Dynamic control of ERG20 expression combined with minimized endogenous downstream metabolism contributes to the improvement of geraniol production in Saccharomyces cerevisiae

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Abstract

Background: Microbial production of monoterpenes provides a promising substitute for traditional chemical-based methods, but their production is lagging compared with sesquiterpenes. Geraniol, a valuable monoterpene alcohol, is widely used in cosmetic, perfume, pharmaceutical and it is also a potential gasoline alternative. Previously, we constructed a geraniol production strain by engineering the mevalonate pathway together with the expression of a high-activity geraniol synthase.

Results: In this study, we further improved the geraniol production through reducing the endogenous metabolism of geraniol and controlling the precursor geranyl diphosphate flux distribution. The deletion of OYE2 (encoding an NADPH oxidoreductase) or ATF1 (encoding an alcohol acetyltransferase) both involving endogenous conversion of geraniol to other terpenoids, improved geraniol production by 1.7-fold or 1.6-fold in batch fermentation, respectively. In addition, we found that direct down-regulation of ERG20 expression, the branch point regulating geranyl diphosphate flux, does not improve geraniol production. Therefore, we explored dynamic control of ERG20 expression to redistribute the precursor geranyl diphosphate flux and achieved a 3.4-fold increase in geraniol production after optimizing carbon source feeding. Furthermore, the combination of dynamic control of ERG20 expression and OYE2 deletion in LEU2 prototrophic strain increased geraniol production up to 1.69 g/L with pure ethanol feeding in fed-batch fermentation, which is the highest reported production in engineered yeast.

Conclusion: An efficient geraniol production platform was established by reducing the endogenous metabolism of geraniol and by controlling the flux distribution of the precursor geranyl diphosphate. The present work also provides a production basis to synthesis geraniol-derived chemicals, such as monoterpene indole alkaloids.

Keywords: Monoterpene, Geraniol, Geranyl diphosphate, ERG20, Saccharomyces cerevisiae

Background

In nature, isoprenoids are a large group of diverse compounds, that are biosynthesized via both the cytosolic mevalonate (MVA) pathway and the plastidial 2C-methyl-D-erythritol 4-phosphate (MEP) pathway in plants and most bacteria, or the mevalonate (MVA) pathway in animals and eukaryotes [1]. The universal precursors of isoprenoids, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are derived from both pathways. Among them, monoterpenes are a particularly interesting subset of this family, which has been widely used as flavors, fragrances and pharmaceuticals as well as potential fuels [2, 3]. Traditionally, monoterpenes
and their derivatives are produced from plants in low amounts, and the extraction process from plants is costly and highly dependent on the availability of raw materials. Engineering microbial organisms for monoterpene synthesis provides a potential effective route for their production.

Monoterpene geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol; C_{10}H_{18}O), an acyclic monoterpene, is typically generated from aromatic plants and has many applications in perfume, pharmaceutical, and other chemical industries [4]. Geraniol is also considered as a promising biofuel due to its high energy content, low hygroscopicity and low volatility in comparison with ethanol [5].

