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ORIGINAL ARTICLE

Enhanced (*S*)-linalool production by fusion expression of farnesyl diphosphate synthase and linalool synthase in *Saccharomyces cerevisiae*

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Abstract

Aims: In order to improve the availability of geranyl diphosphate (GPP) in the mevalonate pathway for enhancing (*S*)-linalool production in *Saccharomyces cerevisiae*.

Methods and Results: A (*S*)-linalool synthase (LIS): AaLS1 from *Actinidia arguta* was coexpressed with FPPS with different peptide linkers to redirect the flux from geranyl diphosphate (GPP) to (*S*)-linalool production in *S. cerevisiae*. The strain with the best peptide linker ($(GGGGS)_3$), produced 101·55 ± 2·97 $\mu g\ l^{-1}$ (*S*)-linalool, a 69·7% increase compared to those with two independent LIS and FPPS expressed. In a 3-l fermenter, the (*S*)-linalool titre was further improved to 240·64 ± 5·31 $\mu g\ l^{-1}$.

Conclusions: The results demonstrate that the fusion proteins catalysing consecutive steps in a metabolic pathway significantly improved the (*S*)-linalool production with GPP as precursor.

Significance and Impact of the Study: The fusion protein strategy co-expressing AaLS1 and FPPS, assembled with a long peptide linker made *S. cerevisiae* produced the highest reported (*S*)-Linalool titre to date.

Introduction

Isoprenoids are the most diverse groups of plant natural products, consisting of ~40 000 structurally different compounds (Withers and Keasling 2006). A lot of monoterpenes in plant have useful functions in human health and have been widely used in the food and perfume industries (Misawa 2011). However, many functional monoterpenes are naturally produced in very low concentrations, hindering the industrial applications (Lee *et al.* 2009). In addition, the seasonal harvest of those rare plants and potential existence of pesticides and mycotoxin contaminations are the serious issues caused by the extraction of these phytochemicals from plants (Bakkali *et al.* 2008). Chemical synthesis of isoprenoids is very tedious and has a complicated downstream process, because the products have chiral structures with temperature sensitive chemical bonds, low boiling points and high vapour pressures (Dudareva and Pichersky 2008). However, the above problems could be solved by microbial fermentation by engineered micro-organisms grown on the renewable substrates. Among the monoterpenes, (*S*)-Linalool is the most important one with a pleasant floral scent and it has been found in many plants with a commercial potential, especially in food and perfume industries (Cheng *et al.* 2012).

Recently, metabolic engineering has been used for the production of functional isoprenoids (Mahmoud and Croteau 2002; Muntendam *et al.* 2009). The rational organization of pathway enzymes is a powerful and promising strategy to increase the efficiency of the biosynthetic system and the metabolic yield of the target product (Siddiqui *et al.* 2012). Fusion of different enzymes catalysing sequential reactions can generate a larger protein with two or more functions. Furthermore, the fusion protein can catalyse consecutive steps more

efficiently than a simple mixture of the individual free enzymes in a metabolic pathway (Orita *et al.* 2007; Wang *et al.* 2007). The proximity of fusion enzymes is beneficial for modulating the efficiency of sequential reactions (Jorgensen *et al.* 2005; Conrado *et al.* 2008).

While a lot of studies on isoprenoids have been carried out in *Escherichia coli*, the trends concerning the production of these products show a more even distribution between these two hosts with an increasingly more prevalent use of *Saccharomyces cerevisiae*. This trend might be attributed to the fact that *S. cerevisiae* as an eukaryotic organism has the advantage of being more suitable than *E. coli* for the expression of membrane bound plant cytochrome P450 enzymes along with their respective reductase (Kirby and Keasling 2009) required for the functionalization of a series of isoprenoids (Kirby and Keasling 2009). A further advantage is the possibility to harness different subcellular compartments (Farhi *et al.* 2011). Moreover, yeast can withstand reduced pH and high osmotic pressure and is not susceptible to phage infections.

In *S. cerevisiae*, the fusion protein of *Arabidopsis thaliana* 4-coumaroyl-CoA ligase (At4CL1) and *Vitis vinifera* stilbene synthase (VvSTS) improved the catalytic efficiency of independent reactions threefold (Wang *et al.* 2011). Fusion of the patchoulol synthase (PTS) of *Pogostemon cablin* and the farnesyl diphosphate synthase (FPPS) of *S. cerevisiae* channelled more metabolic flux to patchoulol production (Albertsen *et al.* 2011). These studies highlight the advantage of fusion expression of proteins in improving the activities of heterologous enzymes and might facilitate the redirection of flux from a natural pathway to a specially designed metabolic branch.

In *S. cerevisiae*, geranyl diphosphate (GPP) is the universal precursor of monoterpenes and a few secondary metabolites (Fig. 1) (Chandran *et al.* 2011). The main point of improving a monoterpene product is to improve the availability of GPP by monoterpene synthase. To improve the availability of GPP for (*S*)-linalool synthase (LIS), two LIS genes from *Actinidia auguta* (*AaLS1*) and *Actinidia polygama* (*ApLS1*) (Chen *et al.* 2010) were linked to FPPS, respectively, for enhancing (*S*)-linalool production in *S. cerevisiae*.

