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Overproduction of geraniol by enhanced precursor supply in *Saccharomyces cerevisiae*

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ABSTRACT

Monoterpene geraniol, a compound obtained from aromatic plants, has wide applications. In this study, geraniol was synthesized in *Saccharomyces cerevisiae* through the introduction of geraniol synthase. To increase geraniol production, the mevalonate pathway in *S. cerevisiae* was genetically manipulated to enhance the supply of geranyl diphosphate, a substrate used for the biosynthesis of geraniol. Identification and optimization of the key regulatory points in the mevalonate pathway in *S. cerevisiae* increased geraniol production to 36.04 mg L^{-1}. The results obtained revealed that the IDI1-encoded isopentenyl diphosphate isomerase is a rate-limiting enzyme in the biosynthesis of geraniol in *S. cerevisiae*, and overexpression of MAF1, a negative regulator in tRNA biosynthesis, is another effective method to increase geraniol production in *S. cerevisiae*.

1. Introduction

Geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol) is an acyclic monoterpene alcohol with the chemical formula $C_{10}H_{18}O$, which is widely used in the perfume industry due to its pleasant odor (Chen and Viljoen, 2010). In addition, geraniol is known to exhibit insecticidal and insect repellent properties, and has also been developed as a chemo-prevention agent for the treatment of cancer (Kim et al., 2011). However, geraniol is naturally produced in limited quantities (Bakkali et al., 2008). Therefore, biosynthesis of such rare and valuable compounds in microorganisms by metabolic engineering strategies is a feasible route to fulfill the increasing commercial demand.

Monoterpenes are derived from two common building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized through the mevalonate pathway in *Saccharomyces cerevisiae*. Two molecules of IPP and one molecule of DMAPP can be condensed to one molecule of geranyl diphosphate (GPP) (Withers and Keasling, 2007) (Fig. 1). GPP is the precursor of monoterpenes such as (*S*)-linalool, geraniol, and cineole (Herrero et al., 2008), and the existence of intracellular GPP pool in *S. cerevisiae* has been verified in previous research (Oswald et al., 2007).

Although metabolic engineering of *S. cerevisiae* for monoterpene production has shown great potential, only a few monoterpene synthases have been characterized and expressed in *S. cerevisiae*, with very low productivity (Fischer et al., 2011; Oswald et al., 2007). The restrained monoterpene productivity is mainly due to the weak flux of the mevalonate pathway in *S. cerevisiae* (Rico et al., 2010). Acetyl-CoA is known to be the substrate of the mevalonate pathway. It has been reported that increased acetyl-CoA supply by overexpression of the key rate-limiting enzymes in the pyruvate dehydrogenase bypass could increase amorphadiene production by 2.8-fold (Shiba et al., 2007).

Metabolic engineering of the mevalonate pathway has been frequently carried out to increase the carbon flux in *S. cerevisiae*, and has been mainly focused on: (1) increasing mevalonate supply by overexpressing a truncated 3-hydroxyl-3-methylglutaryl-CoA reductase gene (*tHMGR*) (Dai et al., 2012; Engels et al., 2008; Scalcinati et al., 2012) or a mutated $HMG2^{K6R}$ (Ignea et al., 2011); (2) reducing the biosynthesis of squalene by replacing its strong native promoter with an weaker or inducible one (Asadollahi et al., 2008; Paradise et al., 2008; Scalcinati et al., 2012); (3) increasing the intracellular GPP pool by mutating the conserved region of farnesyl diphosphate synthase (FPPS) (Fischer et al., 2011; Karsta et al., 2004); and (4) overexpressing *upc2.1* allele (regulating ergosterol biosynthesis) to reduce the flux to sterol biosynthesis (Engels et al., 2008; Ro et al., 2006; Shiba et al., 2007).