In addition, a recent study reported that geraniol can undergo an 11-step heterologous biosynthetic pathway to form strictosidine, which is the common intermediate for production of the anticancer agent monoterpene indole alkaloids (MIAs) [6]. Given its low yield in aromatic plants, geraniol has been successfully synthesized in Escherichia coli and Saccharomyces cerevisiae through metabolic engineering strategies [7–11]. S. cerevisiae has been used widely as a cell factory to produce a diversity of terpenes, and many of them have achieved significant yields [12–15]. However, monoterpene production has been achieved only at low levels thus far [8, 16, 17]. There are two challenges for high level monoterpene production. One is the availability of intracellular geranyl diphosphate (GPP) precursor, and the other is the toxicity of monoterpene to cells [18, 19]. Most of monoterpene are generated from the precursor GPP which is formed by condensation of one molecule of IPP with one molecule of its isomer DMAPP (Fig. 1). Unlike plants, S. cerevisiae does not supply enough GPP for production due to the lack of a specific GPP synthase (GPPS). In S. cerevisiae, the ERG20 gene of the endogenous MVA pathway encodes a farnesyl pyrophosphate synthase (FPPS) that, despite having both GPP and FPP synthase activity, only releases a very low amount of GPP from its catalytic site [20]. To increase the flux to GPP, a set of ERG20 mutants was constructed to screen for GPPS preference mutants. ERG20K197G, the most effective mutant, improved geraniol production to 5 mg/L while expressing geraniol synthase (GES) from Ocimum basilicum [8]. Liu et al. obtained 36 mg/L geraniol by overexpressing two key rate-limiting genes, tHMG1 encoding a truncated version of 3-hydroxy-3-methylglutaryl coenzyme A and IDI1 encoding IPP isomerase, to enhance MVA pathway flux and by overexpressing BTS1, a negative regulator of MOD5 encoding tRNA isopentenytransferase, to decrease the flux to tRNA biosynthesis [10]. In addition, Ignea et al. demonstrated that the double mutant Erg20p (F96W-N127W) had a strong dominant negative ability to decrease the FPPS function of Erg20p, increasing production of the monoterpene sabine by 10.4-fold to 0.53 mg/L in an industrial diploid strain [16]. In our previous work, we further improved geraniol production through enhancing MVA pathway flux, screening different sources of GESs and GPPSs, and designing fusion proteins of GES and GPPS. Geraniol of 293 mg/L production was achieved in fed-batch cultivation, which is the highest monoterpene production in S. cerevisiae ever reported [21]. However, it is still lower than the production of many other terpenes in S. cerevisiae [12, 22, 23].

In addition to the low GPP pool, the toxicity to microorganisms of monoterpene also largely limits their microbial production [2, 24]. To alleviate the toxicity of monoterpene, a two-phase fermentation system has been extensively applied by adding a non-toxic extractive solvent (e.g., dodecane) [2]. In addition, monoterpenols, usually undergo biotransformation to other terpenoids in aromatic plants, as well as in some wine yeasts [25, 26]. A recent study demonstrated that geraniol is converted into citronellol under the catalysis of the enzyme OYE2, and it is acetylated by ATFI encoding alcohol acetyltransferase during wine fermentation by S. cerevisiae [27].
Intracellular bioconversion of geraniol may be a self-defense response to avoid the toxicity of monoterpenes. In addition, introduction of a heterologous biosynthetic pathway and the overexpression of an endogenous pathway often lead to the accumulation of some intermediate metabolites, that may interfere with the native regulation of metabolic flux and increase metabolic burden, resulting in overproduction of some biosynthetic enzymes and accumulation of some toxic intermediate metabolites. Therefore, a dynamic control system that can sense environmental changes such as metabolic intermediates or nutrient concentrations can be used to avoid the toxicity of intermediate metabolites [28–30].

Previously, we achieved a significant increase in geraniol production through expressing geraniol synthase from *Valeriana officinalis* and regulating GPP synthesis [21]. In this study, we further engineered strains through dynamic control of ERG20 to fine-tune the GPP flux combined with minimized geraniol endogenous conversion. Combining these strategies together with *LEU2* auxotrophic complementation, the final geraniol production reached 1.69 g/L in fed-batch fermentation, which is the highest reported production in yeast.