Materials and methods

Strains and culture conditions

Saccharomyces cerevisiae strains used in this study are listed in Table 1. *Saccharomyces cerevisiae* strains were grown in YPD medium (1% yeast extract, 2% tryptone, 2% glucose) or YNB medium (0·5% ammonium sulphate, 0·17% YNB salt, 1% glucose). When necessary,

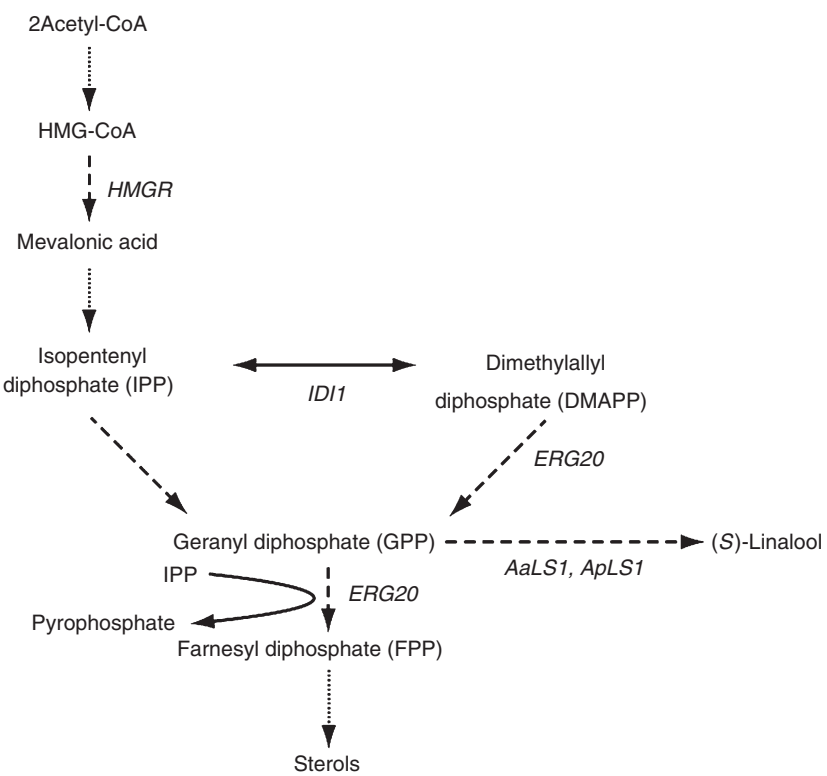

Figure 1 Isoprenoid pathway in *Saccharomyces cerevisiae*, including the branch point to (*S*)-linalool (Chen *et al.* 2010; Chandran *et al.* 2011). Dotted arrows indicate that more than one reaction is required to convert the substrate to the product indicated. Dashed arrows indicate the engineered steps. HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

Table 1 Strains and plasmids used in this study

Strains and plasmids	Descriptions	Source
CEN.PK2	*MAT a/α ura3-52/ura3-52; trp1-289/trp1-289; leu2-3, 112/leu2-3,112; his3Δ 1/his3Δ 1; MAL2-8^C/MAL2-8^C; SUC2/SUC2*	EUROSCARF
pY26-TEF-GPD	Shuttle expression vector with two strong constitutive promoters.	Li *et al.* (2008)
pY26-AaLS1	P_{GPD}-*AaLS1*	This study
pY26-ApLS1	P_{GPD}-*ApLS1*	This study
pY26-AaLS1-ERG20	P_{GPD}-*AaLS1*P_{TEF1}-*ERG20*	This study
pY26-ERG20-SF-AaLS1	P_{GPD}- *ERG20*-SF-*AaLS1**	This study
pY26-ERG20-MF-AaLS1	P_{GPD}- *ERG20*-MF-*AaLS1*	This study
pY26-ERG20-LF-AaLS1	P_{GPD}- *ERG20*-LF-*AaLS1*	This study
pY26-AaLS1-SF-ERG20	P_{GPD}-*AaLS1*-SF-*ERG20*	This study
pY26-AaLS1-MF-ERG20	P_{GPD}-*AaLS1*-MF-*ERG20*	This study
pY26-AaLS1-LF-ERG20	P_{GPD}-*AaLS1*-LF-*ERG20*	This study

*Linker abbreviations: SF, short flexible; MF, middle flexible; LF, long flexible.

YNB medium was supplied with auxotrophic amino acids at 50 μg ml^{-1}. *Escherichia coli* JM109 was used as the host for cloning experiments and plasmid propagation. *Escherichia coli* was grown in LB medium (0·5% yeast extract, 1% tryptone, 1% NaCl) with or without 100 μg ml^{-1} ampicillin. For solid medium, 2% agar was added.

Molecular work

The plasmids used in this study are listed in Table 1 and the primers used for DNA manipulation are listed in Table 2. The DNA fragments of *AaLS1*and *ApLS1* were PCR-amplified from the cDNAs of *A. auguta* and *A. polygama* using primers AaLS1-F/R and ApLS1-F/R, and then were inserted into the *Eco*RI-*Cla*I sites of pY26-TEF-GPD to generate pY26-AaLS1 and pY26-ApLS1 respectively.

Site mutagenesis of FPPS

ERG20 gene was amplified by the primers ERG20-F and ERG20-R (Table 2) from the genome of *S. cerevisiae*, and the PCR product was 1059 bp. The mutagenesis of *ERG20* was created by using the primers ERG20^{K197E}-F and ERG20^{K197E}-R, targeting K197 position (Fernandez *et al.* 2000) of *ERG20* gene (based on the above PCR product). According the protocol described by the manufacturer (Sangon Biotech, Shanghai, China), lysine (K) of K197 was changed to glutamic acid (E), whose condon was changed from AAG to GGT. The genes with mutations were sequenced to find the correct ones, named ERG20K^{197E}. The above DNA fragments were cloned into the vector pY26-AaLS1, forming plasmid pY26-AaLS1-ERG20^{K197E}.