Metabolic engineering of *S. cerevisiae* for increased monoterpene production has rarely been reported in the literature, and the

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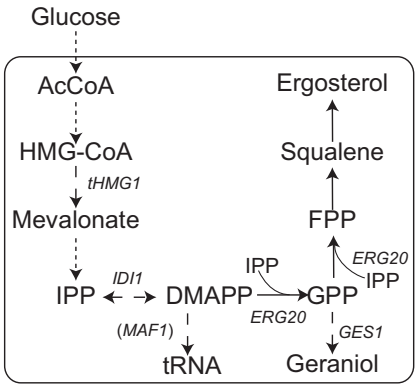

Fig. 1. *Simplified mevalonate pathway in S. cerevisiae, including the branch point to geraniol.* Dotted arrows indicate more than one reaction and dashed arrows indicate the engineered steps. Abbreviations: AcCoA, acetyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; *GES1*, gene encoding geraniol synthase.

rate-limiting points predominantly remain unclear. Therefore, further research to identify and optimize the key regulatory points could facilitate monoterpene production in *S. cerevisiae*. In the present study, microbial production of monoterpene geraniol was achieved through integrated expression of geraniol synthase and enhancement of the mevalonate pathway in *S. cerevisiae*. As a result of the genetic manipulations carried out, the final yield of geraniol increased to 36.04 mg L^{-1}.

2. Materials and methods

2.1. Strains and medium

Escherichia coli JM109 (Takara, Shiga, Japan) was used as the recipient strain for cloning manipulation and plasmid amplification. The cells were cultured at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with or without 100 μg L^{-1} of ampicillin.

S. cerevisiae CEN.PK2-1C (*MAT a, ura3-52, trp1-289, leu2-3,112, his3Δ1, MAL2-8^C, SUC2*), used for genetic manipulation, were obtained from EUROSCARF (Frankfurt, Germany). The strains used in this study are listed in Table 1. Engineered *S. cerevisiae* strains were cultured in yeast extract-peptone-dextrose (YPD) medium (2% glucose, 2% tryptone, and 1% yeast extract) or yeast nitrogen base (YNB) medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% glucose, supplemented with 50 μg mL^{-1} of each of the required amino acids). For solid media, 2% agar was added. A single colony of the transformants was inoculated into a 250-mL flask with 25 mL of YNB medium, and was incubated at 200 rpm and 30 °C for 24 h. This provided seed cultures for inoculation into 500-mL flasks containing 50 mL of fresh YNB medium, which were incubated for 48 h before determining geraniol production.

2.2. Plasmid construction

The yeast expression plasmid pY26-TEF1-GPD1 was provided by Li et al. (2007). The plasmids pRS303, pRS304 and pRS305 were provided by Sikorski and Hieter (1989). The wild-type geraniol synthase encoding gene *GES1* (GenBank Accession No. AY362553) from basil (*Ocimum basilicum*) and codon-optimized *GES1* (*coGES1*) were synthesized by GenScript (Nanjing, China). The sequences of the two genes are provided in the supplementary data. The catalytic domain of *HMG1* (*tHMG1*), *IDI1*, *MAF1*, and *ERG20*^{K197G} were PCR-amplified from yeast genomic DNA, and all primers used for plasmids construction are summarized in Table 2.

The verified vectors were transformed into *S. cerevisiae* using the *Fast*-Yeast Transformation regent (G-Biosciences, St Louis, MO), according to the manufacturer's instructions. All plasmids used in this study are listed in Table 3. The strains harboring integrated plasmids containing auxotrophic selection markers were screened using corresponding auxotrophic YNB plates, and were verified by PCR analysis.

2.3. Geraniol identification and quantification

Geraniol was identified and quantified as previously described by Fischer et al. (2011). Gas chromatography–mass spectrometry (GC–MS) was performed on a GC–MS system (Shimadzu Co., Kyoto, Japan) equipped with a DB-5 capillary column (30 m × 250 μm i.d., 0.25-μm film thickness; Agilent J&W Scientific, Folsom, CA). The amount of geraniol was determined using linear calibration curves with an R^2 value of 0.99 over a concentration range of 0–100 mg mL^{-1}.

2.4. Squalene quantification

Sterols were extracted as previously described by Liu et al. (2013). Squalene concentration was determined with the same instrumentation used for geraniol quantification. Samples from 1-μL splitless injection mode were separated using a GC oven with a temperature program of 80 °C for 1 min, followed by a 10 °C min^{-1} increase to 280 °C, and a hold for 20 min. Squalene concentration

Table 1
Strains used in this study.