**Results**

**Improving geraniol production by minimizing endogenous bioconversion**

Geraniol is the main precursor of monoterpenoids in aromatic plants [4]. It also has many derivatives including nerol, neral, geranylgeraniol, geranial, citronellol and terpenyl acetates [31]. Previous studies have demonstrated that wine yeasts are able to convert geraniol into other monoterpenoids, which influence the sensory properties of wine [26, 32]. During *S. cerevisiae* fermentation, Oye2p is the main enzyme involved in the conversion of geraniol to citronellol, and Atf1p is the main contributor to synthesis of terpenyl acetates from geraniol [27]. In our early study, an efficient *S. cerevisiae* strain YZG13-GE1 was constructed to produce geraniol from glucose through engineering of geraniol synthesis and optimizing GPP synthesis. To further improve geraniol production, we attempted to minimize endogenous metabolism of geraniol in *S. cerevisiae*. Thus, we deleted OYE2 or ATF1 in YZG13-GE1 to create YZG14 or YZG15, respectively. Batch fermentation was carried out in a two-phase fermentation system using dodecane as extractive solvent. Geraniol was produced at 285.9 mg/L by YZG14 with OYE2 deletion, which was improved by 1.7-fold compared with the control strain (YZG13-GE1), and 259.8 mg/L was produced by YZG15 with ATF1 deletion, which was improved by 1.6-fold compared with the control strain (YZG13-GE1) (Fig. 2a). The conversion of geraniol to citronellol was decreased by 55% in YZG14 (∆oye2) (Additional file 1: Figure S1). In addition, we further investigated the effect of double deletion of OYE2 and ATF1 in YZG13-GE1 on geraniol production. The results showed that geraniol production dramatically decreased to 32.2 mg/L in YZG16 with OYE2-ATF1 deletions (Fig. 2a). Cell growth was not affected in either single gene deletion strain (∆oye2 or ∆atf1) (Additional file 1: Figure S2), and geraniol yields were 57.8 mg/g DCW and 58.2 mg/g DCW, a 1.8-fold and 1.7-fold increase, respectively (Fig. 2b; Table 1). However, double deletion of OYE2 and ATF1 decreased the final biomass by 35%, and geraniol yield significantly dropped to 9.6 mg/g DCW (Fig. 2; Table 1).

We also compared geraniol production in YZG14 (∆oye2), YZG15 (∆atf1) and YZG16 (∆oye2∆atf1) in fed-batch cultivation using glucose as sole carbon source.
cell growth of YZG14 (Δoye2) and YZG15 (Δatf1) exhibited a similar growth profile compared with the reference strain YZG13-GE1. YZG14 (Δoye2) and YZG15 (Δatf1) produced 421.7 and 371.1 mg/L of geraniol, which represented approximately 2.5-fold and 2.2-fold increases relative to YZG13-GE1 (256.4 mg/L), respectively (Fig. 3). However, geraniol production was dramatically decreased in double deletion of OYE2 and ATFI1 strain, only producing 46.1 mg/L geraniol (Fig. 3d).

### The effect of ERG20 expression control on geraniol production

In *S. cerevisiae*, synthesis of both the monoterpene precursor GPP and sesquiterpene precursor FPP is catalyzed by a single enzyme, Erg20p, with FPP synthase preference. In the absence of a specific GPP synthase, many efforts have been made to increase the intercellular GPP pool. Expression of heterologous GPP synthase does not increase the GPP-derived products significantly [16, 21]. Although engineering Erg20p into a GPP synthase increased sabinene and geraniol production by 10.4-fold and 2.8-fold, respectively, the titer is still much lower than that of FPP-derived sesquiterpenes, demonstrating the importance of down-regulating FPP synthase [10, 16]. In this study, the native ERG20 promoter was first replaced with either the weak BTS1 promoter or the copper repressible CTR3 promoter. YZG13-GE1 containing the native ERG20 promoter was used as a reference strain. The transcription level of ERG20 using the BTS1 promoter or CTR3 promoter was reduced by 90 and 75% (Fig. 4a), respectively, in SD-URA-HIS medium with 2% glucose. Unexpectedly, the replacement of the ERG20 promoter with that of BTS1 or CTR3 decreased geraniol production by 75.6 and 70.3%, resulting in 40.37 and 48.85 mg/L, respectively (Fig. 4b). The BTS1 and CTR3 promoters also decreased the final biomass by 60 and 40% (Fig. 4c), respectively. Previously, we also found that overexpression of ERG20 reduced geraniol production [21]. These results demonstrated that both down-regulation and up-regulation of ERG20 have a negative impact on geraniol production. The dynamic control of ERG20 was then attempted by replacing the ERG20 promoter with the glucose-sensing HXT1 promoter. This promoter strength is higher than that of ERG20 promoter when glucose is present in the medium (Fig. 4a), but it is repressed when the glucose concentration is low or absent (Additional file 1: Figure S3). We found that the geraniol titer and final biomass were not markedly affected by HXT1 promoter replacement in batch fermentation, and the yield of geraniol increased slightly (37.9 mg/g DCW vs. 32.7 mg/g DCW) (Table 1). Considering that the HXT1 promoter provides both high-glucose induction and low-glucose repression, we attempted both glucose and glucose/ethanol (1:7) mixture feeding strategies to control ERG20 expression in fed-batch fermentation. Compared with the control strain, HXT1 promoter replacement did not increase geraniol production when pure glucose was fed; geraniol production only increased slightly (data not shown). In contrast, when a glucose/ethanol (1:7) mixture was fed, the geraniol production achieved a 176% increase in the HXT1 promoter replacement strain compared with the control strain, leading to 650.8 mg/L (Fig. 4d).