Construction of the plasmids with the peptide linkers

ERG20^{K197E} fragments were amplified from genomic DNA of *S. cerevisiae* by using primer pairs E1-F/R, E2-F/R, E3-F/R, E4-F/R, E5-F/R, E6-F/R with Pyrobest DNA polymerase (Takara, Dalian, China). *AaLS1* fragments were also amplified from genomic DNA of *S. cerevisiae* by using primer pairs L1-F/R, L2-F/R, L3-F/R, L4-F/R, L5-F/R and L6-F/R.

The PCR products of E1-F/R and L1-F/R were mixed equimolarly and PCR-amplified with primer pairs E1-F/L1-R to obtain the fusion protein genes with GGGS fragment as the linker. All the related fusion protein genes were constructed as described above with the corresponding primer pairs. The resulting fusion genes were digested and inserted into the *Eco*RI-*Cla*I sites of pY26-TEF-GPD to generate the expression vectors for fusion protein expressions. All of the plasmids were transformed into *S. cerevisiae* CEN.PK2 using the *Fast*TM-Yeast Transformation regent (G-Biosciences, St Louis, MO).

(*S*)-Linalool production

The engineered *S. cerevisiae* strains were cultured in YNB-supplemented medium overnight. Then the cells were transferred to 250-ml flasks containing 50 ml YPD medium at an initial optical density at 600 nm (OD600) of 0·05. *Saccharomyces cerevisiae* was cultured at 30°C with continuous shaking at 200 rev min^{-1}.

Bioreactor fermentation was performed in a BioFlo 115 3-l fermenter (Eppendorf, Enfield, CT) with a 1·5-l working volume at 30°C, 400 rev min^{-1} and 2·0 vvm. The pH was controlled automatically at 5·5 with the addition of 4 mol l^{-1} NaOH or 2 mol l^{-1} hydrochloric acid solutions. Samples were obtained every 4 h to measure the OD600 and (*S*)-linalool concentration.

Gas chromatography–mass spectrometry analysis

(*S*)-Linalool concentrations were identified and quantified by gas chromatography-mass spectrometry (GC-MS). Headspace emission was performed using a Turbomatrix 16 headspace autosampler (HS) equipped with a 16-vial magazine and a single vial oven (PerkinElmer, Waltham, MA). Aliquots of 5 ml of the samples were placed in 20-ml vials containing 2 g of NaCl. Tests were performed at 95°C and a thermostatting time of 30 min. GC–MS

Table 2 Primers used in this study

Primers	Sequence (5′–3′)	RE*	Linker
AaLS1-F	CGGAATTCATGGCCAGCTTCAACAGGT	*Eco*RI	/
AaLS1-R	CCATCGATTCAAGAAGAACTATCATAGAGCATT	*Cla*I	/
ApLS1-F	CGGAATTCATGGCCAGCTTCCACCGG	*Eco*RI	/
ApLS1-R	CCATCGATTCAAGAAGAACTATCATAGAGCATTGC	*Cla*I	/
ERG-F	ATTTGCGGCCGCATGGCTTCAGAAAAAGAAATTAG	*Not*I	/
ERG-R	TCCCCGCGGCTATTTGCTTCTCTTGTAAACTTTG	*Sac*II	/
ERG20^{K19E}-F	TCATAGTTACTTTCGAAACTGCTTACTATTC		
ERG20^{K197E}-R	AGGAGTGCTTCTTTAGGGAGAACT		
ERG20^{K197G}-F	TCATAGTTACTTTCGGTACTGCTTACTATTC		
ERG20^{K197G}-R	AGGAGTGCTTCTTTAGGGAGAACT		
E1-F	GGAATTCATGGCTTCAGAAAAAGAAATTAG	*Eco*RI	/
E1-R	GCTGGCCATAGAACCACCACCTTTGCTTCTCTTGTAAACTTTG		GGGS
L1-F	GGTGGTGGTTCTATGGCCAGCTTCAACAGG		GGGS
L1-R	CCATCGATTCAAGAAGAACTATCATAGAGCAT	*Cla*I	/
E2-F	GGAATTCATGGCTTCAGAAAAAGAAATTAG	*Eco*RI	/
E2-R	ACCAGAACCACCACCAGAACCACCTTTGCTTCTCTTGTAAACTTTGTTC		$(GGSG)_2$
L2-F	GGTGGTTCTGGTGGTGGTTCTGGTATGGCCAGCTTCAACAGG		$(GGSG)_2$
L2-R	CCATCGATTCAAGAAGAACTATCATAGAGCAT	*Cla*I	/
E3-F	GGAATTCATGGCTTCAGAAAAAGAAATTAGGAGAGAGAG	*Eco*RI	/
E3-R	GCTACCTCCGCCACCACTTCCACCGCCTCCAGAACCTCCTCCACCTTTGCTTCTCTTGTAAACTTTG		$(GGGGS)_3$
L3-F	CGGTGGAAGTGGTGGCGGAGGTAGCATGGCCAGCTTCAACAGG		$(GGGGS)_3$
L3-R	CCATCGATTCAAGAAGAACTATCATAGAGCAT	*Cla*I	/
L4-F	GGAATTCATGGCCAGCTTCAACAGGT	*Eco*RI	/
L4-R	TGAAGCCATAGAACCACCACCAGAAGAACTATCATAGAGCATTGTC		GGGS
E4-F	GGTGGTGGTTCTATGGCTTCAGAAAAAGAAATTAG		GGGS
E4-R	CCATCGATCTATTTGCTTCTCTTGTAAACTTTG	*Cla*I	/
L5-F	GGAATTCATGGCCAGCTTCAACAGGT	*Eco*RI	/
L5-R	ACCAGAACCACCACCAGAACCACCAGAAGAACTATCATAGAGCATTGTC		$(GGSG)_2$
E5-F	GGTGGTTCTGGTGGTGGTTCTGGTATGGCTTCAGAAAAAGAAATTAG		$(GGSG)_2$
E5-R	CCATCGATCTATTTGCTTCTCTTGTAAACTTTG	*Cla*I	/
L6-F	GGAATTCATGGCCAGCTTCAACAGGT	*Eco*RI	/
L6-R	GCTACCTCCGCCACCACTTCCACCGCCTCCAGAACCTCCTCCACCAGAAGAACTATCATAGAGCATTGTC		$(GGGGS)_3$
E6-F	CGGTGGAAGTGGTGGCGGAGGTAGCATGGCTTCAGAAAAAGAAATTAG		$(GGGGS)_3$
E6-R	CCATCGATCTATTTGCTTCTCTTGTAAACTTTG	*Cla*I	/

