Enhanced Production of a Plant Monoterpene by Overexpression of the 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Catalytic Domain in *Saccharomyces cerevisiae*[▽][†]

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Linalool production was evaluated in different *Saccharomyces cerevisiae* strains expressing the *Clarkia breweri* linalool synthase gene (*LIS*). The wine strain T_{73} was shown to produce higher levels of linalool than conventional laboratory strains (i.e., almost three times the amount). The performance of this strain was further enhanced by manipulating the endogenous mevalonate (MVA) pathway: deregulated overexpression of the rate-limiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) doubled linalool production. In a haploid laboratory strain, engineering of this key step also improved linalool yield.

Monoterpenes are a class of isoprenoids of increasing industrial and clinical interest usually produced by plants. They are used as aromatic additives in the food and cosmetics industries and are also important components in wine aroma. Moreover, certain monoterpenes display antimicrobial, antiparasitic, and antiviral properties as well as a plethora of promising health benefits (for recent reviews, see references 2, 7, 15, 28, and 30 and references cited therein). To date, many studies have focused on plant metabolic engineering of monoterpene production (for selected reviews, see references 1, 14, 19, 29, and 35 and references cited therein), and few studies have been carried out on microorganisms (9, 21, 22, 34, 38). Efficient microbial production of these metabolites could provide an alternative to the current methods of chemical synthesis or extraction from natural sources. In this regard, a considerable number of studies have shown the utility of *Saccharomyces cerevisiae* as a valuable platform for sesquiterpene, diterpene, triterpene, and carotene production (references 5, 10, 23, 26, 30, 31, 32, and 33 and references cited therein). However, all the efforts dedicated to the improvement of isoprenoid yields in *S. cerevisiae* have been performed using conventional laboratory strains, and there are no studies concerning natural or industrially relevant isolates.

In recent years, many genes that encode plant monoterpene synthases (MTS), a family of enzymes which specifically catalyze the conversion of the ubiquitous C_{10} intermediate of isoprenoid biosynthesis geranyl pyrophosphate (GPP) to monoterpenes, have been characterized. Such is the case with the *LIS* gene (codes for *S*-linalool synthase) of *Clarkia breweri*, the first MTS-encoding gene to be isolated (13). In contrast to plants, *S. cerevisiae* cannot produce monoterpenes efficiently,

mainly due to the lack of specific pathways involving MTS. However, GPP is formed as a transitory intermediate in the two-step synthesis of farnesyl pyrophosphate (FPP), catalyzed by FPP synthase (FPPS) (Fig. 1), and some natural *S. cerevisiae* strains have been shown to possess the ability to produce small amounts of monoterpenes (8). Whether this occurs through unspecific dephosphorylation of a more available endogenous pool of GPP and subsequent bioconversions is not known. In addition, it has recently been established that *S. cerevisiae* has enough free GPP to be used by exogenous monoterpene synthases to produce monoterpenes under laboratory and vinification conditions (22, 34).

Here we present the process for selecting and optimizing yeast strains for foreign monoterpene production. We have chosen the *C. breweri LIS* gene as a prototype because, when heterologously expressed in *S. cerevisiae*, it specifically results in the production of linalool (3,7-dimethyl-1,6-octadien-3-ol; a floral scent and bioactive acyclic monoterpene identified in numerous fruits and flowers) and no other by-products (22). Two *S. cerevisiae* strains of different origins have been selected and their endogenous mevalonate (MVA) pathways engineered to enhance the production of linalool. These strategies might be employed to produce any other recombinant monoterpene in *S. cerevisiae* by expressing the appropriate monoterpene synthase.