Previously, ethanol has been used as carbon for the production of hydrocortisone, amorpha-4,11-diene and resveratrol [33–35]. In this study, we further compared the impact of feeding different ratios of glucose and ethanol on geraniol production using YZG19 (P₂HXT₁-ERG20). As shown in Fig. 5b, the cell growth of YZG19 (P₂HXT₁-ERG20) gradually improved as the ethanol percentage in glucose/ethanol mixture increased, and the final production of geraniol also gradually increased. The highest production of geraniol was obtained when pure ethanol was fed, producing 867.7 mg/L of geraniol (Fig. 5a). Based on the results described above, the dynamic control of ERG20 expression under the HXT1 promoter together with optimizing the glucose and ethanol ratio improved the production of geraniol by 3.8-fold.

### Combining the dynamic control of ERG20 and reduction of endogenous conversion together with LEU2 complementation enabled a further increase in geraniol production

To further improve geraniol production, we deleted the OYE2 gene in YZG19 (P₂HXT₁-ERG20) to generate YZG20 (Δoye2, P₂HXT₁-ERG20), which produced 984.6 mg/L of geraniol in fed-batch fermentation by feeding ethanol, a 14% increase over YZG19 (P₂HXT₁-ERG20) (Fig. 6a). A recent study showed that leucine metabolism may be networked with isoprenoid biosynthesis, and leucine prototrophy was found to improve diterpenoid miltiradiene

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Table 1 Geraniol yield of the engineered strains in batch fermentation

| Strains*  | Dry cell weight (g/L) | Geraniol yield (mg/g DCW) |
|----------|-----------------------|--------------------------|
| YZG13-GE1 | 5.02 ± 0.31           | 32.69 ± 2.01             |
| YZG14 (Δoye2) | 4.94 ± 0.28           | 57.83 ± 3.24             |
| YZG15 (Δatf1) | 4.47 ± 0.22           | 58.16 ± 2.59             |
| YZG16 (Δoye2Δatf1) | 3.36 ± 0.34           | 9.58 ± 0.96              |
| YZG17 (PBS1-ERG20) | 1.96 ± 0.10           | 20.58 ± 0.91             |
| YZG18 (PCTR3-ERG20) | 2.94 ± 0.23           | 16.61 ± 1.21             |
| YZG19 (PHXT1-ERG20) | 3.92 ± 0.26           | 37.87 ± 2.55             |

* Duplicate experiments were performed for each strain, and the error bars represented the standard deviation.
We further engineered a strain with complemented LEU2 auxotrophy, and final geraniol production was enhanced up to 1.69 g/L, a further 71% increase over YZG20 (Δoye2, P_HXT1-ERG20). Meanwhile, we noticed that LEU2 complementation also improved cell growth and geraniol yield (Fig. 6b). The final geraniol production in this study was improved by 5.8-fold compared with our previous study.

