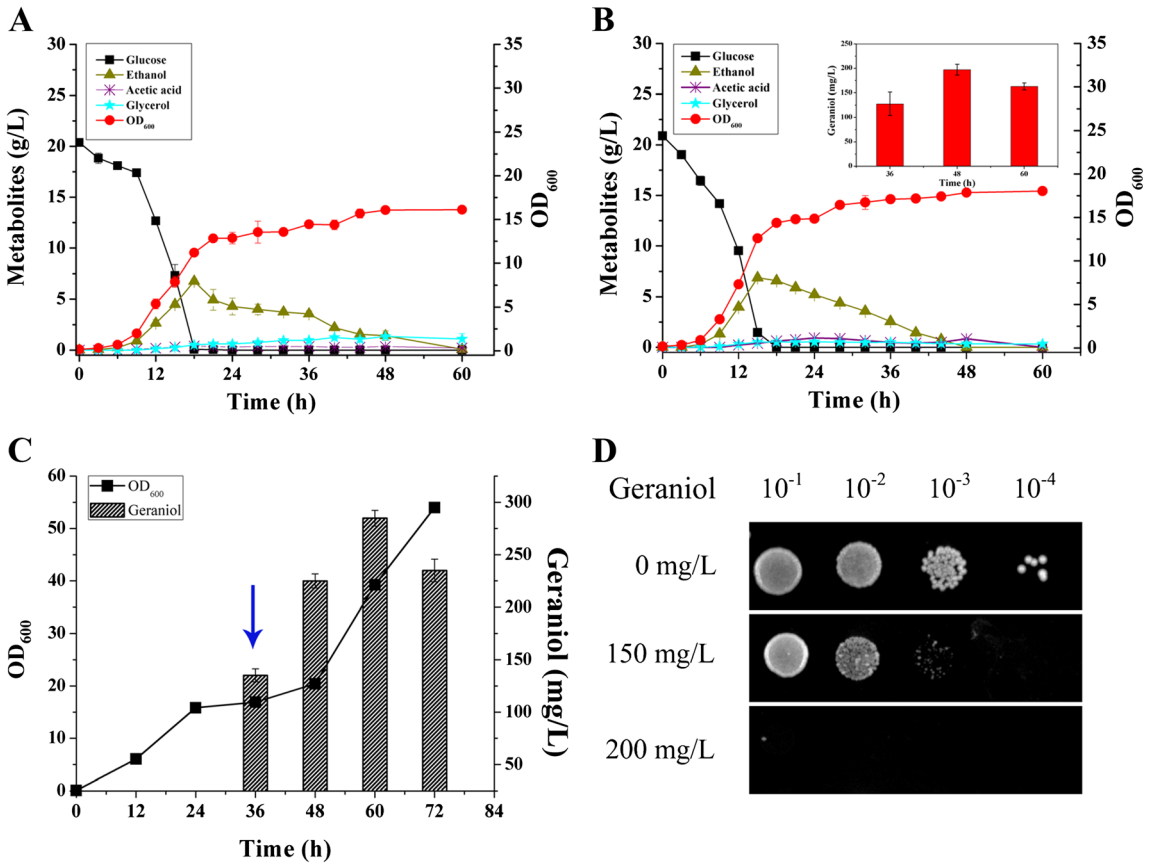

Fig. 6 Fermentation profiles of the batch and fed-batch cultivations. **a** The batch cultivation profile of the control strain YZG00. **b** The batch cultivation profile of the geraniol-producing strain YZG13-GE1. **c** The fed-batch cultivation profile of the geraniol-producing strain YZG13-GE1. The feed was started with the specific feed rate of 0.1/h at 36 h. **d** The growth assay of the CEN.PK102-5B strain in the presence of different geraniol concentrations. The values shown are the average values from duplicates, and the *error bars* represent standard deviations