Strains	Genotype	Plasmid description	Source
CEN.PK2-1C	*MATa; ura3, trp1, leu, his3*		EUROSCARF
JN01	*MATa; ura3, trp1, leu, his3*	pY26-TEF1-GPD1 2 μ *URA3*	This study
JN02	*MATa; ura3, trp1, leu, his3*	pY26/GES1 2μ *URA3* P_{GPD1}-*GES1*	This study
JN03	*MATa; ura3, trp1, leu, his3*	pY26/coGES1 2μ *URA3* P_{GPD1}-*coGES1*	This study
JN04	*MATa; ura3, trp1, leu, his3*:: P_{TEF1}-*coGES1*	pRS303/coGES1 *HIS3* P_{TEF1}-*coGES1*	This study
JN05	*MATa; his3, ura3, trp1, leu2*::P_{TEF1}-*tHMG1*	pRS305/tHMG1 LEU2 P_{TEF1}-*tHMG1*	This study
JN06	*MATa;* P_{TEF1}-*coGES1*::*his3, ura3, trp1, leu2*	pY26/IDI1 2μ *URA3* P_{GPD1}-*IDI1*, pRS303/coGES1 *HIS3* P_{TEF1}-*GES1*	This study
JN07	*MATa;* P_{TEF1}-*coGES1*:: *his3, trp1*::*IDI1, ura3, leu2*	pRS303/coGES1 *HIS3* P_{TEF1}-*coGES1*, pRS304/IDI1 *TRP1* P_{TEF1}-*IDI1*	This study
JN08	*MATa; trp1, ura3, his3*::*coGES1, leu2*	pY26/IDI1 2μ *URA3* P_{GPD1}-*IDI1* pRS303/coGES1 *HIS3* P_{TEF1}-*coGES1*	This study
JN09	*MATa;* P_{TEF1}-*tHMG1*::*leu2, ura3, his3*::*coGES1, trp1*	pY26/IDI1-MAF1 2μ *URA3* P_{GPD1}-*IDI1*, P_{TEF1}-*MAF1*, pRS305/tHMG1 *LEU2* P_{TEF1}-*tHMG1*, pRS303/coGES1 *HIS3* P_{TEF1}-*coGES1*	This study
JN10	*MATa;* P_{TEF1}-*tHMG1*::*leu2, ura3, his3*::*coGES1, trp1*	pY26/IDI1-MAF1-ERG20^{K197G} 2μ *URA3* P_{GPD1}-*ERG20*^{K197G}-*IDI1* P_{TEF1}-*MAF1*, pRS305/tHMG1 *LEU2* P_{TEF1}-*tHMG1*, pRS303/coGES1 *HIS3* P_{TEF1}-*coGES1*	This study

Table 2
Primers used for PCR.

Gene	F/R	Primer sequence (5′-3′)*
tHMG1	F	GGACTAGTATGGACCAATTGGTGAAAACTG
	R	CGGGATCCTTAGGATTTAATGCAGGTGACGG
IDI1	F	CGGGATCCATGACTGCCGACAACAATAGTAT
	R	CGGAATTCTTATAGCATTCTATGAATTTGCC
ERG20^{K197G}-*IDI1*	P1	GGATCCATGGCTTCAGAAAAAGAAATTAGG
	P2	CTATTGTTGTCGGCAGTCATACCACTACCCTATTTGCTTCTCTTGTAAACT
	P3	AGTTTACAAGAGAAGCAAATAGGGTAGTGGTATGACTGCCGACAACAATAG
	P4	GAATTCTTATAGCATTCTATGAATTTGCC
MAF1	F	GCGGCCGCATGAAAGTATGTTATCACTCT
	R	CCGCGGCTACTGTAGGGATTCTTCTTG
ERG20^{K197G}	F	TCCCCGCGGCTATTTGCTTCTCTTGTAAACTT
	R	ATTTGCGGCCGCATGGCTTCAGAAAAAGAAATTAGG

*Underlined bases represent restriction endonuclease cleavage sites.

was calculated using the standard purchased from Sigma-Aldrich (Paradise et al., 2008).