**Discussion**

Engineering *S. cerevisiae* as a cell factory is a promising and attractive route for rapid and inexpensive biosynthesis of terpenoids [12, 13, 22, 23, 34]. Over the last few years, most work has focused on the production of sesquiterpenes in microorganisms including *S. cerevisiae*. For instance, production of the sesquiterpene amorpha-4,11-diene, the precursor for the antimalarial agent artemisinin, has reached 40 g/L using engineered *S. cerevisiae* in fed-batch fermentation [12]. Both monoterpenes and sesquiterpenes are derived from the MVA pathway. However, monoterpenes production in *S. cerevisiae* is much lower than that of sesquiterpenes, mainly due to the poor GPP precursor supply and the toxicity of monoterpenes in yeast. In this work, we further engineered production of the monoterpene geraniol through reduction of endogenous geraniol conversion, dynamic control of ERG20 expression and leucine biosynthesis complementation based on YZG13-GE1, a geraniol-producing strain from our previous work [21]. Final geraniol production of 1.69 g/L was obtained, which to our knowledge is the highest level reported for engineered yeast.

Monoterpenes are highly toxic to many microorganisms. Cells may reduce their toxicity through the conversion of monoterpenes to other lower toxicity compounds.

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**Fig. 3** Fed-batch fermentation of YZG13-GE1 (control) (a), YZG14 (Δoye2) (b), YZG15 (Δatf1) (c) and YZG16 (Δoye2Δatf1) (d). Aerobic fed-batch fermentations were carried out by feeding 400 g/L glucose at 0.1/h feed rate. The data shown are representative of duplicate experiments, and the error bars represent the standard deviation.
by various specific or non-specific enzymes. In our previous study, we confirmed that 180 mg/L of geraniol in culture could strongly inhibit yeast cell growth [21]. Although two-phase extractive fermentation could effectively alleviate monoterpene toxicity in situ, the conversion of monoterpenes to other terpenoids is not avoided completely. In \textit{S. cerevisiae}, a mechanism was proposed in which geraniol was first oxidized to geranial by \textit{OYE2} encoding an NADPH oxidoreductase and its homologous gene \textit{OYE3}, belonging to the old yellow enzyme family, and then was reduced to citronellol [25, 27, 37]. However, the oxido-reductases involved in the reaction of geranial to citronellol are still unknown. Of the two old yellow enzymes, \textit{OYE2} was the main contributor to the reduction reaction. Except for citronellol, terpenyl acetates including citronellyl acetate and geranyl acetate are another group of geraniol-derived metabolites catalyzed by \textit{ATF1} encoding alcohol acetyltransferase. In our study, deletion of \textit{OYE2} or \textit{ATF1} led to a 1.8-fold increase of geraniol production. Interestingly, the \textit{ATF1} deletion strain increased citronellol production compared with the control strain (Additional file 1: Figure S1). A similar phenomenon was observed in a previous study, which indicated that there may be a relationship between the physiological functions of \textit{OYE2} and \textit{ATF1}. Although the old yellow enzymes in yeast may have a physiological role.
in detoxification of unsaturated metabolites and reactive oxygen species, both OYE2 and ATF1 are thought to be involved in sterol metabolism [38, 39]. Therefore, functional complementation in the regulation of sterol metabolism between OYE2 and ATF1 may be a reason for the lower cell growth and geraniol production in the double deletion strain.