of monoterpene synthase. To construct a monoterpene geraniol biosynthesis pathway in *S. cerevisiae*, heterogeneous GESs were introduced. We selected the three GESs that yielded the highest fidelities of geraniol production in vitro from seven characterized GESs, including ObGES, which was used for geraniol synthesis in all previously reported studies (Fischer et al. 2011; Liu et al. 2013a; Oswald et al. 2007). Terpene synthases generally contain a signal peptide at their amino-termini for plastid localization, which is cleaved after the protein is inserted into the plastid (Bohlmann et al. 1998). When a plant terpene synthase is expressed in yeast, its signal peptide might affect its enzymatic activity. Our results showed that GESs without the signal peptide had higher enzymatic activities. Among them, the *tVoGES*-expressing strain showed the highest geraniol production, which was fivefold greater than that of a previously reported ObGES. Therefore, we selected a *tVoGES*-expressing strain for further engineering.

We subsequently enhanced the supply of GPP by regulating the MVA pathway. In *S. cerevisiae*, GPP is synthesized by the condensation of IPP and DMAPP, and FPP is then formed by adding one more IPP. The ratio of IPP and DMAPP largely affects the synthesis of GPP and FPP. Idi1p is an isomerase that balances the ratio of IPP and DMAPP. After overexpressing the Idi1p-encoding *IDI1* gene in YZG04, geraniol production increased by 52 %. This suggests that the increase in DAMPP that resulted from *IDI1* overexpression increases the GPP pool. In addition, overexpressing a *tHMG1* gene encoding HMG-CoA reductase, a key rate-limiting enzyme of the MVA pathway, increased carbon flux through the MVA pathway.

After overexpressing *tHMG1*, the accumulation of squalene, a carbon flux indicator of the MVA pathway, significantly increased (34-fold) (Fig. 3b), whereas geraniol production only increased by 85 %, indicating that the lack of available GPP for monoterpene production became the main limiting factor in *S. cerevisiae*. Given that the majority of geraniol is produced in plants, we investigated the capability of plant GPPSs to produce GPP in *S. cerevisiae*. In plants, three classes of GPPSs have been identified according to sequence analyses (Schmidt and Gershenzon 2008). Heteromeric GPPSs, such as *M. piperita* GPPS, are composed of small and large subunits, (Chang et al. 2010). These kinds of GPPSs show no activity if either subunit is lacking, but together they produce GPP and geranylgeranyl diphosphate (GGPP) in the presence of excess IPP. Homomeric GPPSs are divided into two classes. One,

which is found in conifers such as *A. grandis* and *P. abies*, has high fidelity and only produces GPP (Burke and Croteau 2002). The other class, which is found in *Arabidopsis thaliana*, was initially identified as a GPPS, but a recent study suggested that this enzyme may be a polyprenyl pyrophosphate synthase that produces products ranging from C25 to C45 (Hsieh et al. 2011). We selected three high-fidelity homomeric GPPSs, AgGPPS2, PaGPPS2, and CrGPPS, which produce only GPP in vitro, and expressed them in *S. cerevisiae* strain YZG06. Unexpectedly, the expression of three plant GPPSs all decreased geraniol production, similar to endogenous Erg20p overexpression. We presumed that the GPPSs may show FPPS activity in vivo; however, the exact reason of their negative effect on geraniol production needs to be confirmed by further experiments. Engineering native yeast Erg20p to yield GPPS activity, as opposed to FPP synthase activity, is another strategy to improve the GPP pool. Previous studies have reported the alteration of the enzymatic property of an avian FPP synthase (FPPS1), as well as yeast Erg20p, using similar protein engineering strategies (Fischer et al. 2011; Ignea et al. 2014). Among the Erg20p mutants, the Erg20p(K197G), Erg20p(K197S), Erg20p(N127W), and Erg20p(F96W-N127W) mutants showed much greater GPPS activity relative to FPP synthase activity. In the present study, we selected the Erg20p(K197G) and Erg20p(F96W-N127W) mutants and expressed them in geraniol-producing strains. Overexpressing Erg20p(F96W-N127W) led to the highest improvement of geraniol production. These results demonstrated that the native yeast Erg20p variants with high GPPS activity synthesized GPP more effectively than plant GPPSs in *S. cerevisiae*. In addition, we found that both up-regulating and down-regulating the expression of native *ERG20* decreased geraniol production, and down-regulating native *ERG20* expression decreased cell growth. Although Erg20p is a FPP synthase, its native expression level may be necessary to maintain cell growth and GPP synthesis to a certain extent.