3. Results

3.1. Expression of geraniol synthase in S. cerevisiae

To evaluate the expression of geraniol synthase, the geraniol synthase encoding gene *GES1* was expressed in *S. cerevisiae* CEN.PK2-1C using an episome plasmid pY26-TEF-GPD, generating the engineered *S. cerevisiae* strain JN02. The GC–MS results verified the production of intracellular and extracellular geraniol by JN02. No geraniol production was detected in the control strain JN01 carrying empty pY26-TEF-GPD (Fig. 2). The functional expression of wild-type geraniol synthase in JN02 yielded 0.52 mg L^{-1} of geraniol. Expression of *coGES1* in JN03 increased geraniol production to 0.73 mg L^{-1}, which was 40.4% higher than that observed in JN02. Furthermore, integration of *coGES1* into the genome of JN04 increased geraniol production by 26.9%, when compared with that produced by JN02 (Fig. 3).

3.2. Overexpression of key rate-limiting enzymes of the mevalonate pathway

To increase the carbon flux to the mevalonate pathway, *tHMG1* encoding a key rate-limiting enzyme, HMG-CoA reductase, was overexpressed in *S. cerevisiae*, and the resultant strain was named as JN05. Overexpression of *tHMG1* in JN05 increased geraniol production to 1.57 mg L^{-1}, which was 1.38-fold higher than that produced by its parental strain JN04. Interestingly, the copy number of *IDI1* encoding IPP isomerase in *S. cerevisiae* appeared to be an important factor affecting the final yield of geraniol. Overexpression of *IDI1* using a multi-copy plasmid in JN06 yielded 1.62 mg L^{-1} of geraniol, which was 1.45-fold higher than its parental strain JN04. In contrast, only 0.98 mg L^{-1} of geraniol was produced (50.0% increase) when an extra copy of *IDI1* was introduced into the genome of JN07. These results suggested that IPP isomerase was a rate-limiting enzyme in the biosynthesis of monoterpene in *S. cerevisiae*. Combined overexpression of *IDI1* and *tHMG1* in JN08 further increased geraniol production to 6.77 mg L^{-1} (Fig. 3).

Table 3
Plasmids used in this study.

Plasmids	Description	Source
pMD18-T simple	Amp^r	Takara
pY26-TEF-GPD	2μ *URA3* P_{TEF}, P_{GPD}	Li et al. (2007)
pRS303	*HIS3* P_{TEF1}	Sikorski and Hieter (1989)
pRS304	*TRP1* P_{TEF1}	Sikorski and Hieter (1989)
pRS305	*LEU2* P_{TEF1}	Sikorski and Hieter (1989)

3.3. Overexpression of MAF1 in S. cerevisiae

In *S. cerevisiae*, biosynthesis of both tRNA and GPP requires DMAPP, which is a common substrate, and *MAF1* encodes a negative regulator in the biosynthesis of tRNA (Kaminska et al., 2002). To channelize DMAPP for the biosynthesis of GPP, *MAF1* was co-overexpressed with *IDI1* in JN08, generating the strain JN09. This manipulation resulted in a 102% increase in geraniol production in the engineered strain above that noted in its parental strain (Fig. 3).

3.4. Redistribution of GPP and farnesyl diphosphate biosynthesis in S. cerevisiae

FPPS encoded by *ERG20* is required for the biosynthesis of both GPP and farnesyl diphosphate (FPP), and a previous study reported that the K197 mutation in FPPS resulted in an increased GPP pool in *S. cerevisiae* (Fischer et al., 2011). Thus, to enhance GPP supply in the proximity of geraniol synthase, *ERG20*^{K197G} was overexpressed in JN09, generating the strain JN10, and consequently, a further increase in geraniol production to 36.04 mg L^{-1} was detected (Fig. 3). These results showed that overexpression of *ERG20*^{K197G} could increase the GPP pool in *S. cerevisiae* through redistribution of the biosynthesis of GPP and FPP.

3.5. Alterations in squalene concentration

To evaluate the effect of genetic manipulations on squalene biosynthesis, which is the downstream product of geraniol, the squalene content of the engineered strains was determined and compared with the control (JN01) and wild-type strain (CEN.PK2-1C). The squalene concentration in the engineered strains JN05, JN06, JN07, JN08, JN09, and JN10 exhibited 2.8-, 6.0-, 0.6-, 16.4-, 24.9-, and 40.3-fold increase, respectively, when compared with that noted in JN04 (Fig. 4).