In the geraniol-producing engineered *S. cerevisiae*, the reaction catalyzed by ERG20 is a key branch point towards either geraniol synthesis or downstream ergosterol synthesis for cell growth. Although ERG20 mutations could improve monoterpene production, the reduction of intercellular FPP synthesis damaged cell growth [8, 16]. In this study, the weak promoter \( P_{BTS1} \) and the copper-repressible promoter \( P_{CTR3} \) were first tested for ERG20 down-regulation to redirect GPP flux. The transcriptional level of ERG20 in the promoter replacement strains was decreased to a one-fifth to one-sixth, and reduced cell growth was also observed when geraniol synthase and the MVA pathway were overexpressed in promoter replacement strains (Fig. 4; Additional file 1: Figure S4). The enhancement of the MVA pathway together with ERG20 down-regulation possibly led to the accumulation of toxic intermediates such as DMAPP or GPP. The toxicity of isoprenoid precursors has also been reported in *E. coli* and *Bacillus subtilis* [40–42]. Previously, we found that although overexpression of ERG20 did not affect growth, it decreased geraniol production. Therefore, neither up-regulation or down-regulation of ERG20 was able to increase geraniol production. A suitable ERG20 expression strategy that can weaken the downstream FPP flux but not impair cell growth to further improve geraniol production in engineered yeast is needed. To achieve this goal, dynamic control of ERG20 is proposed, which is likely to not only balance metabolism between product formation and cell growth, but also prevent the accumulation of toxic metabolites. Recently, several dynamic control systems have been developed and applied in metabolic engineering, i.e., a biosensor (sensor-regulator) system [43], a sequential control system based on the medication GAL regulation system combined with the HXT1 promoter [23] and a novel CRISPR-Cas9 interference system [44]. To avoid early accumulation of toxic intermediates and to better balance the utilization of GPP, \( P_{HXT1} \), a glucose-sensing promoter, was employed to dynamically control ERG20 expression in our study. An HXT1 promoter replacement strain (YZG19) did not contribute to improve geraniol production in batch or fed-batch fermentation using glucose as sole carbon, but its advantage appeared when the glucose concentration was low (feeding glucose/ethanol mixture carbon). The geraniol production improved stepwise when the glucose percentage decreased in the glucose/ethanol mixture (Figs. 4, 5). These results indicate that the dynamic control of ERG20 expression by the HXT1 promoter can balance the flux distribution between cell growth and monoterpene synthesis, thus improving geraniol production with low glucose feeding (Fig. 5). In addition, ethanol feeding possibly improved the geraniol production to some extent due to supplying acetyl-CoA precursors in a more direct metabolic pathway. An ethanol feeding strategy has also been successfully applied in amorphadiene and resveratrol production [34, 35].

Finally, strategies combining the OYE2 deletion and \( P_{HXT1} \)-controlled ERG20 expression further improved...
geraniol production to 984.6 mg/L (Fig. 6). A previous report showed that leucine metabolism may be networked with sterol biosynthesis, and that leucine was disassimilated to form HMG-CoA in *Leishmania mexicana* [45]. In *S. cerevisiae*, the leucine biosynthetic pathway may act as a bypass pathway to supplement additional HMG-CoA. Therefore, we further constructed the prototrophic strain YZG21 by complementing the auxotrophic marker *LEU2*. The *LEU2* complementation ultimately improved the geraniol production to 1.69 g/L, meanwhile also improving cell growth and yield. A similar result was observed in a previous study on miltiradiene production [36].

Although a relatively high level of geraniol was obtained in our work, the cytotoxicity of monoterpenes is still considered as a problem that hinders further improvement of monoterpenes production. Improving the resistance of microorganisms to monoterpenes still needs to be addressed to further enhance the monoterpenes production. In addition, diploidization is another potential strategy for improving biomass and productivity given that diploid strains generally grow faster and tolerate higher stresses compared with haploid strains [36, 46]. More importantly, constructing a plasmid-free geraniol overproducing strain by integration of all pathway components into the genome is necessary for application in industrial processes.

**Conclusions**

In summary, we engineered geraniol biosynthesis through the reduction of endogenous geraniol conversion and dynamic control of *ERG20* expression. In addition, we further identified the importance of *LEU2* complementation to geraniol synthesis. Ultimately, 1.69 g/L of geraniol was achieved when these approaches were combined in fed-batch fermentation with pure ethanol feeding. The present work provides a good platform for the production of geraniol and its derived chemicals.