MATERIALS AND METHODS

Strains, culture media, and growth conditions. *Escherichia coli* DH5α [*endA1 hsdR17 gyrA96 thi-1 relA1 supE44 recA1 ΔlacU169* (φ80 *lacZ*ΔM15)] was used for cloning experiments and plasmid propagation. *S. cerevisiae* strains used in this study are listed in Table S1 in the supplemental material. *E. coli* was maintained in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with or without 100-μg ml^{-1} ampicillin. *S. cerevisiae* strains were maintained in YPD rich medium (1% yeast extract, 2% bacteriological peptone, 2% glucose) or SD minimal medium (0.17% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI], 2% glucose, 0.5% ammonium sulfate) with or without 20-mg liter^{-1} uracil. When necessary, SD media were supplemented with 20-mg liter^{-1} adenine, 20-mg liter^{-1} histidine, 20-mg liter^{-1} tryptophan, and 30-mg liter^{-1} leucine. For solid media, 1.5% agar was added. To determine linalool yields of recombinant yeasts, aliquots from overnight cultures of selected transformants grown in SD medium lacking uracil were transferred to 250-ml flasks containing

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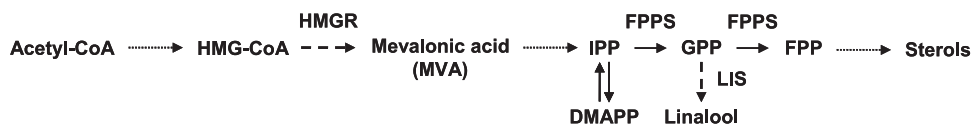
FIG. 1. Simplified isoprenoid pathway in *S. cerevisiae*, including the branch point to linalool. Dotted arrows indicate that more than one reaction is required to convert the substrate to the product indicated. Dashed arrows indicate the engineered steps. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; HMGR, HMG-CoA reductase; FPPS, FPP synthase; LIS, linalool synthase.

50 ml of YPD medium at an initial optical density at 600 nm (OD_{600}) of 0.05. Yeast cultures were grown with continuous shaking (200 rpm) at 30°C, and aliquots of the cultures were taken at different times. For plasmid stability analyses, transformants were grown under both selective (SD medium) and nonselective (YPD medium) conditions, and the colonies growing under each condition were counted.

DNA manipulations, transformations, and Northern analyses. General DNA manipulations were performed according to standard protocols (41). Transformations of yeast strains were done using lithium acetate to permeabilize the cells as previously described (18, 37). Transformants were selected and maintained on SD medium plates without uracil. *S. cerevisiae* total RNA was isolated as described previously (3). Northern analyses were carried out essentially as described previously (41). Agarose gels were loaded with 12.5 μg of total glyoxylated RNA per track. Membranes (Hybond-N⁺; Amersham Biosciences) were stained with 0.03% methylene blue as a loading control, washed, and hybridized. Hybridizations were done using a digoxigenin (DIG)-dUTP-labeled fragment generated by PCR (oligonucleotides LIS-A and LIS-B [see Table S2 in the supplemental material]) according to the manufacturer's instructions (Roche).

Construction of plasmids. The $TDH3_p::LIS::PGK1_t$ expression cassette containing the *C. breweri LIS* cDNA (GenBank accession no. U58314 [13]) was isolated from pG1-Lis (21) as a 3.8-kb PstI-NaeI fragment, which was subsequently subcloned into YEplac195 (17) that had been previously digested with PstI and SmaI, thus creating YEplac195-Lis. To construct the $PGK1_p::_{cat}HMG1::CYC1_t$ expression cassette (${_cat}HMG1$ codes for the catalytic domain of the isozyme 3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase 1, or $_{cat}$Hmg1), we first synthesized by PCR each of the three components of the expression unit using genomic DNA from a yeast strain derived from S288c and the primers indicated in Table S2 in the supplemental material. The three PCR fragments were individually subcloned into pGEM-T Easy. A 1.6-kb NcoI-BamHI fragment containing the $_{cat}HMG1$ allele and a 1.49-kb SalI-NcoI fragment containing the *PGK1* promoter ($PGK1_p$) were ligated into YEplac195, which had previously been digested with SalI and BamHI, and the intermediate plasmid YEplac195-Hmg18 was thus generated. To construct YEplac195-Hmg1$^{531-1054}$, the *CYC1* terminator obtained as a 0.3-kb BamHI-EcoRI fragment ($CYC1_t$) was subcloned into YEplac195-Hmg18, which had previously been digested with BamHI and EcoRI. In order to transfer the *LIS* expression cassette into the YEplac195-Hmg1$^{531-1054}$ vector, the 3.8-kb PstI-NaeI fragment from pG1-Lis was treated with T4 DNA polymerase and ligated into the YEplac195-Hmg1$^{531-1054}$ vector, which had previously been digested with SalI and blunt ended using T4 DNA polymerase. Plasmid YEplac195-LisHmg1$^{531-1054}$ was thus generated. Fragments obtained by PCR were checked by sequencing to ensure the absence of PCR-induced mutations. PCR amplifications were carried out using the Expand high-fidelity system (Roche). Plasmids are indicated in Table S1 in the supplemental material.