4. Discussion

Wild-type *S. cerevisiae* cannot produce monoterpenes due to the deficiency of the enzyme monoterpene synthase with the exception of a few wine-making yeast strains have been found to be capable of producing less than 5 μg L^{-1} of monoterpenes such as (*S*)-linalool and α-terpineol (Carrau et al., 2005). While expression of geraniol synthase could result in geraniol accumulation in *S. cerevisiae*, increased copy number of the gene encoding geraniol synthase in *S. cerevisiae* (strain JN03 and JN04) exhibited limited effect on geraniol production (Fig. 3), indicating that geraniol synthase was not the key factor in increasing geraniol production. Thus, it could be deduced that low geraniol production was mainly caused by precursor deficiency. It has been reported that GPP, the precursor of monoterpene geraniol, is produced through the mevalonate

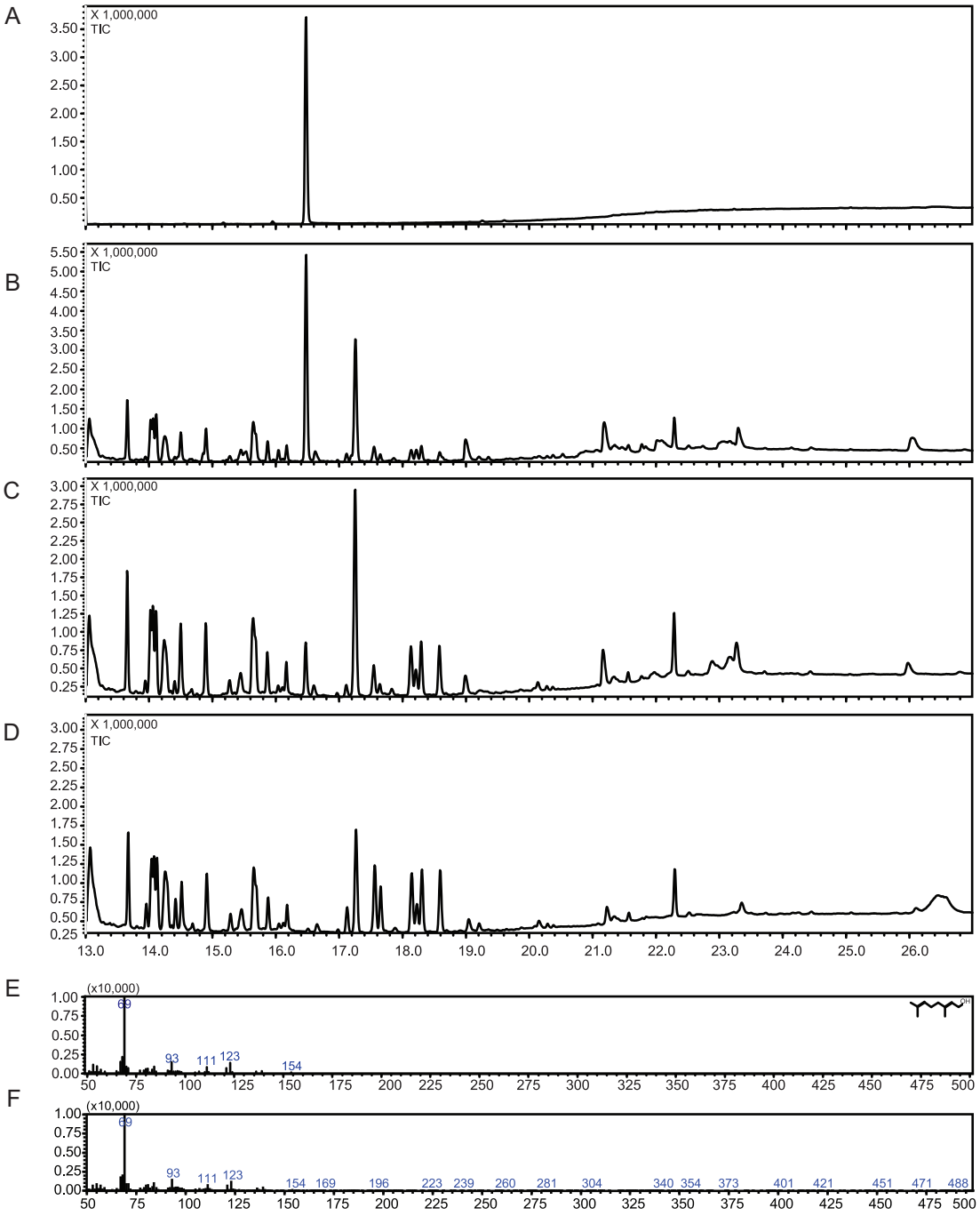

Fig. 2. *Production of geraniol in engineered S. cerevisiae*. The strain was cultivated in YNB medium for 48 h. GC–MS analysis of intracellular and extracellular geraniol produced by JN02 (with *GES1* gene integrated into the chromosome) and the parent strain JN01 (control) was performed. Geraniol was used as a standard for comparison. (A) standard; (B) extracellular geraniol; (C) intracellular geraniol; (D) control; (E) mass spectrum of the standard; (F) mass spectrum of JN02.

pathway in *S. cerevisiae* (Herrero et al., 2008). However, the activities of the mevalonate pathway enzymes are strictly regulated at different levels (Maury et al., 2005), and the key rate-limiting steps in monoterpene biosynthesis are mostly unknown. The aim of the present study was to screen and optimize the key regulatory points to increase geraniol production in *S. cerevisiae*, as well as to analyze the effect of manipulations on squalene accumulation.