**Methods**

**Medium**

The *E. coli* strain Tans5α was used for gene cloning and grown in Luria–Bertani medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with 100 mg/L ampicillin at 37 °C. The engineered yeast strain YZG13-GE1 constructed in our previous work was used as the parent strain for further engineering [21]. Yeast cells were cultivated in yeast extract-peptone-dextrose (YPD) medium (20 g/L glucose, 20 g/L tryptone, and 10 g/L yeast extract), SD-URA or SD-URA-HIS medium (20 g/L glucose, 1.7 g/L yeast nitrogen base, and 5 g/L \((\text{NH}_4)_2\text{SO}_4\); synthetic complete drop-out medium without uracil and/or histidine). Geneticin (G418) at 400 mg/L was added to the culture medium for gene deletion and promoter replacement.

**DNA manipulation and strain construction**

All yeast strains constructed in this study are listed in Table 2. The reference strain is YZG13-GE1, which is derived from CEN.PK102-5B and harbored pZGV6-GE1 (2μ ori, URA3, *P_{TEF1}*-tVoGES-(GGGS)-*ERG20* WW) and pZMVA4 (2μ ori, HIS3, *P_{TEF1}*-tHMGI, *P_{PGK1}*-IDI1, *P_{TEF1}*-UPC2.1) [21]. The primers for all DNA fragment amplifications are listed in Additional file 1: Table S1.
To replace the ERG20 promoter with different promoters, integration cassettes were constructed via fusion PCR using loxp-kanMX-loxp as the selection marker. The BTS1 promoter (from $-1$ to $-333$), CTR3 promoter (from $+30$ to $-1116$), and HXT1 promoter (from $-1$ to $1190$) replaced the ERG20 promoter in the chromosome [23, 47, 48]. Similarly, the deletions of genes ATF1 and OYE2 were also performed through homologous recombination using loxp-kanMX-loxp as the selection marker. The LEU2 cassette was integrated into the YPRCτ3 site of the chromosome. The DNA fragments were transformed into S. cerevisiae CEN.PK102-5B by the standard lithium acetate method [49], and the transformants were selected on YPD agar plates supplemented with 400 mg/L geneticin (G418). The plasmid pSH47 (purchased from Euroscarf) was transformed into S. cerevisiae to remove the selection marker. The engineered strains were then transformed with plasmids pZGV6-GE1 and pZMVA4, resulting in a series of geraniol-producing strains (Table 2).

### Batch and fed-batch fermentation for geraniol production

To determine the performance of the recombinant yeast strains, batch fermentation and fed-batch fermentation were performed in a 1-L Infors-HT fermenter (Infors AG, Bottmingen, Switzerland). To prepare seed cultures, the strains were grown for 24 h in 5 mL of SD-URA-HIS medium, and then inoculated into fresh SD-URA-HIS medium at an OD$_{600}$ of 0.2 and cultivated for 12 h. For batch fermentation, a seed culture was inoculated into a 1-L Infors-HT fermenter (Infors AG, Bottmingen, Switzerland) containing 0.6 L of SD-URA-HIS medium at an initial OD$_{600}$ of 0.2. Batch cultures were conducted at 30 °C, with the agitation rate at 600 rpm and an airflow rate of 1 vvm. The pH was maintained at 5.0 by automatic addition of 2.5 M NaOH. Dissolved oxygen was maintained above 30% saturation throughout cultivation by setting the stirring speed rate. For fed-batch cultivation, strains were first grown in a 400-mL batch culture at 30 °C with shaking at 600 rpm, an airflow rate of 1 vvm and pH 5.0. After the glucose and ethanol produced in the batch phase were depleted, feeding solution containing 400 g/L glucose, 100 g/L (NH$_4$)$_2$SO$_4$, 34 g/L yeast nitrogen base, 1.3 g/L CSM-Ura-His, and 2 g/L leucine was fed to the fermenter at a controlled specific feed rate of 0.1/h. Feeding of a glucose/ethanol mixture or pure ethanol was also used for strains in which the ERG20 promoter was replaced with the HXT1 promoter to control ERG20 expression. In both batch and fed-batch cultures, 20% dodecane was added to the medium after cultivating for 12 h for geraniol extraction. Independent duplicate cultures were conducted for each strain.