*Underlined sequences are the corresponding sites for restriction enzymes (RE).

analysis was performed using a GC–MS-QP2010 Ultra (Shimadzu, Tokyo, Japan) equipped with an Rtx-Wax capillary column (30 m × 0·25 mm i.d., 0·25-μm film thickness) (Restek, Bellefonte, PA) using helium as carrier gas at a flow rate of 1 ml min^{-1}. The operating conditions were as follows: injector temperature, 250°C; oven temperature program, 60°C for 0·5 min followed by a linear gradient to 100°C at 8°C min^{-1}, then to 200°C at 30°C min^{-1} for 3 min; acquisition parameters: *m/z*: 71, 93, 121. The mass spectrometer was run in selected ion monitoring (SIM) mode by electron impact ionization at 70 eV (Oswald *et al.* 2007). (*S*)-Linalool concentrations were calculated using standard solutions and were given as the averages of results from at least three independent cultures. Calibration curves were determined using the same protocol by exposure the headspace of YPD spiked with standards of final concentrations of 1, 10, 100 and 1000 μg l^{-1}.

Statistical analysis

All the experiments were performed at least in triplicate. Student's *t*-test was employed to investigate statistical differences. Samples with *P* values of <0·05 were considered statistically different (Zhang *et al.* 2007).

Results

Construction of recombinant *Saccharomyces cerevisiae* strains expressing the LIS genes

AaLS1 and *ApLS1* encoding FPPS, were found in *Actinidia arguta* and *A. polygama* and enantioselective gas chromatography revealed that both terpene synthases produced only (S)-(+)-linalool. In order to find which one was better for linalool production, both genes were estimated in this study.

AaLS1 and *ApLS1* were cloned into the replicating shuttle vector (Fig. 2a) under the control of the strong constitutive *GPD* promoter (Fig. 2c) and transformed into the diploid strain CEN.PK2. The transformants were isolated from YNB minimum medium supplied with amino acids (histidine, leucine, tryptophan without uracil). The accumulation of (*S*)-linalool was analysed by GC–MS. The results revealed the presence of (*S*)-linalool in the culture supernatants while (*S*)-linalool was not detected in the strain with empty vector.

The titre of (*S*)-linalool ($59{\cdot}85 \pm 4{\cdot}05$ $\mu g\ l^{-1}$) in the strain expressing *AaLS1* gene was three times higher than the one expressing *ApLS1*. The GC–MS profiles of both strains indicated that (*S*)-linalool was produced. The recombinant strains expressing plasmid pY26-AaLS1 had the peak with the same retention time as the (*S*)-linalool standard. Mass spectrum of the product detected in the recombinant yeast strain yielded a fragmentation pattern identical to the (*S*)-linalool standard (Fig. 3).

Effect of overexpression of the FPPS and LIS fusion proteins on (*S*)-linalool production

The K197E mutated FPPS was found to prefer producing GPP (Fernandez *et al.* 2000) and in this study, the FPPS lysine (K) of K197 was changed to glutamic acid (E) by site mutagenesis. To investigate whether fusion expression of heterologous LIS with the host enzyme may channel the flux more efficiently towards (*S*)-linalool production than expression of the corresponding free enzymes, *S. cerevisiae* CEN.PK2 harboured plasmids expressing free FPPS and LIS, FPPS-LIS and LIS-FPPS respectively (Fig. 2b).

Because the construction of functional fusion proteins often requires a peptide linker sequence that can reduce folding interference from each other, and changes in linker length and structure would affect overall performance of a fusion protein (Crasto and Feng 2000; Lu and Feng 2008), it was essential to optimize the peptide linkers for more efficient fusion proteins (Fig. 2b).