Previous reports revealed that overexpression of a key rate-limiting enzyme, HMG-CoA reductase, could enhance the carbon flux to the mevalonate pathway in *S. cerevisiae* (Kirby et al., 2008; Rico et al., 2010). *S. cerevisiae* has two copies of genes encoding HMG-CoA reductase, *i.e.*, *HMG1* and *HMG2*. Hmg1p has been reported to be stable and responsible for more than 83% of the enzyme activity in wild-type cells under aerobic conditions (Basson et al., 1986; Hampton et al., 1996). The activity of Hmg1p has been transcriptionally repressed by sterols downstream of the mevalonate pathway (Dimster-Denk et al., 1999), and a truncated *HMG1* (*tHMG1*) has been noted to circumvent this feedback (Kirby et al., 2008). In the present study, overexpression of *tHMG1* in JN05 showed a significant increase in geraniol production (Fig. 3) and squalene accumulation (Fig. 4). The results obtained indicated that the mevalonate pathway was strengthened and more GPP was synthesized.

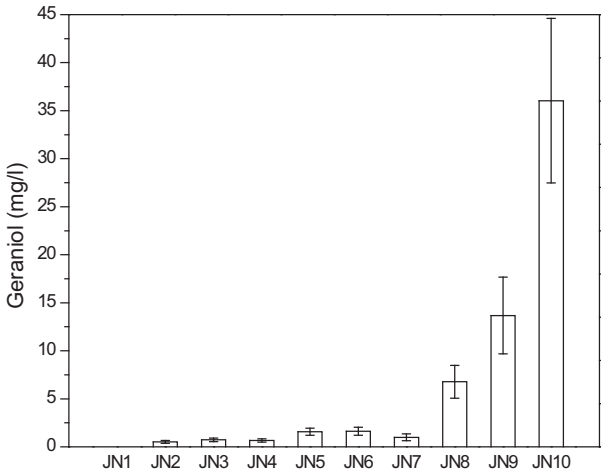

Fig. 3. *Production of geraniol by different S. cerevisiae strains.* All strains were cultivated in YNB medium lacking corresponding amino acids for 48 h. The results are presented as the means and standard deviations of at least three different experiments. Statistically significant differences ($p < 0.05$) were determined by Student's t test.

In *S. cerevisiae*, the gene *IDI1* (Fig. 1) encodes an isomerase that catalyzes the isomerization of IPP to DMAPP by enhancing the electrophilicity of the isoprene unit by at least a billion folds (Durbecq et al., 2001). Biosynthesis of one molecule of GPP requires two molecules of IPP and one molecule of DMAPP (Withers and Keasling, 2007). However, in nature, the ratio of IPP and DMAPP does not appear to be equal. For example, IPP and DMAPP are produced in a ratio of 5:1 in *E. coli* (Rohdich et al., 2002). Upon altering the balance between IPP and DMAPP, an increase in GPP and monoterpene geraniol production is expected. In the present study, an increase in geraniol production and squalene accumulation in JN08, harboring multi-copy *IDI1* in the cytoplasm, was detected. Besides, overexpression of an extra *IDI1* in the genome of JN07 resulted in much lower geraniol production and squalene concentration than JN08 (Figs. 3 and 4). These results indicated that *IDI1*-encoded IPP isomerase was the rate-limiting enzyme in monoterpene geraniol production.