Monoterpene analysis. Linalool was analyzed by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) and GC-mass spectrometry (MS) as described previously (22). 2-Octanol (0.2 μg) was used as an internal standard. Linalool concentrations were calculated using standard solutions and are given as the averages of results from at least three independent cultures. Calibration curves were determined using the same protocol by exposure of the polydimethylsiloxane (PDMS) filament to the headspace of YPD spiked with standards at final concentrations of 1, 10, 100, 1,000, and 10,000 μg liter^{-1}. Compounds were identified by comparing retention times and mass spectra with those of standard compounds (Sigma, St. Louis, MO) using a Hewlett-Packard (HP) capillary gas chromatograph, model 5890, series II, equipped with an HPInnowax column (length, 15 m; inside diameter, 0.25 mm; film thickness, 0.25 mm) and an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Agilent Technologies, Waldbronn, Germany), under the same chromatographic conditions.

LIS enzyme assays. To determine linalool synthase activities, we adapted the protocol described for *Lactococcus lactis* (21). Yeast cells were grown for 24 h as

described above. Twenty-milliliter aliquots were centrifuged (4,000 rpm, 15 min, 4°C), and the resulting pellets were maintained frozen until use. To make cell extracts, pellets were resuspended in 1 ml ice-cold linalool assay buffer (LAF) containing 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol (DTT), 10% (vol/vol) glycerol, 20 mM MgCl₂, 5 mM MnCl₂, 1 mM sodium ascorbate, and protease inhibitor cocktail (Roche). Cells were disrupted by bead beating (4 times for 30 s each cycle) using 1 ml glass beads (diameter, 0.5 mm), and the resulting supernatant was recovered after centrifugation (12,000 rpm, 5 min, 4°C). The protein concentrations of extracts were measured using the Bio-Rad/Bradford assay employing bovine serum albumin (BSA) as the standard. Supernatant volumes corresponding to protein contents of 2 and 4 mg were adjusted with LAF to yield final volumes of 1 ml and transferred to 10-ml Pyrex tubes. Twenty microliters of nonradioactive GPP (from a 2.6 mM solution; Sigma) was added, vials were closed with Teflon septa, and the reaction mixtures were incubated at 30°C with gentle shaking (100 rpm) for 30 min. Subsequently, 0.2 g NaCl was added and the reaction products were collected with a PDMS fiber and measured as described above. Linalool (~83 μg liter^{-1}) was detected in the control vial (GPP alone). This background amount was subtracted from the linalool produced in the enzymatic assays. Linalool was not detected as a product of GPP derived from the endogenous phosphatase activity of the microbial host, since the yeast strains transformed with empty plasmid (no *LIS* gene) produce linalool levels similar to that of the control. Under the assay conditions used, linalool production was proportional to the amount of protein present in the assays. Enzyme assays were performed in duplicate.