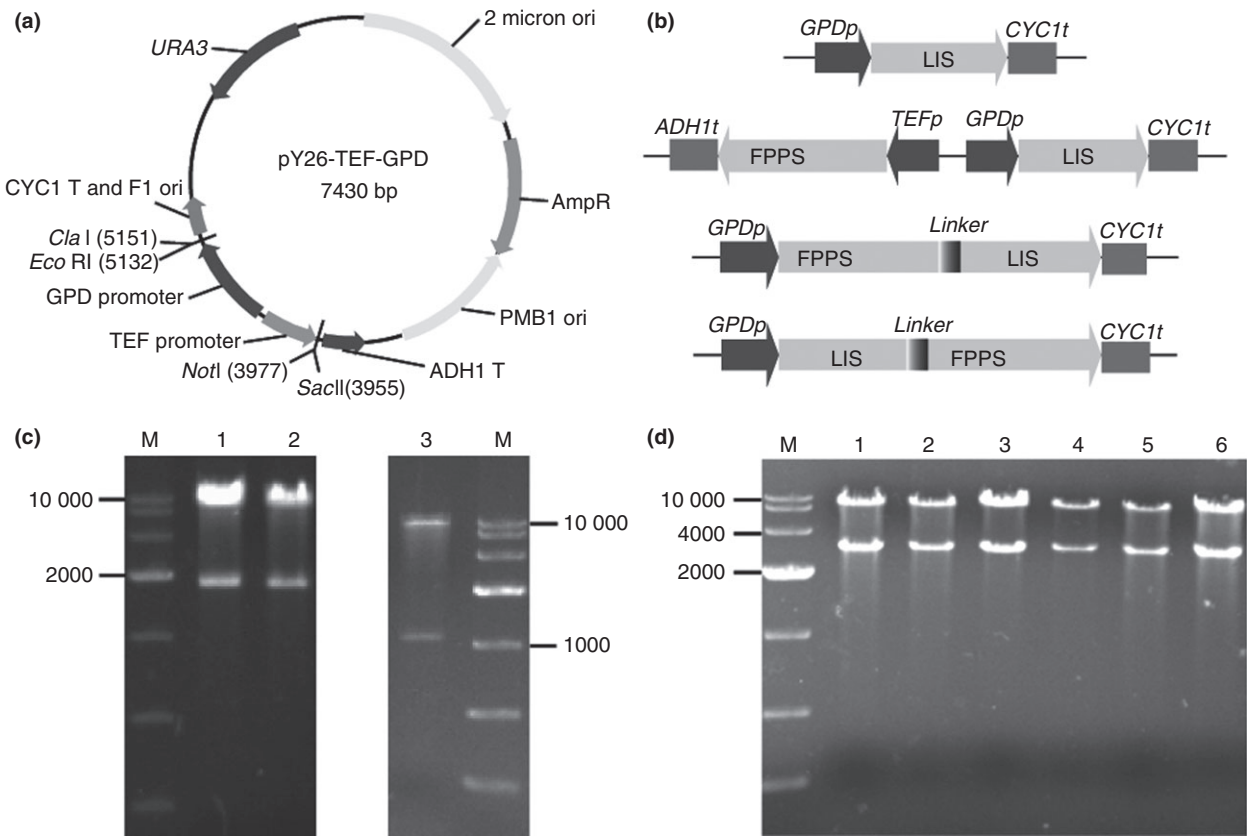

Figure 2 Construction of expression vectors. M: DNA marker DL10000. (a) Structure of the plasmid pY26-TEF-GPD using the promoters GPD and TEF. (b) Structures of the FPPS and LIS proteins expressed in *Saccharomyces cerevisiae*. (c) (1) pY26-AaLS1 after cutting with *Eco*RI and *Cla*I, *AaLS1* gene (1725 bp); (2) pY26-ApLS1 after cutting with *Eco*RI and *Cla*I, *ApLS1* gene (1725 bp); (3) pY26-AaLS1-ERG20 after cutting with *Sac*II and *Not*I, *ERG20* gene (1059 bp). (d) (1–6): the plasmids pY26-ERG20^{K197E}-SF-AaLS1, pY26-ERG20^{K197E}-MF-AaLS1, pY26-ERG20^{K197E}-LF-AaLS1, pY26-AaLS1-SF-ERG20^{K197E}, pY26-AaLS1-MF-ERG20^{K197E}, pY26-AaLS1-LF-ERG20^{K197E} after cutting with *Eco*RI and *Cla*I respectively.