DMAPP is a common competitive substrate for both tRNA and GPP in *S. cerevisiae* (Fig. 1), and *MAF1* encodes a negative regulator of Mod5p, the tRNA isopentenyltransferase. Previous studies

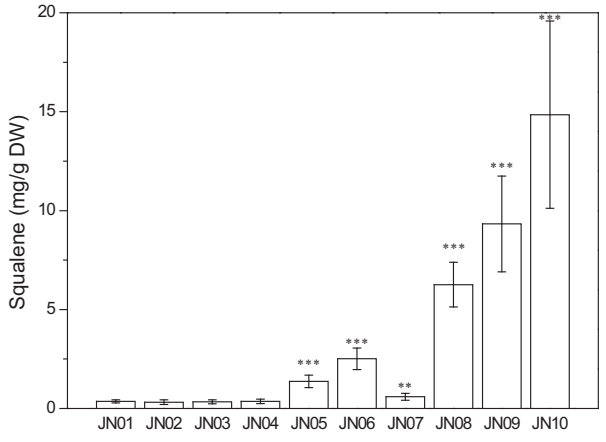

Fig. 4. *Concentration of squalene in different S. cerevisiae strains.* All strains were cultivated in YNB medium lacking corresponding amino acids for 48 h, and squalene was quantified. The results are presented as the means and standard deviations of at least three different experiments. Statistically significant differences ($p < 0.05$) were determined by Student's t test, and two and three asterisks indicated $p < 0.001$ and $p < 0.001$, respectively.

have demonstrated that deletion of *MAF1* in *S. cerevisiae* increased the tRNA level and decreased ergosterol concentration, which also caused cell growth inhibition as a result of insufficient ergosterol supply (Kaminska et al., 2002). It has been observed that overexpression of *MAF1* does not cause RNA supply deficiency, and it has been proved that single overexpression of *MAF1* (>10-fold) in *S. cerevisiae* does not reduce the level of pre-tRNA (Desai et al., 2005). In the present study, overexpression of *MAF1* increased geraniol production, revealing that *MAF1* overexpression could enhance the DMAPP flux to GPP biosynthesis by restraining the flux to tRNA biosynthesis.

Unlike plants, *S. cerevisiae* lacks a specific GPP synthase, and both GPP and FPP are synthesized by one enzyme, namely, FPPS encoded by *ERG20* (Withers and Keasling, 2007). It has been reported that strong FPPS activity could convert GPP to FPP immediately, releasing a trace amount of free GPP to the cytoplasm, which could be used as a substrate for synthesis of monoterpenes (Oswald et al., 2007). Furthermore, the K197G mutation in Erg20p has been noted to increase the release of GPP and enhance the GPP pool in *S. cerevisiae* (Fischer et al., 2011). In the present study, overexpression of $ERG20^{K197G}$ in JN10 resulted in a sharp increase in geraniol production and a moderate increase in squalene accumulation, showing that the genetic manipulation helped to redistribute the biosynthesis of GPP and FPP, resulting in an increase in GPP synthesis.

In summary, the present study demonstrated the feasibility of overproducing geraniol in engineered *S. cerevisiae* through metabolic engineering of the mevalonate pathway. The dramatic increase achieved by manipulating the mevalonate pathway enzymes confirmed the great potential for GPP accumulation. Although marked increase in geraniol production was attained in this study, the weak flux to the mevalonate pathway suggests that the weak precursor supply to the mevalonate pathway is the most important factor for the enhancement of monoterpene biosynthesis in *S. cerevisiae*. Therefore, it is necessary to identify the bottleneck in the mevalonate pathway and maximize the GPP supply for efficient production of monoterpenes in *S. cerevisiae*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.10.017.

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