RESULTS AND DISCUSSION

Expression of the *C. breweri LIS* gene in different laboratory strains of *S. cerevisiae* results in similar linalool yields. As the genetic background of the host may have dramatic consequences with regard to recombinant monoterpene production, we first evaluated the performance of six *S. cerevisiae* strains of different genotypes and ploidies. It has been suggested (24) that *MAT***a** strains might synthesize more FPP, as this compound is needed for prenylation of the mating pheromone (4). Indeed, the yields of the sesquiterpene epicedrol (synthesized in one step from FPP) in *MAT***a** strains are twice those in *MAT*α strains (24). We therefore studied the effect of mating type on linalool production. The *LIS* expression cassette ($TDH3_p::LIS::PGK1_t$) from plasmid pG1-Lis (22) was subcloned into the 2μm shuttle vector YEplac195 (17) and used to transform *ura3*-auxotrophic strains (derived from S288) of opposite mating types, BQS252 (*MAT***a** *ura3-52*) (16) and BQS253 (*MAT*α *ura3-52*), using the LiAc/ss-DNA/PEG method (18). Two uracil-prototrophic transformants of each genetic background were thus isolated, YR174 and YR175 (*MAT***a**) and YR296 and YR297 (*MAT*α), and their abilities to excrete linalool were compared. Yields of linalool were similar in both mating types (average, 21.92 ± 1.75 μg liter^{-1}) (Fig. 2A), suggesting that the potential diversification of the MVA pathway to provide prenyl groups for the mating pheromone does not improve GPP availability. We then used the **a** mating type for further analyses. As expected, linalool was not de-
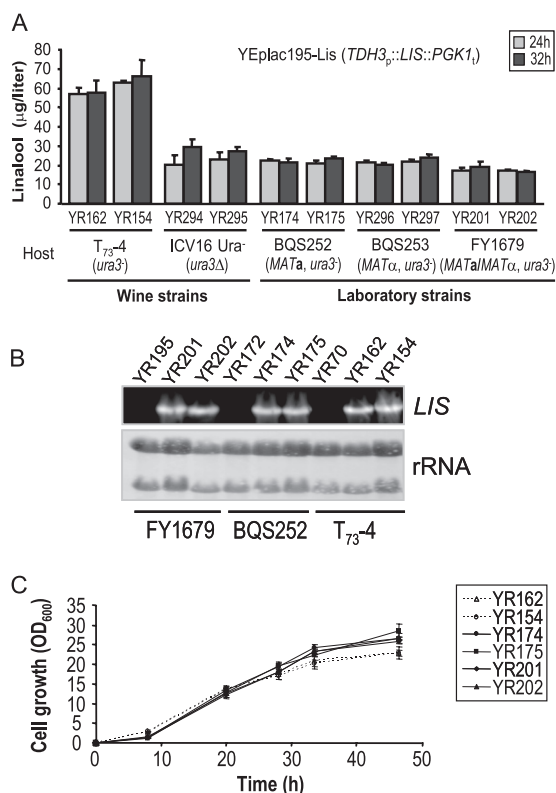
FIG. 2. Comparison of levels of linalool production by different host strains. (A) Analysis of linalool yields in different strains of *S. cerevisiae*. Cultures were sampled at the times indicated, and linalool was quantified. Results are presented as the means and standard deviations from at least three different experiments. (B) Northern blot of total RNA extracted from recombinant yeasts producing linalool and the complemented control strains (24 h). rRNA is shown as a loading control. (C) Growth curves of yeast strains transformed with YEplac195-Lis. These cultures were used to quantify linalool (A).

tected in the complemented control strains YR172 and YR304 (BQS252 and BQS253 transformed with YEplac195).