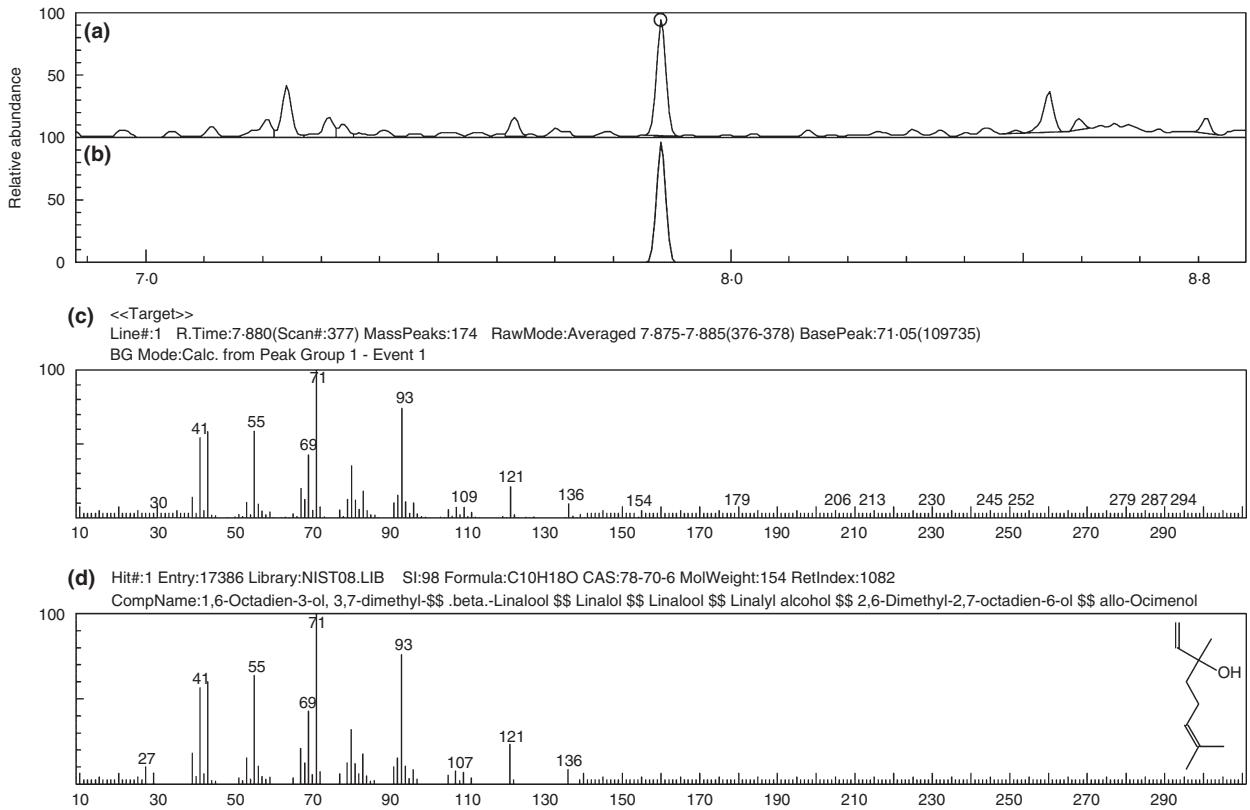

Figure 3 Functional expression of the LIS in *Saccharomyces cerevisiae*. (a) Presence of (*S*)-linalool in cell cultures of the recombinant strain expressing plasmid pY26-AaLS1, analysed and identified by GC spectrum. The Circle indicate retention time and peaks of (*S*)-linalool; (b) The GC spectrum of (*S*)-linalool standard. (c) The mass spectrum of the product detected in the recombinant yeast strain, which yielded a fragmentation pattern identical to that of (*S*)-linalool; (d) The mass spectrum of the (*S*)-linalool standard sample.

A set of linkers was designed for the fusion of two proteins: short flexible (SF): GGGS, middle flexible (MF): $(GGSG)_2$ and long flexible (LF): $(GGGGS)_3$. The strain with the plasmid expressing LIS-FPPS had a higher (*S*)-linalool titer than one expressing FPPS-LIS. The strain with the plasmid pY26-AaLS1-LF-ERG20^{K197E}, which had a long flexible linker achieved the highest titer of (*S*)-linalool: 101·55 ± 2·97 $\mu g\ l^{-1}$, a 69·7% increase compared with the ones with two independent enzymes (Fig. 4).

(*S*)-Linalool production by batch fermentation

Because it was reported that LIS required a divalent cation co-factor for their activities and it preferred Mg^{2+} (Chen *et al.* 2010). Under the optimum divalent cation condition, the production of (*S*)-linalool by the strain with pY26-AaLS1-LF-ERG20^{K197E} had been further improved to 134·23 ± 6·32 $\mu g\ l^{-1}$, which was a 32·2% increase compared to the common YNB medium. In order to find the best titre of (*S*)-linalool produce by CEN.PK2 with pY26-AaLS1-LF-ERG20^{K197E}, the fermentation was performed in a well-controlled fermenter with the optimized parameters. The highest concentration of (*S*)-linalool in the engineered strains was observed 36 h later in log phase. Under the optimal condition, the final (*S*)-linalool by the strain with pY26-AaLS1-LF-ERG20^{K197E} was further improved to 240·64 ± 5·31 $\mu g\ l^{-1}$, 69·1% higher than that of the strain with free LIS (Fig. 5a). The cell growth also showed that the production of (*S*)-linalool did not have significant impact on the cell growth of *S. cerevisiae* (Fig. 5b). The special aroma of (*S*)-linalool could be easily identified during the later culture process, showed not only the accumulation of (*S*)-linalool, but also a considerable amount of (*S*)-linalool was evaporated from the culture broth.

Discussion

(*S*)-Linalool production could be enhanced by expressing the fusion proteins of FPPS and (*S*)-linalool synthase (LIS) in *S. cerevisiae*. The close proximity of fusion proteins was to regulate the metabolic flux in the branch

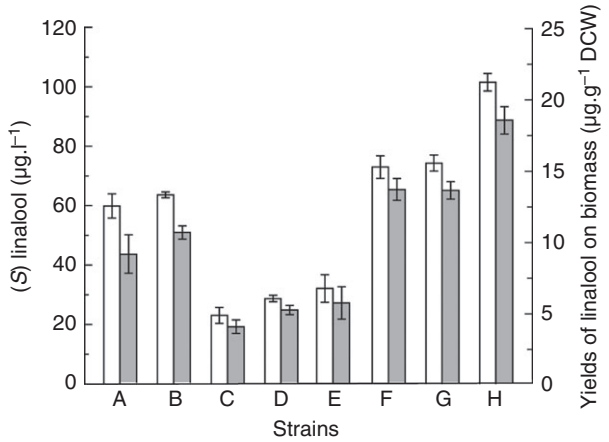

Figure 4 (*S*)-Linalool production of strains expressing LIS and FPPS as individual enzymes or fusion proteins. The content of (*S*)-linalool (μg l^{-1}) is (*S*)-linalool production by recombinant yeasts harbouring plasmids overproducing the various enzymes. The final (*S*)-linalool yield (μg l^{-1} $OD600^{-1}$) is the content of (*S*)-linalool at the same biomass. The strains were cultivated for 48 h in YPD medium and extracted at stationary growth phase. The optical density of cell culture measured at 600 nm (OD600) was used to quantify biomass. (a) The strain was transformed with plasmid pY26-AaLS1; (b) the strain was transformed with plasmid pY26-AaLS1-ERG20^{K197E}; (c–h) the strains were transformed with plasmids pY26-ERG20^{K197E}-SF-AaLS1, pY26-ERG20^{K197E}-MF-AaLS1, pY26-ERG20^{K197E} -LF-AaLS1, pY26-AaLS1-SF-ERG20^{K197E}, pY26-AaLS1-MF-ERG20^{K197E}, pY26-AaLS1-LF-ERG20^{K197E} respectively. The data represent the averages ± standard deviations of at least three independent clones. White columns: concentrations of (*S*)-linalool; grey columns: yields of (*S*)-linalool on biomass.