We also designed different Erg20p(F96W-N127W)-tVoGES fusion proteins. It has been reported that the formation of protein complexes between enzymes that catalyze consecutive synthesis steps in a metabolic pathway is helpful for substrate channeling. The fusion of two or more such enzymes may avoid the loss of intermediates and alleviate feedback inhibition (Srere 1987). We investigated the effects of different linkers and fusion proteins on geraniol production, and found that the strain harboring tVoGES-GGGS-Erg20p(F96W-N127W) exhibited the highest geraniol production. The active sites of this fusion protein may be in closer proximity than those of the other fusions, thereby facilitating more efficient substrate channeling. It is worth mentioning that the accumulation of squalene also decreased by approximately 15 % compared with the strain overexpressing a single enzyme. This suggests that the tVoGES-GGGS-Erg20p(F96W-N127W) fusion improved the GPP flux during geraniol production and decreased GPP flux down the squalene synthesis pathway (Liu et al. 2013a).

Geraniol production by YZG13-GE1 was 197 mg/L in batch cultivation; however, it no longer increased after reaching 293 mg/L in fed-batch cultivation, and cell growth was also affected. We found that in a quantitative growth assay, cell growth was significantly inhibited when the geraniol concentration was greater than 200 mg/L. It seems that the geraniol titer cannot be further improved through fed-batch cultivation because of the toxicity of geraniol; therefore, geraniol toxicity is another barrier for its production. It has been reported that monoterpenes are highly toxic to many microorganisms, including *S. cerevisiae*, and their toxicity results from their altering membrane properties (e.g., pinene and D-limonene) (Liu et al. 2013b; Uribe et al. 1985) or damaging the cell wall (e.g., limonene) (Brennan et al. 2013). Because monoterpenes are lipophilic compounds, they are sparingly soluble in water and cause phase toxicity, rather than molecular toxicity. Although two-phase extractive fermentation can alleviate monoterpene toxicity in *S. cerevisiae*, phase toxicity still occurs beyond a solvent's solubility (Brennan et al. 2012). Adaptive evolution was performed to improve yeast tolerance to monoterpenes and $tTCB3p^{1-989}$, a truncated protein involved in endoplasmic reticulum-plasma membrane tethering, was identified as a genetic target by which to alleviate phase toxicity (Brennan et al. 2015). Therefore, the combination of two-phase extraction fermentation and strain engineering is an effective strategy to further improve monoterpene production in *S. cerevisiae*.

In this study, we constructed yeast strains for efficient geraniol production by regulating GPP flux and selecting efficient geraniol synthases. We first increased GPP synthesis by overexpressing the key limiting enzyme in the MVA pathway. The comparison of heterologous GPPSs and endogenous Erg20p variants revealed that the endogenous Erg20p variants with high GPPS activity had better performance in terms of increasing the GPP pool. In addition, down-regulating the expression of wild-type Erg20p did not have a positive effect on geraniol production. Geraniol production reached 293 mg/L, the highest reported production in yeast to date, via global regulation of the MVA pathway and fusing a GES and Erg20p(F96W-N127W). The platform developed here can be readily used to synthesize other monoterpenes. However, the toxicity of geraniol obviously limits its production; therefore, monoterpene tolerance needs to be improved during the subsequent development of cell factories.

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Author contributions JZ and JH designed the experiments. JZ and CL carried out the experiments. JZ, XB, YS, and JH analyzed the data. JZ and JH wrote the manuscript. All of the authors have read and approved the final manuscript.

Compliance with ethical standards

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

Competing interests The authors declare that they have no competing interests.

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