We also tested linalool production by W303-1A (*MAT*a; laboratory strain from a different origin) (43) transformed with the same replicative *LIS* expression vector (YEplac195-Lis 2μm *URA3*) as that used to transform BQS252 and BQS253. Absolute linalool production was around 50% lower in recombinant W303-1ALis strains (YR270 and YR272) than in recombinant BQS252Lis (average, 10.90 ± 2.04 μg liter^{-1}). However, due to the lower level of growth of W303-1A transformed with YEplac195-Lis than that of BQS252Lis and BQS253Lis, when linalool production is measured in reference to OD values, the productivities of these three genetic backgrounds were quite comparable (averages, 1.43 ± 0.27 μg liter^{-1} OD$_{600}$$^{-1}$ and 1.37 ± 0.22 μg liter^{-1} OD$_{600}$$^{-1}$, respectively, at 32 h). These results suggest that haploid laboratory yeast strains have similar abilities to produce monoterpenes. However, in comparison with the linalool levels produced by recombinant wine strains under similar growth conditions (22), the productivity of these laboratory strains is significantly lower.

Wine yeast strain T$_{73}$-4 has a greater capacity for recombinant monoterpene biosynthesis than other tested strains. In order to improve wine quality as well as the elaboration pro-

cesses, wine yeast strains have been selected for a very specialized phenotype and are genetically complex, usually being polyploid or aneuploid (6). To elucidate whether the better ability to convert endogenous GPP into monoterpenes is a common feature in oenological strains of *S. cerevisiae*, two different wine strains, T$_{73}$-4 (nearly diploid [37], derived from T$_{73}$ Lallemand) and ICV16*ura3*Δ (derived from ICV16 Fermicru Primeur, DSM [25]), as well as the laboratory diploid strain FY1679 (*MAT*a/*MAT*α *ura3-52*/*ura3-52*; isogenic to BQS252 and BQS253), were transformed with YEplac195-Lis and two uracil-prototrophic transformants of each genetic background were isolated. Figure 2A shows that linalool production by recombinant FY1679Lis strains (YR201 and YR202) was not greater than that of recombinant BQS252Lis and BQS253Lis, indicating that diploidy alone does not improve linalool levels. In contrast, linalool yields for recombinant wine strains were greater than in their laboratory counterparts, indicating that these genetically complex backgrounds may be more appropriate for producing heterologous monoterpenes. Notably, the inherent capacities of the two industrial strains differ, and production of linalool by recombinant T$_{73}$-4Lis cells (YR154 and YR162; average, 60.94 ± 6.15 μg liter^{-1}) was about 2-fold greater than that of recombinant ICV16Lis (YR294 and YR295). The reasons for the differing efficacies of yeast hosts are not obvious, and further studies would be necessary to satisfactorily explain this. Differences in transgene transcription are unlikely to be a primary factor, as the *LIS* gene is similarly expressed in recombinant BQS252Lis, FY1679Lis, and T$_{73}$-4Lis strains (Fig. 2B). Also, greater linalool production cannot be associated with higher cell densities, as T$_{73}$-4Lis strains reached slightly lower (around 15%) optical densities than recombinant BQS252 and FY1679 (Fig. 2C). Moreover, as described previously for the wine strain T$_{73}$-PG1LIS URA (22), both recombinant laboratory (YR175) and wine (YR162 and YR295) yeast strains accumulated linalool during the early and rapid phases of culture growth (see Fig. S1 in the supplemental material). In addition, linalool synthase activities did not differ greatly between recombinant wine strain ICV16Lis or T$_{73}$-4Lis (588 ± 222 and 617 ± 154 μg liter^{-1} mg total protein^{-1}, respectively) and laboratory strain BQS252Lis (636 ± 240 μg liter^{-1} mg total protein^{-1}), despite the different levels of linalool produced by them. Taken together, these results suggest that redirection of the endogenous isoprenoid pathway toward linalool production depends on the genetic background of the hosts, which could affect GPP availability. The low level of linalool accumulation in laboratory strains could reflect other restricting conditions, including limited coupling between the introduced linalool synthase and the endogenous GPP pool or even the limiting availability of a cofactor. Use of the T$_{73}$ strain as a production host for recombinant monoterpenes and eventually other isoprenoids is an improvement for increasing yield per unit of culture volume.