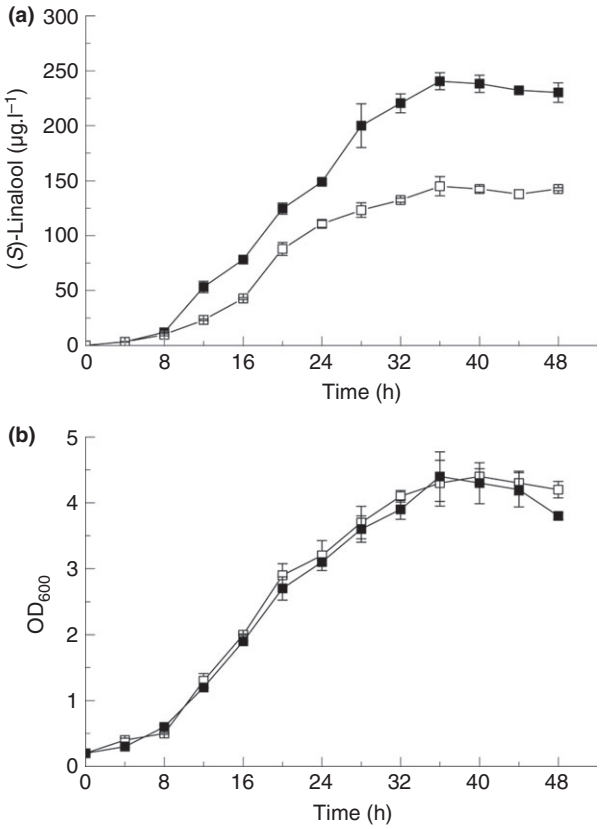

Figure 5 (*S*)-linalool production in fermenter. (a) (*S*)-linalool production of strains with pY26-AaLS1-ERG20^{K197E} (open square) and pY26-AaLS1-LF-ERG20^{K197E} (solid square). (b) OD600 of strains with pY26-AaLS1-ERG20^{K197E} (open square) and pY26-AaLS1-LF-ERG20^{K197E} (solid square).

point of the mevalonate pathway. By altering linker length and orientation of the two active sites, optimal spatial organization of the two enzymes was achieved.

Heterologous expression of a functional synthase gene in *S. cerevisiae* caused the unstable metabolic intermediates, generated by the host enzymatic machinery. The efficiency of forming the final product was influenced by loss of intermediates through diffusion, degradation or conversion by competitive enzymes (Conrado *et al.* 2008). The local concentration of intermediates in the vicinity of sequentially acting enzymes may be low. A typical way to avoid such problems was optimizing the expression levels of the key enzymes in the mevalonate pathway of *S. cerevisiae* to redirect the metabolic flux towards the desired product (Shiba *et al.* 2007; Rico *et al.* 2010). This approach focused mainly on altering the metabolic enzymes in the host metabolic pathway, but the heterologous enzymes introduced into the hosts were rarely engineered. Fortunately, the problem was solved by the construction of fusion proteins catalysing consecutive steps in a metabolic pathway (Tokuhiro *et al.* 2009).

In the mevalonate pathway, GPP was used as the precursor for synthesis of monoterpene. Most of the GPP molecules bonded with FPPS for subsequent conversion to FPP, which was the substrate for several classes of essential yeast metabolites (Fischer *et al.* 2011). When heterologous monoterpene synthase was introduced into the cell, it competed with FPPS for the substrate of GPP to produce the final product. Maintaining the concentration of GPP in the immediate vicinity of the monoterpene synthase was achieved by constructing fusion genes of FPPS and LIS.

The bi-functional fusion proteins with LIS at the N-terminal or C-terminal of FPPS connected by linker peptides have both (*S*)-linalool and FPPS activities. The fusion enzymes were more efficient than the two individual enzymes. This was the reason why fusion proteins introduced into the cell factory increase the concentration of LIS around the transient intermediate GPP, facilitating the movement of GPP from one active site to the next and reducing the loss of GPP to competing pathways (Albertsen *et al.* 2011; Wang *et al.* 2011).

The activity of the fusion protein was influenced by the orientation of the two active sites in the fusion protein and the linker length, but the effect was dependent on the specific properties of the enzymes in the fusion protein (George and Heringa 2003). The linker length and orientation of the genes were optimized for functionality to improve product yields. Two fusion configurations differentially affected (*S*)-linalool production. The fusion of LIS to the N-terminal end of FPPS (LIS-FPPS) produced higher (*S*)-linalool yields than the fusion of FPPS to the N-terminal end of LIS (FPPS-LIS). In this case, the long linker $(GGGGS)_3$ led to higher production of (*S*)-linalool. It is possible that a longer linker might localize the active sites at an appropriate physical distance, which was important and sufficient for improving an engineered pathway.

In summary, in order to increase the availability of GPP, several fusion proteins of FPPS from *S. cerevisiae* and LIS genes from *A. arguta* with different linkers were generated. By using the optimum linker, the production of (*S*)-linalool reached $101{\cdot}55 \pm 2{\cdot}97$ $\mu g\ l^{-1}$, representing an increase in 69·7% compared with that of two independent enzymes. In the well-controlled bioreactor, the (*S*)-linalool titre was further improved to $240{\cdot}64 \pm 5{\cdot}31$ $\mu g\ l^{-1}$, which is the highest reported to date. The results demonstrate that the promise of fusion protein catalysing consecutive steps in a metabolic pathway in the improvement of monoterpene production with GPP as precursor.

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Conflict of Interest

No conflict of interest declared.