### RNA extraction and quantitative real-time PCR

Total RNA was prepared from 40 mL exponentially growing cell cultures using the UNIQ-10 Trizol RNA extraction kit (Sangon Biological Engineering, Shanghai,
China). The samples were digested using DNase I treatment (TakaRa, Dalian, China) to avoid DNA contamination. The treated total RNA was used for cDNA synthesis using the PrimeScript RT-PCR Kit (TakaRa, Dalian, China). qPCR was performed using the SYBR Green Master Mix Kit (Roche Molecular Biochemicals, Germany). The ACTI gene was chosen as the internal control gene. The relative transcription levels of genes were analyzed using the 2−ΔΔCT method.

Analysis of metabolites by HPLC

At each 12-h interval, the cultures were sampled and centrifuged at 12,000 rpm for 5 min. The filtered samples were analyzed using an HPLC equipped with an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA, USA) at 45 °C with a mobile phase of 5 mM H2SO4 at a flow rate of 0.6 mL/min. The peaks of metabolites including glucose, ethanol, acetic acid and glycerol were detected by refractive index (RI) and ultraviolet (UV) detectors.

Characterization of geraniol and citronellol by GC–MS

To quantify titers of geraniol and citronellol in different cultures, 1 mL of the upper layer (dodecane/culture mixture) was sampled and concentrated at 13,000 rpm for 5 min to separate the dodecane phase, and then the dodecane layer was transferred into a GC vial and stored at −20 °C for analysis. The residual geraniol was extracted again by adding 10% (v/v) dodecane into the medium. Geraniol and citronellol were identified using a GC–MS system (Shimadzu Co., Kyoto, Japan) equipped with an HP-5 ms capillary column (30 m × 0.25 mm × 0.25 μm), an AOC-20i auto-injector, and a QP-2010 mass detector, and the operational conditions were as follows. One microliter of each dodecane sample was injected into the system with a split ratio of 10 and the carrier gas helium was set at a constant flow rate of 0.78 mL/min. The oven temperature was first maintained at 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 mass spectrometer was set to SIM acquisition mode, scanning m/z ions within the range 40–500 for identification of geraniol and citronellol. The total run time was 30 min. Standard compounds of geraniol and citronellol (Sigma-Aldrich) were dissolved in dodecane and used to plot standard curves for quantification.

Biomass determination

Optical density at 600 nm (OD600) was measured using a spectrophotometer (Eppendorf AG, 22331 Hamburg, Germany). Dry cell weight (DCW) was obtained from OD600 measurement after calibration as indicated before (1 g/L biomass = 0.246 × (OD600) − 0.0012) [50].

Additional file

Additional file 1: Table S1. Primers used in this study. Figure S1. The conversion of geraniol to citronellol in control strain and deletion strains. The data shown are representative of duplicate experiments, and the error bars represent the standard deviation. Figure S2. The effect of OYE2 on/ and ATF1 deletion on cell growth in batch fermentation. All strains harbored pZGV6-GE1 and pZMVA4 plasmids for geraniol production. The data shown are representative of duplicate experiments, and the error bars represent the standard deviation. Figure S3. Transcription level of ERG20 controlled by different promoters in SD-URA-HIS medium with different concentrations glucose. The cells were collected when OD600 reached 0.6 to extract mRNA for ERG20 mRNA and transcription determination. The data shown are representative of triplicate experiments, and the error bars represent the standard deviation. Figure S4. The effect of ERG20 expression controlled by different promoters on cell growth in batch fermentation. All strains harbored pZGV6-GE1 and pZMVA4 plasmids for geraniol production. The data shown are representative of duplicate experiments, and the error bars represent the standard deviation.

Authors’ contributions

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

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file 1.

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