Deregulated overexpression of a truncated *HMG1* allele increases linalool production in yeasts. Since another restriction on monoterpene production could be precursor supply, we also wanted to know whether engineering key steps within the MVA pathway affects linalool yields. The major regulatory control point of this pathway (Fig. 1) in many organisms, including yeasts, is the formation of mevalonic acid (MVA) from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), a step
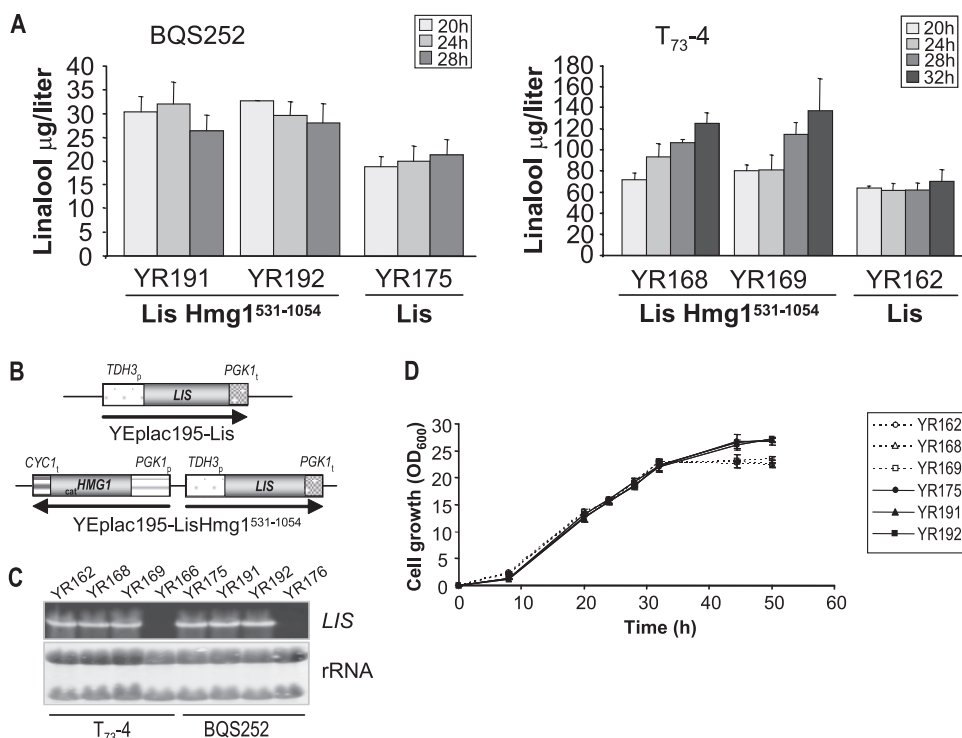
FIG. 3. Increase of linalool production by overproducing $_{cat}$Hmg1. (A) Histograms showing linalool content in the supernatants of the control and two independent transformants. Cultures were sampled at the times indicated. Values are averages from at least three independent experiments. Error bars indicate standard deviations. (B) Schematic diagram of the gene cassettes in the two plasmids used to transform *S. cerevisiae*. (C) Expression analysis of the introduced *LIS* transgene by Northern hybridization using a DIG probe. rRNA is shown as a loading control. (D) Growth curves of yeast strains transformed with YEplac195-LisHmg1$^{531-1054}$. The same cultures used to build panel A were evaluated.

catalyzed in *S. cerevisiae* by HMG-CoA reductases 1 and 2 (for reviews, see references 20 and 31). Deregulated overexpression alone of truncated *HMG1* alleles ($_{cat}$*HMG1* codes for the catalytic domain of the isozyme HMG-CoA reductase 1, or $_{cat}$Hmg1) has successfully been employed to increase the rate of flow through the MVA pathway and thus improve the production of squalene (an intermediate of ergosterol synthesis) and amorphadiene (the sesquiterpene precursor of the antimalarial drug artemisin) in *S. cerevisiae* (11, 36, 39), as well as carotenoids in *Candida utilis* and *Neurospora crassa* (42, 44). However, overproduction of $_{cat}$Hmg1 alone cannot be considered a precedent for success in improving monoterpene yields, as this strategy failed when employed trying to improve the yields of the sesquiterpene epicedrol (24) and the triterpene β-amyrin (27). Moreover, certain strategies that have been seen to work well in laboratory strains have failed in industrial ones (12). Bearing in mind the above, along with the fact that GPP is a transient intermediate of FPPS in the formation of FPP, it is difficult to predict whether an increase in the flow through of the pathway would result in a concomitant increase of GPP availability.