References

Albertsen, L., Chen, Y., Bach, L.S., Rattleff, S., Maury, J., Brix, S., Nielsen, J. and Mortensen, U.H. (2011) Diversion of flux toward sesquiterpene production in *Saccharomyces cerevisiae* by fusion of host and heterologous enzymes. *Appl Environ Microbiol* **77**, 1033–1040.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008) Biological effects of essential oils-a review. *Food Chem Toxicol* **46**, 446–475.

Chandran, S.S., Kealey, J.T. and Reeves, C.D. (2011) Microbial production of isoprenoids. *Process Biochem* **46**, 1703–1710.

Chen, X.Y., Yauk, Y.K., Nieuwenhuizen, N.J., Matich, A.J., Wang, M.Y., Perez, R.L., Atkinson, R.G. and Beuning, L.L. (2010) Characterisation of an (*S*)-linalool synthase from kiwifruit (*Actinidia arguta*) that catalyses the first committed step in the production of floral lilac compounds. *Funct Plant Biol* **37**, 232–243.

Cheng, B.-H., Lin, C.-Y., Yeh, T.-F., Cheng, S.-S. and Chang, S.-T. (2012) Potential source of S-(+)-linalool from *Cinnamomum osmophloeum* ct. *linalool* leaf: essential oil profile and enantiomeric purity. *J Agric Food Chem* **60**, 7623–7628.

Conrado, R.J., Varner, J.D. and DeLisa, M.P. (2008) Engineering the spatial organization of metabolic enzymes: mimicking nature's synergy. *Curr Opin Biotechnol* **19**, 492–499.

Crasto, C.J. and Feng, J. (2000) LINKER: a program to generate linker sequences for fusion proteins. *Protein Eng* **13**, 309–312.

Dudareva, N. and Pichersky, E. (2008) Metabolic engineering of plant volatiles. *Curr Opin Biotechnol* **19**, 181–189.

Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., Abeliovich, H. and Vainstein, A. (2011) Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab Eng* **13**, 474–481.

Fernandez, S.M.S., Kellogg, B.A. and Poulter, C.D. (2000) Farnesyl diphosphate synthase. Altering the catalytic site to select for geranyl diphosphate activity. *Biochemistry* **39**, 15316–15321.

Fischer, M.J.C., Meyer, S., Claudel, P., Bergdoll, M. and Karst, F. (2011) Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol Bioeng* **108**, 1883–1892.

George, R.A. and Heringa, J. (2003) An analysis of protein domain linkers their classification and role in protein folding. *Protein Eng* **15**, 871–879.

Jorgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S. and Moller, B.L. (2005) Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. *Curr Opin Plant Biol* **8**, 280–291.

Kirby, J. and Keasling, J.D. (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. *Annu Rev Plant Biol* **60**, 335–355.

Lee, S.Y., Kim, H.U., Park, J.H., Park, J.M. and Kim, T.Y. (2009) Metabolic engineering of microorganisms: general strategies and drug production. *Drug Discov Today* **14**, 78–88.

Li, A., Liu, Z., Li, Q., Yu, L., Wang, D. and Deng, X. (2008) Construction and characterization of bidirectional expression vectors in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **8**, 6–9.

Lu, P. and Feng, M.G. (2008) Bifunctional enhancement of a beta-glucanase-xylanase fusion enzyme by optimization of peptide linkers. *Appl Microbiol Biotechnol* **79**, 579–587.

Mahmoud, S.S. and Croteau, R.B. (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends Plant Sci* **7**, 366–373.

Misawa, N. (2011) Pathway engineering for functional isoprenoids. *Curr Opin Biotechnol* **22**, 627–633.

Muntendam, R., Melillo, E., Ryden, A. and Kayser, O. (2009) Perspectives and limits of engineering the isoprenoid metabolism in heterologous hosts. *Appl Microbiol Biotechnol* **84**, 1003–1019.

Orita, I., Sakamoto, N., Kato, N., Yurimoto, H. and Sakai, Y. (2007) Bifunctional enzyme fusion of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase. *Appl Microbiol Biotechnol* **76**, 439–445.

Oswald, M., Fischer, M., Dirninger, N. and Karst, F. (2007) Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7**, 413–421.

Rico, J., Pardo, E. and Orejas, M. (2010) Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **76**, 6449–6454.

Shiba, Y., Paradise, E.M., Kirby, J., Ro, D.K. and Keasing, J.D. (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab Eng* **9**, 160–168.

Siddiqui, M.S., Thodey, K., Trenchard, I. and Smolke, C.D. (2012) Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *FEMS Yeast Res* **12**, 144–170.

Tokuhiro, K., Muramatsu, M., Ohto, C., Kawaguchi, T., Obata, S., Muramoto, N., Hirai, M., Takahashi, H. *et al.* (2009) Overproduction of geranylgeraniol by metabolically engineered *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **75**, 5536–5543.

Wang, J.H., Tsai, M.Y., Lee, G.C. and Shaw, J.F. (2007) Construction of a recombinant thermostable-amylase-trehalose synthase bifunctional enzyme for facilitating the conversion of starch to trehalose. *J Agric Food Chem* **55**, 1256–1263.

Wang, Y., Yi, H., Wang, M., Yu, O. and Jez, J.M. (2011) Structural and kinetic analysis of the unnatural fusion protein 4-coumaroyl-coA ligase::stilbene synthase. *J Am Chem Soc* **133**, 20684–20687.

Withers, S.T. and Keasling, J.D. (2006) Biosynthesis and engineering of isoprenoid small molecules. *Appl Microbiol Biotechnol* **73**, 980–990.

Zhang, J., Fu, R.Y., Hugenholtz, J., Li, Y. and Chen, J. (2007) Glutathione protects *Lactococcus lactis* against acid stress. *Appl Environ Microbiol* **73**, 5268–5275.