Based on these considerations and looking to increase linalool production, we decided to study the effects of overexpressing the $_{cat}$*HMG1* allele (coding for a mutated soluble form of the protein lacking the first 530 amino acid residues and having an engineered Met start codon before Asp531) in *S. cerevisiae* host cells (BQS252 and T$_{73}$-4) that produce different levels of linalool. To deregulate the *HMG1* gene and avoid

recombination between tandemly repeated copies (such as promoter or terminator sequences) that could be looped out by excisional recombination (40), the truncated *HMG1* gene was present in YEplac195-LisHmg1$^{531-1054}$ under the control of the strong constitutive promoter *PGK1* and the *CYC1* terminator (Fig. 3B). Plasmid YEplac195-LisHmg1$^{531-1054}$ was used to transform the selected strains, and the recombinants YR191 and YR192 (BQS252 background) and YR168 and YR169 (T$_{73}$-4 background) were isolated. Figure 3A shows that coexpression of $_{cat}$*HMG1* and *LIS* in T$_{73}$-4 was associated with an increase in linalool production to 132.66 ± 24.72 μg liter^{-1}, while in BQS252 the linalool content was 30.19 ± 3.88 μg liter^{-1}. Thus, in comparison with the control strains YR162 (T$_{73}$-4Lis) and YR175 (BQS252Lis), 2- and 1.5-fold increases in linalool production were achieved. Differences found in the kinetics of linalool production in BQS252Lis$_{cat}$Hmg1 and T$_{73}$-4Lis$_{cat}$Hmg1 could explain differences in the final linalool titers. Whereas the highest accumulation of linalool in the engineered laboratory strains (YR191 and YR192) was observed at 20 h, as occurs in BQS252Lis and T$_{73}$-4Lis, the production of linalool in T$_{73}$-4Lis$_{cat}$Hmg1 progressively increased over the whole time course of the experiment (Fig. 3A). Linalool was not detected in strains overexpressing $_{cat}$*HMG1*. Levels of expression of the *LIS* transgene were similar in the engineered yeasts (Fig. 3C), indicating that that greater linalool titers are not associated with enhanced expression of the *LIS* transgene. All transformants reached ODs similar to those of the control strains (YR175 and YR162), and

the rates of growth were identical (Fig. 3D), indicating that neither the amount of linalool nor the manipulations introduced within the isoprenoid pathway apparently produce deleterious effects on yeast growth under such conditions. Taken together these results suggest that the pool of GPP increases in both genetic backgrounds as a consequence of overproducing $_{cat}$Hmg1 and also that the window in which GPP is available for monoterpene production is wider in the engineered wine T_{73}-4 strain. A previous report showed that overexpression of the catalytic domain of Hmg1 resulted in a dramatic increase in squalene production (11, 36), whereas our study shows a moderate increase in linalool yield. These results may suggest for the enzymes involved in the biosynthesis of ergosterol (i.e., FPPS) a higher affinity for GPP than for LIS.

In conclusion, we describe that pathway optimization and host selection resulted in an increased synthesis of recombinant linalool (up to 6-fold). The *S. cerevisiae* strains developed in this study can serve, in principle, as platform hosts to make any of the hundreds of known monoterpenes by expressing the appropriate MTS in place of linalool synthase. The levels that we obtained are still low for industrial applications. However, they are sufficient for testing the function of novel monoterpene synthase-encoding genes as well as conducting structural studies on the monoterpenes produced. Moreover, our engineered strains are promising for further development.

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